TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Mikrobielle Ökologie

Biodiversity and control of *STAPHYLOCOCCUS AUREUS* in the dairy chain

Klaus Mathias Gutser

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Agrarwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. J. Bauer
Prüfer der Dissertation:
1. Univ.-Prof. Dr. S. Scherer
2. Univ.-Prof. Dr. M. Wagner
(Veterinärmedizinische Universität Wien/ Österreich)

Die Dissertation wurde am 15.11.2011 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 29.03.2012 angenommen.

Declaration

This dissertation describes my original work except where acknowledgement is made in the text. It does not exceed the page limit and is not substantially the same as any work that has been, or is being submitted to any other university for any degree, diploma or any other qualification.

Klaus Mathias Gutser
October 2011

Acknowledgments

These studies were carried out at the dairy educational- und research establishment MLF Wangen (destrict Baden-Württemberg, Germany) and at the veterinary university of Vienna, Department of milk hygiene (Austria). I also appreciate the priviledge of visting two times the Robert Koch insititute by Prof. Wolfgang Witte und collaborators, as well Dr. Jürgen Wallmann at BGVV Berlin, Dr. Peter Krabisch and Dr. Peter Gangerl at TGD Bayern in Grub, Prof. Rogger Stephan at ETH Zürich, Dr. Peter Zangerl at Bundesanstalt für Alpenländische Milchwirtschaft Rotholz (Austria), Dr. Alfred Rammelmeier at Bundesanstalt für Milchwirtschaft Wolfpassing (Austria) and Dr. Unglaub at TGD Aulendorf (destrict Baden-Württemberg, Germany). They all were ready to help or gave me their strain collections for this study.

I have four persons to thank for this thesis; Professor Siegfried Scherer at TUM Weihenstephan in Freising and Professor Martin Wagner at veterinary university Vienna, as well Dr. Richard Gerlach and Dr. Jochen Buck at MLF Wangen. Warm thank to you for encouragement and support. First and foremost in this context, I wish to thank Prof. Siegfried Scherer and Prof. Martin Wagner for giving me the opportunity to work on this project, constructive suggestions and for the possibility of graduation through the assumption of the examination and Co-examination.

I want to thank the financial supporters of this work IWI, foundation of Baden-Württemberg as well Gebr. Baldauf § Co. Lindenberg. Special thanks here to Dr. Martin Grauer, the project manager of IWI Baden-Württemberg. He trusted and encouraged me to go-a head with my work.

Many people have contributed to my work. Dr. Josef Hüfner is thanked for his comments, inspirational meetings and sharing his knowledge and experience with me. I express my sincere thanks to Dr. Ingeborg Hein and Dr. Beatrix Stessl and all collaborators from the department of milk hygiene at veterinary university Vienna, for offering help and countless working hours with the statistical analysis of data. An important part of this work was done at the farms. I am obliged to all farmers taking part in this research. I really appreciate well the contribution of the farms taking part to mastitis detection and sampling experiments.

Finally, I can never express enough gratitude to my “extended” family, relatives and friends for simply being there for me, supporting me whenever nedded. My parents have helped me enormously with everything. Special thanks go to my friends Dr. Immo and Emmanuela Trinks and Dr. Christian Sorg. They need a lot of their own leisure for reading the thesis. Their great work was indispensable for an understandable English or giving solutions for phrasing difficult contents.

Zusammenfassung

„Biodiversität und Kontrolle von *Staphylococcus aureus* in der Milchwirtschaft“

Die Biodiversität und Kontrolle von *Staphylococcus aureus* (*S. aureus*) aus bovinen Mastitiden bilden den Schwerpunkt dieser Arbeit. Die in den letzten 40 Jahren abgelaufenen Veränderungen in der Milchwirtschaft hin zu industriellen Produktionsweisen war sehr wesentlich begleitet durch eine Verschiebung der Flora von Mastitiserregern, die in Monitoring-Programmen bisher unzureichend berücksichtigt wurden. Die Fragestellungen dieser Arbeit basieren entscheidend auf einem umfangreichen Literaturstudium bis in die 1930er Jahre bezüglich der Aussagekraft der Methoden für den Nachweis von *S. aureus*. Um die reale Bedeutung von *S. aureus* für die Qualität von Rohmilch und deren Produkte abschätzen zu können, ist eine kritische Bewertung ungelöster methodischer Probleme für Probenahme, Isolierung und Typisierung notwendig. Die gute Korrelation mit PFGE und FTIR bestätigen die hohe Spezifität der Phagentypisierung sowie die Bedeutung der phänotypischen elektiv Kriterien.

Neben Stammsammlungen aus Monitoring-Programmen standen 12 Rohmilch verarbeitende Betriebe mit Herden von hoher oder niedriger Prävalenz zur Verfügung, in denen zur Bestimmung der *S. aureus*- Populationen modifizierte Methoden verwendet wurden: Die Probenahme berücksichtigte alle Kolonie morphologisch verschiedenen Typen in Vor-, Haupt- und Nachgemelk der laktierenden Kühe, Tankmilch und Käse. Eine detaillierte Auswertung der Zellgehalte, Ausscheidungsraten im Vor-, Haupt- und Nachgemelk erklärt Defizite einer üblichen Vorgehensweise mit ausschließlicher Untersuchung von 0.01 ml Milch im Vorgemelk bezüglich Einschätzung des Krankheitsverlaufs der Mastitis. Die in den verschiedenen Gemelken ermittelten Ausscheidungsraten ermöglichen einerseits die Unterscheidung zwischen kontaktösen Mastitiden und Umwelt-Mastitiden. Andererseits tragen sie zum Verständnis der Dynamik subklinischer Mastitiden bei und damit zu einer verbesserten Kontrolle der Krankheitserreger und epidemiologischer Gefährdungspotenziale.

Aus den Korrelationen zwischen *S. aureus*- Ausscheidungsraten und den Eiweiß-, Fett- und Harnstoffgehalten der Milch wird die Relevanz von Fütterungsfehlern besonders für spontane Infektionen im Strichkanal bestätigt.

Die Prävalenz von 80 % der *S. aureus* Typen mit einer hohen Sensitivität für Phagen des bovinen Phagensatzes hat sich in Deutschland seit 40 Jahren nicht verändert. Jedoch wurde die früher vorliegende hohe Sensitivität (ca. 65%) für Phagen der Gruppe IV mit dem Einsatz von Penicillin vor 40 Jahren auf ca.17 % reduziert. Der Anteil Penicillin resistenter Stämme in Phagengruppe IV stieg von < 10 % auf ca. 50 % an.

Die *S. aureus*- Subpopulation *var. bovis* wurde in eine inhomogene Gruppe mit vielen phänotypisch unterschiedlichen Typen unterteilt. Dies gilt insbesondere für Herden mit einem hohen Einsatz von Antibiotika aufgrund häufig auftretender Mastitiden. Mit der Eliminierung der Phagengruppe IV stieg die Anzahl Eigelb-negativer und β- Hämolyse fehlender *S. aureus* an. Diese erwähnten Veränderungen sind auf die Zunahme der bovinen Phagentypen 119, 78 und 96 (AC1) mit Anteilen von 20, 17 und 7 % zurückzuführen. Die Phagentypen 119 und 96 waren ausschlaggebend für den Anteil von 31 % an Clumpingfaktor negativen Isolaten mit schwacher Koagulaseaktivität.

Aus den Untersuchungen in den 12 Rohmilch verarbeitenden Betrieben lässt sich die Bedeutung der Probennahme und auch die begrenzte Aussagekraft von Monitoring-Programmen, basierend auf nur einem Isolat pro Herde, belegen. Sanierungsmaßnahmen verdrängten nur kontaktöse *S. aureus*- Typen. Clumpingfaktor negative Typen verringerten sich auf Anteile von < 4 % und davon zeigten 31 % der Stämme keine oder schwache Hämolyse. Unter 7 % gehörten den Phagentypen 119, 78 oder Phagengruppe IV an und nur 18 % der Stämme zeigten eine hohe Sensitivität gegenüber bovinen Phagen. Viele *S. aureus*-Stämme verhielten sich wie Erreger von Umweltmastitiden und waren oft nur nachweisbar in Milchproben > 0.01 ml bei Abwesenheit anderer *S. aureus*- Stämme oder Beprobung des Haupt- oder Nachgemelks. Sechs dieser elf Stämme waren *sea* positiv und gehörten zugleich den humanen Biotypen an. Milch von Kühen mit hohen Zellgehalten enthielt nur SEC oder SED positive *S. aureus.*

Sanierungsmaßnahmen können in Milchviehherden offensichtlich das Risiko für das Auftreten SEA produzierender *S. aureus* ohne β-Hämolyse fördern. Die Präsenz dominierender Enterotoxin produzierender Stämme kann mit Hilfe Realtime PCR geklärt werden. Mittels Anreicherung der Tankmilch und Einbeziehung der applizierten Antibiotika sollte der Nachweis Antibiotika resistenter Stämme auch in niedrigen Konzentrationen (siehe MPN-Methode) möglich sein, so dass rechtzeitig Vorsorgemaßnahmen eingeleitet werden können. Zudem profitieren Monitoring-Programme von dieser Kenntnis. Eine konsequentere Nutzung elektronischer Datenbanken für epidemiologische Untersuchungen, basierend auf international standardisierten Methoden, mit Einbeziehung phänotypischer Eigenschaften sollten weltweit Vergleiche der *S. aureus* - Populationen erleichtern.

Summary

"Biodiversity and control of *Staphylococcus aureus* in the dairy chain"

The diversity of *Staphylococcus aureus* (*S. aureus*) populations and the efficiency of controlling *S. aureus* in bovine mastitis influencing the biodiversity of *S. aureus* in raw milk are the central questions of the thesis. The evolution of modern milk production ("industrial techniques") is the main influencing factor when discussing about the shift of mastitis pathogens during the last 40 years. The questions of this thesis were determined by both a far-reaching study of literature going back to the 1930´s and extending empirical studies in dairy herds focused on *S. aureus* detection by a large range of methods. National monitoring programs constitute a base when discussing about the shift of *S. aureus* in dairy herds. Careful considerations about various unsolved methodological problems in sampling, culturing and typing however are necessary to improve our picture about the prevalence of *S. aureus* in raw milk and raw milk products. The high agreement of typing results in one *S. aureus* collection with 30 phages of the BPS, HPS and single additional phages with PFGE and FITR confirmed the high specificity and discriminatory efficiency of phage typing and show the strong concern of the common selective criteria for *S. aureus* population analysis with special regard to enterotoxigenic or antibiotic resistant isolates. Missing or only weak haemolysis are problems for culturing phage type 116 or other penicillin resistant bovine *S. aureus* types on BA.

A detailed analysis of the SCC, shedding patterns in different milk fractions (foremilk, main milk and stripping samples) explains the common approach investigating 0.01ml foremilk with respect to the pathogenesis of mastitis. Twelve farms producing raw milk products, separated in herds with a high and low *S. aureus* prevalence, were part of the project. An optimized sampling procedure considering each detectable morphologically different colony of *S. aureus* type in foremilk, main milk and stripping of cows, bulk milk and cheese was used for the determination of the *S. aureus* population in these herds. The shedding patterns and bacteriological investigation of different milk fractions were formerly the central question for distinguishing contagious or environmental pathogens and their role in the control and dynamic of mastitis or epidemiological investigations. The relationship between *S. aureus* shedding rates and the ratio of protein-, fat-, and urea content confirmed the critical relevance of feeding especially for spontaneous or teat canal infections.

The prevalence of 80 % *S. aureus* types with strong reactions for phages of the BPS did not change in Germany since the last 40 years.

But about 65 % showed in the past only strong reactions for the phages of phage group IV. The selective pressure of penicillin in bovine mastitis during the last 40 years reduced the prevalence of isolates in phage group IV to 17 % and the penicillin resistance of *S. aureus* in phage group IV increased from < 10 % up to 50 % and more. The *S. aureus variety bovis* was splitted in a high number of subtypes with variable phenotypic characteristics. Especially in herds with mastitis the typical *S. aureus* strain in phage group IV were completely eradicated and replaced with a high number of diverse intermediary biotypes. With the eradication of phage group IV, both the prevalence of non β-haemolytic strains and number of egg-yolk positive strains on BP increased. The still high prevalence of egg-yolk negative and penicillin sensitive isolates are caused by phage type 119 or 78 with a share of about 20 % or 17 % in national monitoring programs. The phage types 78, 116 and 119 had a distribution ≤ 2 % 40 years ago. The isolates with a high sensitivity for phage 119 and phage 96, lead to the current high prevalence of clumping factor negative *S. aureus* isolates with weak coagulase activity.

The large importance of sampling and limitations in monitoring programs, when considering only one strain per herd, were observed in the 12 herds of raw milk cheese producing farms. The effective eradication of the contagious *S. aureus* types in sanitation programs of these herds is confirmed not only by low prevalence of < 7 % isolates, belonging to phage type 119, 78 and phage group IV. In national monitoring programs only 10 % strains show weak or no haemolysis and 31 % are clumping factor negative, whereas 42 % of biotypes in the 12 raw milk producing farms exhibit weak or no haemolysis and only 4 % were clumping factor negative. Many strains had a high similarity to environmental udder pathogens and were often only detectable in > 0.01 ml milk samples in consideration of the *S. aureus* contents and the three milk fractions of cows that are foremilk, main milk and stripping samples. Six of 11 SE producing biotypes in these herds with human phenotypic characteristics were *sea* positive, whereas *S. aureus* of mastitis or quarters with high SCC produced only SEC or SED.

We concluded that the eradication of contagious strains increases the risk for *S. aureus* shedding cows with low SCC and isolates producing SEA with antibiotic resistance and no α- or β-haemolysis on BA. The presence of dominating strains with enterotoxins could be clarified by Realtime PCR. An enrichment of the bulk milk, by using the MPN- technique with the applied antibiotics in corresponding herds, would allow the detection of antibiotic resistant isolates in low concentrations still before they are spread in the whole herd. It would be a more sensitive method for *S. aureus* monitoring programs as well. Additionally consequent electronic data sharing of typing results from international, standardized methods would worldwide facilitate and specify comparisons between *S. aureus* populations.

Content

List of abbreviations

α	α-, complete lysis on Blood Agar
β	β-, not complete lysis (hot-cold lysis) on Blood Agar
µg	microgramm
BA	Blood Agar
BP	Baird-Parker Agar
BP-RPF	Baird-Parker Agar with Rabbit plasma fibrinogen
BPS	international bovine phage set
BMSCC	somatic cell count of the bulk milk
CF	clumping factor
CFU	colony forming units
CMT	California mastitis test
CNS	coagulase-negativ staphylococci
CPS	coagulase-positiv staphylococci
CL	confluent lysis (strongest reaction of phage typing)
DNA	desoxyribonucleic acid
FTIR	Fourier transformed infrared spectroscopy
HCPS	high prevalence of coagulase-positiv staphylococci (*S. aureus*)
HPS	international human phage set since 1974
IDF	International Dairy Federation
Ig	immunoglobuline (A ,G , M)
IMI	intramammary infection
IPS	old international basic phage set
ISO	International Organization for Standardization
h	hours
hlb	β- gene
l	liter
LBP	Liquid Baird-Parker
LCPS	low prevalence of coagulase-positiv staphylococci (*S. aureus*)
log	logarithm
ml	millilitre
mM	milli molar
M	molar
MLEE	multilocus enzyme gel electrophoresis

MLST	multilocus sequence typing
MPN	most probable number
MRSA	Methillicin resistant *S. aureus*
n	total count of samples (number of probes)
OD	optical density
P	probability
PCR	Polymerase Chain Reaction
PFGE	Pulsed - field gel electrophoresis
PT	Type / profile of PFGE
r	correlation or regression coefficient
RTD	Routine Test Dilution (Dilution of phage titre for typing)
RFLP	Restriction Fragment Length Polymorphism
s_r	standard deviation
SAPI	*Staphylococcus aureus* Pathogenic Island
SCC	Somatic Cell Count
SCL	semi confluent lysis (strong reaction of phage typing)
S. aureus	*Staphylococcus aureus*
Str. uberis	*Streptococcus uberis*
Str. agalactiae	*Streptococcus agalactiae*
SE (SEA-SED)	Staphylococci- Enterotoxin (A-D)
se (sea-sejj)	Staphylococci-enterotoxin gene (*a, b, c, d, g, h, i, j*)
spa	DNA sequencing of the polymorphic X region of the protein A gene (used for typing)
sak	staphylokinase gene
scn	genes encoding staphylococcal complement inhibitor (SCIN)
tst	toxic shock syndrome gene
TST	toxic shock syndrome toxin
ST	multilocus sequence type (Type of MSST)
U	units
x_A	arithmetic mean
x_G	geometric mean

List of Figures

List of Tables

1 Introduction

Staphylococcus aureus (*S. aureus*) is regarded as the most important bacterium causing bovine subclinical mastitis (IDF-Bulletin, 2006). Epidemiologically studies with phage typing and genotyping methods show that the only source of *S. aureus* in raw milk is the udder of cows (Davidson, 1961b; Zadoks et al., 2002; Peles et al., 2007). *S. aureus* and *Str. agalactiae* are classified as contagious pathogens that show a rapid spread in a herd resulting in large numbers of infected quarters (Bramley and Dodd, 1984; Smith et al., 1985; Fox and Gay, 1993). *S. aureus* is not only part of the native flora of raw milk and raw milk products. *S. aureus* is as well the bacterium causing the most food poisoning outbreaks worldwide (Zangerl, 1999a; Monrandi et al., 2007).

In contrast to *Str. agalactiae*, only a low correlation (r=0.46) between *S. aureus* counts in the bulk tank milk and the prevalence of positive foremilk samples of lactating cows in the herds was found (Gonzalez et al., 1986). About 60 % of bulk tank milk samples have a *S. aureus* count of ≥100 cfu/ml (Seelemann et al., 1963; Kandler and Alboenaga, 1965a; Zangerl, 1999a). The correlation between the total bacteria count and *S. aureus* count in bulk tank milk is low (Seelemann et al., 1963; Kandler and Alboenaga, 1965b). This is also observed for the *S. aureus* count in the bulk milk and BMSCC (Appendix: Table 8). The Switzerland is according to Busato et al. (2000) the country with lowest SCC in the bulk milk worldwide,with an average of 88,000 SCC/ml but still 62 % of the bulk tank milk samples in Switzerland are *S. aureus* positive ranged from 10 to 3000 cfu/ml, with an extreme value of 100,000 cfu/ml in one sample (Stephan et al., 2002). 32 % of *S. aureus* from bulk tank milk in the Switzerland can produce the enterotoxins A, AD, B, C or D (Stephan et al., 2002).

The distribution and kind of enterotoxins, phenotypic characteristics and typing results are correlated to the pathogenesis of *S. aureus* mastitis (Olson et al., 1970; Lombai et al., 1976 Gudding, 1980). *S. aureus* producing enterotxin A and D are usually correlated only to subclinical mastitits (Olson et al. 1970; Lombai et al., 1976; Zschöck et al., 2005). Untermann et al. (1973), Zschöck et al. (2005) and Gonano et al. (2009) could not detect enterotoxin A producing *S. aureus* in mastitis samples of cows, in contrast to the findings of Mayer (1975), Koiranen and Niskanen (1975), Mochmann et al. (1976) and Stephan et al. (2001). An important argument for the investigation of *S. aureus* in the dairy chain is that *S. aureus* producing frequently entertoxin C from acute mastitis were not milked in the bulk tank milk. This observation could explain differences in the normal distribution of monitoring programs regarding the enterotoxigeneity in dairy herds, while it does not explain entirely negative

results in any study. Only the consideration of differences in bacteriological examination and sampling can explain a high variation of results between mastitis samples in comparison to *S. aureus* of raw milk and raw milk products (Mochmann et al., 1976; Stephan et al., 2001; Monrandi et al., 2007; Gonano et al., 2009).

The bacteriological examination of *S. aureus* is still an unresolved problem regarding the investigation of this pathogen in the dairy chain. According to Munch-Petersen (1970) it is not possible to recover *S. aureus* selectively even in mixtures with coagulase negative staphylococci on an agar plate, independent of the used medium (BA, BP, Neave´s or Chapman medium´s). This could be an explanation why the recovery rate of *S. aureus* of milk samples from single cows increases according to Heeschen et al. (1968) up to 40 % when investigating 0.1 ml foremilk samples with a selective enrichment (liquid baird parker) in form of 0.1 ml foremilk on Blood agar. The varying lecithinase and coagulase activity on Baird parker (BP) respectively BP-RPF (Baird-Parker with rabbit plasma fibrinogen supplement) and the weak haemolytic activity without β-haemolysis on BA (Blood agar) is together with the contaminating flora the distinct methodological problem in this context (Zangerl, 1999a; ISO/FDIS 6888 1/1; ISO/FDIS 6888 1/2; IDF-Bulletin, 2006). A major probem is the low reproducibility in bacteriological examinations. BP has according to the ISO/FDIS 6888 1/1 the lowest reproducibility limit for dairy products when measuring the *S. aureus* levels in cheese (0.47-0.66 in $\log_{10}$ cfu/g) with the highest standard deviation (s_R in $\log_{10}$ cfu/g: 3.24-5.61), in contrast to the BP-RPF according to ISO 6888 1/2 (reproducibility limit in $\log_{10}$ cfu/g: of 0.27-0.32, s_R $\log_{10}$ cfu/g: 1.91-2.94).

The suitability of corresponding methods for the detection of *S. aureus* is correlated to ecological niches. The Vogel Johnson enrichment has according to Fox et al. (1992) the significantly highest recovery rate (45.6 %) and Blood agar has the lowest number of positive *S. aureus* samples (12.5 %), when investigating the skin of cows. Fox et al. (1992) observed the opposite when sampling milking liners. BA however has the highest recovery rate (44.4 %) for milking liners while BP respectively liquid Baird parker the lowest number of positive *S. aureus* samples (16.6 %). This is not only an evidence for the high prevalence of isolates from bovine mastitis on the milking equipment in the dairy chain. It further confirms the great importance of haemolysis for the mastitis diagnostic methods, and explains the only use of BA for mastitis milk samples, whereas BP and BP-RPF are the methods of choice for the detection of *S. aureus* in raw milk and raw milk products.

The reason why Loncarevic et al. (2005; Appendix: Table 45) concluded that different plating media and their corresponding elective criteria suppress or favour the isolation of certain *S.*

aureus types was the missing correlation between genotyping and enterotoxigeneity on Blood agar (BA) and Baird parker with rabbit plasma fibrinogen supplement (BP-RPF). Human *S. aureus* have usually no β-haemolysis, coagulate no bovine plasma and are not more or less sensitive to bovine phages in comparison to bovine isolates (Meyer, 1966a; Hajek and Marsalek, 1971). Human *S. aureus* show in agreement with Pulverer (1966), Gedek (1972) and Farah et al. (1988) only α-haemolysis or weak haemolysis similar to coagulase negative staphylococci (CNS). According to Baird-Parker (1962) the positive egg-yolk reaction is an elective criterion for detecting *S. aureus* from human samples with a high prevalence of CNS. Meyer (1966b) and Hajek and Marsalek (1971, Appendix: Table 20) developed with the phenotypic characteristics of coagulase activity, haemolysis, pigmentation and the sensitivity for human and/or bovine phages, a taxonomic classification of *S. aureus* from different hosts. Meyer (1966a) had three distinct questions, which lead him to try and classify *S. aureus* in various differentiation patterns:

1. Are the strains pathogenically relevant?
2. Does the strain play a role in any epidemiological context?
3. Is the strain interesting for sientific questionings?

Not only do the phenotypic characteristics and sensitivity to phages play an important role in the discussion of zoonoses; the importance of phenotypic characteristics for the selection in susceptibility testing of colonies is one of the reasons for the failure of antibiotic therapy for *S. aureus* in hospitals (Goerke et al., 2007). In two quarters of one cow Sommerhäuser et al. (2003) could detect five different *S. aureus* genotypes together with other types at each sampling time, which also explains the problem of susceptibly testing regarding the antibiotic therapy. The important function of elective criteria, such as β-haemolysis on BA or egg-yolk reaction on BP, is very problematic with respect to the fact that single virolent strains are missed something.

The huge importance of phenotypic characteristics in regard to epidemiological questions is confirmed by the similar discrimination index of biotypes (0.842) in relation to phage typing (0.795) or ribotyping (0.863) (Aarestrup et al., 1995a). About 40 years ago in Germany 65 to 70 % of the whole bovine *S. aureus* population belonged to phage group IV (Meyer, 1966b; Bonin and Blobel, 1967; Gedek, 1972). Only in Denmark, Sweden and USA has been phage group IV on the third position of all isolates sampled (Davidson, 1972). Meyer (1966b) created for the bovine strains in phage group IV the *S. aureus variety bovis*, which were pigmented, egg-yolk negative, β-haemolytic, clumping factor positive, coagulated bovine

plasma which were penicillin sensitive. The prevalence of enterotoxin producing strains in the *S. aureus variety bovis* is very low (Hajek and Marsalek, 1973; Mayer, 1975; Mochmann et al., 1976). *S. aureus* is sensitive to phages of phage group III, which are frequently penicillin resistant, egg-yolk positive, coagulate only human plasma and produce no β- on Blood agar (Edwards and Rippon, 1957; Meyer, 1966b; Gedek, 1972) In contrast to the *S. aureus variety bovis*, those S. *aureus* types with a high sensitivity to phages of phage group III often produce enterotoxin A and are the distinct strains which are involved in food poisoning outbreaks (Parker and Lapage, 1957; Mochmann et al., 1976; Kerouanton et. al, 2007).

According to Kapur et al. (1995) and Smith et al. (2005) many mastitis pathogenic bovine *S. aureus* belong to single clones world wide. However, the large number and low standardisation of typing methods are the distinct reasons for difficulties in comparing the results of international epidemiological investigations. This is observed for phage typing as well as for the younger genotyping methods (Aarestrup et al. 1997; Lange et al., 1999). Instead of the existence of an international bovine phage set, the international basic or human set, containing only phages of human origin, is still used for bovine strain typing (Olson et al., 1970; Adesiun, 1995; Aarestrup et al., 1997; Larsen et al., 2000; Vintov et al., 2003a,b). According to Jørgensen (2005) thirteen different genotyping methods are used for typing *S. aureus*. The most frequently genotyping methods for epidemiological investigations of bovine *S. aureus* are Ribotying (in the 1990s), pulsfield electrophoresis (PFGE) and Restriction Fragment Length Poly-morphisme (RFLP). Furthermore, Helm (1992) demonstrated that different *S. aureus* strains could be distinguished by Fourier transformed infrared spectroscopy (FTIR).

According to Hummel et al. (1992, Appendix: Table 22) the great concern of the clumping factor negative *S. aureus* type is the best example for problems of international epidemiological investigations when using different typing methods in the dairy chain. The clumping factor negative bovine strains are frequently enterotoxin C positive, homogeneous with regards to phenotypical characteristics and belong to one genotype or close related subtypes in each investigation, independent of the genotyping method used (Matsunaga et al., 1993; Fitzgerald et al., 2000; Stephan et al., 2001). According to Olson et al. (1970) all enterotoxin C producing bovine strains belong to the phage complex-80 or phage group I. This bovine strain is only sensitive to phages of phage group I and cannot be distinguished from clumping factor positive strains of phage group I using the human phage set, but belong with the international bovine set to phage type 119 (Hummel et al., 1992). Hummel et al.

(1992) unfortunately did not investigate the enterotoxigeneity of their isolates and Olson et al. (1970) used only the international basic set for typing human staphylococci.

The high prevalence of SEC positive bovine strains is the best example for methodological difficulties in genotyping. All *S. aureus* types with *sec* and *tst* belong according to Jørgensen (2005) to different genotypes when using Multilocus Sequence Typing (MLST), the gold standard of modern typing methods or PFGE (Appendix: Figure 25, Table 41). The heterogeneity of MLST-types is however much lower for *sec*/*tst* positive or negative clones in comparison to the number of PT`s when using PFGE. Apparently the high number of mutations or non-conservative regions in the *S. aureus* genome induces in the PFGE-cluster-anaylsis, according to Jørgensen (2005), a high number of PT`s which belong to single subclusters (Appendix: Figure 25). This proves that many mutations in a clone are responsible for the high discriminatory efficiency of PFGE. Possible related strains can have up to six different fragments according to Tenover et al. (1995). This causes not only a problem in interpreting the results of PFGE.

The high number of non-typable bovine strains and lack of reproducibility when using only human phages on the one hand, and the comparability of results with the basic set with human phages of Blair and Williams (1961) on the other hand, were the reasons why Davidson (1961a) created a set with a selection of phages from the human basic set as well as with bovine phages. The combination of phages from two different hosts in combination with different phage dilutions according to Blair and Williams (1961) is the main methodological problem of phage typing considering the results of Meyer (1967) and Hajek, and Howak (1978) (Appendix: Table 19). For example, all bovine strains belonging with the human set of phage group I, I/96 or 96 still show strong reactions at Routine Test Dilution (RTD) for the bovine phage AC1 (Appendix: Table 19). When phage typing is started as usual with the lowest concentration at RTD, the blocked or weak phage reactions, which would be positive at 100×RTD, remain unnoticed (Bonin and Blobel, 1967; Meyer, 1967).

The most penicillin resistant strains are grouped without the use of bovine phages to phage group III or mixed human phage groups, whereas these strains are classified in phage group IV when using the international bovine phage set (Gedek, 1972; Garcia et al., 1980; Swartz et al., 1985). This is of great concern because of the increasing number of isolates in phage group III 40 years ago when only the results of phage patterns with the human phage set were considered (Vintov et al., 2003a,b). The collections with the same representative sampling plan usually have a similar prevalence of penicillin resistant strains. Monitoring programs in Germany revealed a prevalence of 23.8 % and up to 26.5 % penicillin resistance in *S. aureus*

strains (Krabisch et al., 1999; Wallmann et al., 2003; Vintov et al., 2003a). The still frequent use of penicillin according to Tenhagen et al. (2006) is supposed to be involved in the increasing prevalence of penicillin resistant *S. aureus* in dairy herds. *S. aureus* in herds with a high frequency of mastitis have a penicillin resistance of 48 % (Krabisch et al., 1999). Sabolic et al. (1980) found a similar high prevalence of 53 % penicillin resistance for *S. aureus* in herds with a high prevalence of *Str. agalactiae*.

The lack of controlling bovine mastitis in dairy herds leads to the frequent use of drugs. Since treatment of *Str. agalactiae* infected cows with penicillin was much more successfull than segregating or culling this method was encouraged as the main way to eradicate *Str. agalactiae* (Dodd et al., 1977). Since 1945 these programs have not been copied widely, possibly because in 1955 it became clear that other pathogens such as *Str. uberis* or coliforms, which are difficult or impossible to eradicate by antibiotics, were common (Dodd et al., 1977, Dodd, 1980). More control by modifiying various management practices has according to Dodd et al. (1977) been advocated, but progress has been slow. Coordinated experiments based on teat dipping and dry cow therapy with over 60 herds tested in England and USA reduced the infection rate to 70 % in three years; however, no success was revealed for coliforms (Dodd, 1980). Limitations in this approach were apart from the environmental udder pathogens the poor response to therapy of staphylococcal infections. Staphylococcal infection was also becoming important because of the change over to using milking machines (Dodd et al., 1980). The problems in controlling *S. aureus* mastitis was the main reason why the detection limit of *S. aureus* in raw milk products has recently been elevated from <10,000/g to a more practical level of < 100,000/g (Anonymus, 2005).

The high correlation between the prevalence of *S. aureus*-positive cows and the concentration of *S. aureus* specific antibodies to the number of lactations makes this the best indicator with respect to the control problem of this pathogen (Brown et al., 1974). According to Dodd (1980) the dynamics of infection are one of the main reasons why antibiotic therapy alone fails to provide an effective control, since only 29% of the total infections found in the year were eliminated. Dodd concludes that the new infection rate is ultimately the most important factor in controlling udder pathogens. The main factor governing the rate of change in infection levels however is the duration of the infections and not the new infection rate (Dodd, 1980). New *S. aureus* IMI have frequently only elevated somatic cell count (SCC) without clinical symptoms (Zadoks et al., 2001). The consequence is that new chronic shedders stay for a long time unnoticed and can spread this pathogen in the whole herd.

The conventional bacteriological mastitis diagnostics based on international standards resulted in 43.1 % ± 20.8 % false positives, which were mainly due to teat canal infections simulating mastitis (Giesecke and Viljoen, 1974). The distinct problem in mastitis is the high degree of bacteriological negative samples, which varies from 5 to 50 % (Pearson and Mackie, 1979; Kurzhals et al., 1985; Matilla et al., 1985; Zorah et al., 1993). The prevalence of bacteriological positive quarters never rose above 50 % except for quarters with 10.000.000 SCC/ml (Tolle, 1977). The most important problem in this context, together with the methodological difficulties in bacteriological examinations, is the detection of all *S. aureus* shedding cows at the time of sampling. Dinsmore et al. (1991) suggested that repeated sampling might be necessary because many sampling and culturing methods are unable to detect all infected cows at a single survey**.** According to Zecconi et al. (1997) the number of positive foremilk samples in one dairy herd increased at one timepoint of sampling up to 146 % when investigating 10 ml of centrifuged quarter milk samples with a detection limit of 1 CFU / 10 ml, instead of 0.01 ml with a detection limit of 100 CFU / ml.

Furthermore the common contagious *S. aureus* are repeatedly detected in contrast to *S. aureus* with a high similarity to environmental udder pathogens, which seems to circumvent the control procedures (Sommerhäuser et al., 2003). New *S. aureus* types are frequently only detectable after the treatment or culling of cows, which were positive at initial sampling (Sommerhäuser et al., 2003). The great concern for spontaneous infections increases after the reduction of cows with persisting *S. aureus* mastitis (Griffin et al., 1977). The diversity of the *S. aureus* population in one herd is therefore only observed in control programs running over a longer period of time when using the common approach of 0.01 ml foremilk sampling (Davidson, 1963; Larsen et al., 2000; Sommerhäuser et al., 2003).

Vorbach et al. (2006) suggest that lactation partly evolved as an inflammatory response to tissue damage and infection. The high numbers of bacteriological negative foremilk samples indicate the great importance of phagocytosis in subclinical mastitis and the important role of SCC in the udder cistern. Daley et al. (1991) found a positive correlation between the somatic cell count (SCC) and phagocytosis, resulting in an alternating, asynchronic shedding cycle of *S. aureus* counts (CFU/ml) in relation to SCC. These shedding cycles and the variability in the type of positive milk fraction pose a challenge to the effective diagnosis of *S. aureus* infected udders and might result in a major variance in the predictive values of low prevalence situations (IDF-Bulletin, 2006). At periods with lower SCC, cells are still efficient enough to kill intracellular bacteria, and at this time, bacteria can often be non- detectable (Daley et al., 1991). These findings might also explain quantitative and qualitative differences in DNA-

based real-time PCR compared to traditional microbiological methods for the detection of *S. aureus* in raw milk (Hein et al., 2005; Studer et al., 2008). On the other hand, quarters with low somatic cell counts showed no shedding cycle of somatic cells (Studer et al., 2008).

The fast and easy detection of SCC and its high sensitivity in comparison to udder pathogens are probably the three major reasons why the SCC is considered to be the best general indicator for udder health. The automatic counting of SCC made it possible to screen the bulk milk and entire herds with little effort on a standardized high level for a more sensitive diagnostic of mastitis (Tolle et al., 1966). In 1967, somatic cell count was included as a criterion to define mastitis (Hillerton, 1999). Cows with single quarter shedding ≥100,000 SCC/ml are considered as being ill having subclinical mastitis (Hillerton, 1999). The low SCC level for subclinical mastitis is justified because of the low median SCC, except of *Str. agalactiae* with less than 500,000 SCC/ml (Zeidler et al., 1968; Appendix: Table 5). However, according to Seelemann (1964; Appendix: Table 4) 40 % or more of quarters with less than 100,000-500,000 SCC/ml show negative in the CMT-test, which is still the most important screening method in the veterinary mastitis diagnostic (Busato et al., 2000; Godden et al., 2002). Very important in this context is also the use of SCC from quarter milk sampels or udder total milk samples when controlling subclinical mastitis in dairy herds (Brolund, 1985; Hillerton, 1999).

Nine further factors with except of SCC testing methodology affect according to Reneau (1986) the SCC: The infection status, age of cow, stage of lactation, season, stress (isolation, weather or thermal stress), diurnal variations (depeding on the sampling time), day- to-day-variation, management factors and breed differences. An important observation is that the somatic cell count (Smith and Schulze, 1967; Vries, 1976; Bruckmayer, et al. 2004) and the shedding rate of udder bacteria (Murphy, 1943; Vries, 1976; Sears et al., 1991; Mavrogianni et al., 2006) change during milking. Vries (1976) found correlations between the bacteria species, SCC shedding rate and bacteria count during milking (Appendix: Table 15). Each udder quarter of a cow has additionally a typical profile regarding the shedding rates of pathogens and SCC (Huber, 1970; Vries, 1976). The results of Abo-Elnaga and Kandler (1965) and Vries (1976) show that the *S. aureus* count, respectively bacteria count, can be the highest in the main milk, in comparison to the foremilk and strippings. This is very important for cows with subclinical mastitis shedding, where *S. aureus* remains unnoticed in the main milk and in the bulk tank milk.

The *S. aureus* prevalence in dairy herds, the number of lactations and the lactation month of the cows are distinct influence factors in *S. aureus* monitoring programs in addition to the

sampling procedure, the length of the control program and the pathogenesis of mastitis (Blackburn et al., 1968; Zeidler et al., 1969; Barkema et al., 1998; Zadoks et al., 2001; Milchprüfring Bayern, 2001; Appendix: Figure 2-10). Herd size, age distribution of the cows and the number of lactations are one of the important and heterogeneous parameters when comparing data from different countries, especially in the past when cows were in average usually older. The results of Roberson et al. (1994a,b) show the great concern over the number of lactations but also over the necessity for distinguishing herds with low (LCPS) and herds with a high *S. aureus* prevalence (HCPS) (Appendix: Table 11, 12). Roberson et al. (1994a) however do not differentiate between cows with two and more lactations, and the relation of the lactation average between HCPS and LCPS herds is unknown as well the history of the herds.

The number of lactations is not only very important when discussing the SCC-level of bacteriological healthy quarters and the SCC-level of *S. aureus* positive cows (Blackburn et al., 1968), but the average of number of lactations furthermore determines the *S. aureus* prevalence and history of clinical mastitis with antibiotic treatments (Barkema et al., 1998). Rataja-Schulz et al. (2004) revealed for example a penicillin resistance of 26 % in cows at the first lactation, whereas staphylococci from multiparous cows with two or more lactations had a penicillin resistance of 39 %. A penicillin resistance of about 40 % is according to Sobiraj et al. (1997) in agreement with each of the 63 collections of veterinary practices from all different federal districts in Germany, as well as with the results of Tenhagen et al. (2006) investigating herds with high BMSCC mean of 372,000 SCC /ml.

In summary, the main deficiencies when investigating the biodiversity of *S. aureus* in the dairy chain are the interdependences between traditional election criteria, methodical difficulties in phage typing and the comparability and discriminatory efficiency of different typing methods. A further problem is the shift of the bovine *S. aureus* population during the past 40 years and its association to penicillin. The determination of *S. aureus* prevalence in dairy herds and biodiversity of *S. aureus* at one timepoint of sampling is another important point with respect to difficulties in controlling *S. aureus* in dairy herds. These aspects have to be discussed together with the role of the somatic cell count in bovine mastitis and shedding patterns of *S. aureus* and their function in the pathogenesis and dynamic of bovine mastitis. The present situation can only be understood when the literature and knowledge from the past is included in the discussion.

2 Problem definition and formulation of objectives

The central questions of this thesis are the bovine *S. aureus* population and the control of *S. aureus* in bovine mastitis influencing the biodiversity of *S. aureus* in raw milk. Culturing of *S. aureus* strains, the sampling procedure as well the SCC, as an election criterion for taking samples are crucial sources of error for all questions in this connection. The interdependence of all questions is the reason why each individual problem has to be discussed in a logical order. Therefore the problem definition has been subdivided as following:

1. The biodiversity of *S. aureus* from bovine mastitis

    1.1 The role of traditional election criteria for population analysis in bovine milk

    1.2 Methodological difficulties in phage typing of bovine *Staphylococcus aureus*

    1.3 The bovine *Staphylococcus aureus* population over a period of 40 years and its association to penicillin

    1.4 The comparability and discriminatory efficiency of different common typing methods in the dairy chain

2. Methodological difficulties in controlling the *Staphylococcus aureus* mastitis in dairy herds and effects on population analysis in the dairy chain

    2.1 The determination of *Staphylococcus aureus* prevalence in dairy herds

    2.2 The biodiversity of *Staphylococcus aureus* at the timepoint of sampling

    2.3 The role of the somatic cell count in bovine mastitis

    2.4 Shedding patterns of *Staphylococcus aureus* during milking and their function in the pathogenesis of bovine mastitis

    2.5 The dynamic of bovine mastitis in dairy herds

The determination of the biodiversity of *S. aureus* from bovine mastitis and methodological difficulties in this context are the first questions which are discussed. Only by analysing the influencing factors from the culturing of strains and subsequent investigations of common

typing methods provide a comprehensive picture about the *S. aureus* population in bovine mastitis, raw milk and raw milk products.

First of all a critical examination is necessary considering all advantages and disadvantages of BA, BP and BP-RPF in relation to the *S. aureus* populations sampled. The evidence of possible correlations can not only explain variations regarding the prevalence of single *S. aureus* types between monitoring programs in mastitis and raw milk or raw milk products.

Very important in this context is the correlation between the election criteria on BA, BP and BP-RPF and penicillin resistant or enterotoxin producing isolates. The findings provide the information about possibly necessary methodological modifications, which will help to avoid false negative results or wrong conclusions regarding the investigations of this pathogen in the dairy chain.

All crucial and the most certain information about the *S. aureus* poulation in the 1960´s were based on phage typing. Furthermore, phage typing is the single method when typing hundreds of strains in scale screenings for mastitis control programs with minimal effort (Vintov et al., 2003b). Understanding of the methodological difficulties is therefore, together with the election criteria, one of the most important aspects concerning the biodiversity of bovine *S. aureus*. The discussion about the prevalence and validity of the most relevant phage types of bovine mastitis is together with the distinctive characteristics (penicillin resistance, haemolsyis, egg-yolk and clumping factor reaction) the base for a realistic estimation regarding the shift in the *S. aureus* population over the past 40 years.

Subsequently, *S. aureus* collections from bovine mastitis of the most recent years in Germany are compared to each other with special regard to the biodiversity during the 1960`s. The 1960`s was not only the period when international methods regarding the bacteriological examinations and phage typing were established, but it was as well the period shortly before the systematical sanitation programs of subclinical mastitis based on the BMSCC began. For getting a better and comprehensive picture about the *S. aureus* population 40 years later the following three strain collections were investigated:

a. strains from national monitoring program,

b. strains from acute mastitis or cows with high SCC ,

c. strains from herds with a high occurence of mastitis, guaranteed high biodiversity and which showed the suitability to certain international phages and phage sets.

The low standardization in epidemiological investigations is the reason why the sum of phenotypical and physiological characteristics, enterotoxigeneity, antibiotic resistance and different typing methods (phage typing considering different phage sets, FTIR-cluster analysis and genotyping) has to be considered in epidemiological investigations. Only when considering all the relevant characteristics it is possible to obtain certain information about the suitability and efficiency of the correponding established typing methods. The FTIR-cluster analysis is very useful in this context. It is the only method which takes the chemical components of the entire bacteria cells into account for the calculations of cluster-analysis.

Deficiency in control of bovine mastitis is the second thematic area regarding the biodiversity of *S. aureus* in bovine milk. Deficiency in control of bovine mastitis is the main reason for the limited success rate regarding the contamination of raw milk with *S. aureus*. Methodological difficulties of susceptibility testing are also responsible for the increasing antibiotic resistance which is occuring mainly in dairy herds with a high rate of drug use. Only the investigation of the *S. aureus* biodiversity in farms with herd management that want to avoid *S. aureus* contaminating raw milk, permite information about the possible shift in the *S. aureus* population through sanitation programs. In this context only epidemiological studies show the lack of efficient measurements, which would be necessary for the control of this pathogen in dairy herds.

A study designed to include twelve farms producing raw milk cheese and the consideration of all relevant influencing factors was choosen for a realistic exposure regarding the questions in the control of *S. aureus* in the dairy chain. The twelve selected herds were grouped into HCPS (high prevalence of coagulase-positive *S. aureus*) and LCPS (low prevalence of coagulase-positive *S. aureus*) farms according to the classification of Roberson et al. (1994a). Six herds had a high *S. aureus* prevalence. The remaining six herds belonged to LCPS farms. All important herd management data and results of bacteriological examinations regarding all raw milk and raw milk products were well documented over a period of at least five years.

The definition of *S. aureus* prevalence in dairy herds with consideration to the sampling procedure and with special regards to the age of the cows is the first aspect when investigating methodological difficulties regarding the detection of this pathogen. Foremilk, main milk and strippings samples were finally used for the detection of as many *S. aureus* shedders as possible at the timepoint of sampling. The bacteriological results of the common aproach investigating only 0.01 ml foremilk during the first three sampling rounds were compared to results of a modified sampling procedure including foremilk, main milk and strippings of each cow during the last sampling round. The susceptibility testing and FTIR-cluster analysis was

performed for as many different *S. aureus* phenotyes as possible in each cow from milking (foremilk, main milk and strippings), raw milk and raw milk cheese. A high number of these strains were later on phage typed.

The SCC-values, shedding patterns of *S. aureus* in relation to the pathogenesis of mastitis and dynamic of subclinical mastitis is the second thematic area when discussing the deficiencies in the control of *S. aureus* in dairy herds. The role of the SCC has to be discussed in more detail because of its important function in the diagnosis of bovine mastitis. Subsequently possible correlations beetween shedding patterns of *S. aureus*, SCC-values and the pathogensis of persistent mastitis were investigated. Existing correlations in this context are essential in order to understand the dynamics of bovine mastitis with respect to the prevalence and diversity of *S. aureus* in dairy herds.

Finally the role of *S. aureus* population in bovine masitits is discussed in the overall context considering all relevant influencing factors and changes in the milk production over the past 60 years. The consideration of all important aspects in the concluding general discussion can impart the development and significance of *S. aureus* in the dairy chain.

3 Materials and Methods

3.1 Material (bacterial isolates)

Almost 400 human isolates and about 1400 of bovine and 100 of carpine or ovine origin were part of this study. Only the *S. aureus* collections from special interest are shown in greater detail. The first collection (collection A: Germany) with 213 bovine *S. aureus* isolates was part of a national resistance monitoring program conducted in 2002 (Wallmann et al., 2003). 72 *S. aureus* isolates (collection B: mastitis) from randomised sampling in 72 herds were obtained by a laboratory specialized in udder health in the northeast of Germany in summer 2002. These *S. aureus* are isolates from herds with high somatic cell count (SCC) of about 400.000 SCC/ml in the bulk milk or acute mastitis. The bovine *S. aureus* of the third collection (collection C: Resistant) result from a resistance monitoring program conducted in Bavaria and including 13 multi resistant isolates with penicillin resistance and 55 penicillin resistant isolates from problem herds with a high frequent use of drugs (Krabisch et al., 1999). 28 penicillin sensitive isolates from the herds with a high frequency of mastitis were also phage typed. However in accordance to Gedek and Kopp (1967) they were not directly incorporated into the comparison of collections A and B to show the sensitivity of phages for antibiotic resistant isolates only.

547 *S. aureus* isolates from 10 of the 12 farms were part of the investigations regarding the study-set up for the control in the dairy chain. 406 were isolated in the foremilk, strippings or during milking (the main milk) of single cows. 141 isolates were collected from bulk milk and cheese samples over a period of two years.

3.2 Determination of egg-yolk reaction and haemolysis patterns

The frozen strains which were grown in brain heart bouillon were also streaked out on BP and BA containing 5 % sheep blood agar (Oxoid, Ltd, Hampshire, UK). The egg-yolk reaction on Baird parker was determinated after 24 h and 48 h (ISO 6888 1/1: 1999; Oxoid, Ltd, Hampshire, UK).

The cultures were according to Grün (1968) streaked with the eye in one line on BP and incubated at 37°C. Colony size and telluride reduction were also detected. Haemolysis was determinated according to Skalka et al. (1979). It was distinguished between isolates with no

(k), weak (w), α, β or αβ-haemolysis. The orange pigment of strains on BA was assessed after three days in light at room temperature in order to guarantee the production of pigments.

3.3 Determination of clumping factor and coagulase activity in the tube and BP-RPF

For the clumping factor, the coagulase tube and also for the BP-RPF agar the same rabbit plasma samples were used (Oxoid, Ltd, Hampshire, UK). The determination of the clumping factor was investigated according to Witte et al. (1988). The test in the coagulase tube was performed according to ISO/FDIS 6888-3. Fresh colonies were incubated in brain heart bouillon overnight at 37 °C. The next day 0.1 ml of the brain heart bouillon was added to 0.3 ml rabbit plasma and incubated at 37 °C. The strength of reaction was noted after 1 h, 2 h, 4 h, 6 h and 24 h. The coagulase tube test performed according to ISO/FDIS 6888-3. was considered as positive (+) when the cultures yielded at least 3+ coagulase reactions with a large organized clot (Sperber and Tatini, 1975). The coagulase reactions with only small organized (2+) or unorganized (1+) clots in the tube were considered to be weak positive (w). The diameter of turbidity zones can be used according to Blobel und Schließer (1979) for the detection of the coagualse activity on BP-RPF (Oxoid, Ltd, Hampshire, UK). The isolates of the same brain-heart bouillon were streaked with the eye in one line on BP-RPF and incubated at 37 °C. The coagulase reaction on BP-RPF was determinated after 24 h and 48 h (ISO/FDIS 6888 1/2).

3.4 Antibiotic resistance testing

Antimicrobial drug susceptibility testing of the isolates was performed on Müller-Hinton agar (Oxoid, Ltd, Hampshire, UK) by the disk diffusion method in accordance with Clinical Laboratory Standards Institute guidelines (CLSI, 2005). The antimicrobial agents (Oxoid, Ltd, Hampshire, UK) tested included G (10 U/disk), methillicin (5µg/disk), cefoxitin (30 µg/disk), erythromycin (15 µg/disk), lincomycin (15 µg/disk), tetracyclin (30 µg/disk), sulfamethoxazole/ trimethoprim (23.75/1.25 µg/disk) and streptomycin (25 µg/disk). *S. aureus* ATCC 25923 was used as control strain in every test run.

3.5 Detection of toxins

The ability to produce enterotoxins was tested with the System Vidas Staph enterotoxin II SET2 (ordering number: REF 30705, bioMerieux, Nürtingen, Germany). For the detection of

enterotoxins the same brain heart boullion was used. Strains were tested for the presence of *sea, seb, sec, seg, seh, sei, sej* and *tst* by PCR according to Monday and Bohach (1999), Mehrotra et al. (2000) and MC Lauchlin et al. (2000). Primers were obtained from MWG Biotech (Ebersberg, Germany). The sequences of the oligonucleotide primers used for the specific amplification of enterotoxines and the TST-1 gene are summarized in Table 1, alongside with the predicted PCR product sizes. Primers were combined to obtain two different sets of multiplex PCRs: one set containing primers for *sea, seb, sec* and *sed* (PCR1) and the otherone for *seg, seh, sei,* and *sej* (PCR2). A PCR for the detection of *tst* was performed separately (PCR 3). DNA was amplified by 30 cycles of 95 °C for 60 s, 55 °C for 60 s and 72 °C for 60 s with a final extension at 72 °C for 10 min. The amplification was performed in a GeneAmp PCR System 9700 (Perkin-Elmer, Wellesley, MA) using Platinum Taq DNA polymerase (Invitrogen, Lofer, Austria). The PCR products were resolved by agarose gel electrophoresis and visualized by UV transillumination.

Table 1 Oligonucleotide primers for the amplification of genes encoding staphylococcal enterotoxins

Gene	Primer	Primer sequence (5´ to 3´)	Amplification size(bp)	Reference
sea	GSEAR-1 GSEAR-2	GGT TAT CAA TGT GCG GGT GG CGG CAC TTT TTT CTC TTC GG	102	Mehrotra et al. (2000)
seb	GSEBR-1 GSEBR-2	GTA TGG TGG TGT AAC TGA GC CCA AAT AGT GAC GAG TTA GG	164	Mehrotra et al. (2000)
sec	GSECR-1 GSECR-2	AGA TGA AGT AGT TGA TGT GTA TGG AGA TGA AGT AGT TGA TGT GTA TGG	451	Mehrotra et al. (2000)
sed	GSEDR-1 GSEDR-2	CCA ATA ATA GGA GAA AAT AAA AG ATT GGT ATT TTT TTT CGT TC	278	Mehrotra et al. (2000)
seg	GSEGR-1 GSEGR-2	TGC TAT CGA CAC ACT ACA ACC CCA GAT TCA AAT GCA GAA CC	704	MCLauchlin et al. (2000)
seh	GSEHR-1 GSEHR-2	CGA AAG CAG AAG ATT TAC ACG CCA TAT TCT TTG CCT TTA CCA G	495	MCLauchlin et al. (2000)
sei	GSEIR-1 GSEIR-2	GAC AAC AAA ACT GTC GAA ACT G CCA TAT TCT TTG CCT TTA CCA G	630	MCLauchlin et al. (2000)
sej	GSEJR-1 GSEJR-2	CAT CAG AAC TGT TGT TCC GCT AG CTG AAT TTT ACC ATC AAA GGT AC	142	Monday and Bohach (1999)
tst	GTSSTR-1 GTSSTR-2	ACC CCT GTT CCC TTA TCA TC TTT TCA GTA TTT GTA ACG CC	326	Mehrotra et al. (2000)

3.6 Phage typing

Phage typing was performed at 100×RTD according to Witte (2004). A reaction was considered to be positive when the lysis resulted in ≥ 20 plaques (20-50 plaques + are usually separately noted). At 100×RTD is it possible to distinguish between phage reactions with > 1000 plaques (semiconfluent (SCL) and confluent lysis (CL)) and inhibited lysis with ≥ 50 plaques (Bonin and Blobel, 1967; Witte, 2004). According to Degre (1967) the reproducibility of phage typing is at least as good at 100×RTD as it is at RTD and 1000×RTD. The differentiation of phage types were implemented according to Blair and Williams (1961). The strains were subdivided into the different phage groups according to Davidson (1972; Appendix: Table 34) and Parker (1983). The sensitivity of isolates to phages belonging to the same phage groups were summarized in single “phage profiles”. One phage profile can contain one ore more different different phage types. Even suspensions of 30 phages were placed on one agar plate. This procedure ensures first of all the same conditions for phage typing of all phages and second, it is easier to assess the strength of phage reactions on one agar plate. All phage suspensions came from the Robert Koch institute and all phages were were cheked for the correct RTD. The set of 30 international phages includes all of the important bovine and human phages (Table 2).

Table 2 Origin of phages that belong to international phage sets (BPS= international bovine phage set, IPS= international basic phage set, HPS= international human phage set)

Phage numbers	Reference	Source of Phage	Phage group	Phage sets
29, 52A	Blair and Williams (1961)	human	I	IPS +HPS+BPS
52, 79, 80	Blair and Williams (1961)	human		IPS + HPS
3A	Blair and Williams (1961)	human	II	IPS + HPS+ BPS
55	Blair and Williams (1961)	human		IPS + HPS
116	Nakagawa (1960)	bovine		BPS
6, 42 E, 47, 53, 75	Blair and Williams (1961)	human	III	IPS + HPS+ BPS
54	Blair and Williams (1961)	human		IPS + HPS
84[a]	Jevons et al. (1966)	human		HPS + BPS
85[a]	Jevons et al. (1966)	human		HPS
42D[b]	Blair and Williams (1961)	human	IV	IPS + BPS
102, 107	Davidson (1961)	bovine		BPS
117	Smith (1948)	bovine		BPS
108, 111	Davidson (1961)	bovine		additonal phages
96[a]	Blouse et. al (1972)	human	V	HPS
81[c]	Blair and Williams (1961)	human	not alloted miscellaneous phages (M)	IPS + HPS
187[b]	Blair and Williams (1961)	human		IPS
95[a]	Blouse et. al (1972)	human		HPS
812	Meyer (1966a)	human		additional phage
78	Blair and Williams (1961)	human		BPS
118	Seto et al. (1956)	bovine		BPS
119	Seto et al. (1956)	bovine		BPS

[a] This phage is part of the HPS since 1974.

[b] This phage was part of the international phage set since 1974.

[c] This phage is part of the IPS since 1961.

22 phages are of human origin (phage group I: 29, 52, 52A, 79, 80; phage group II: 3A, 55; phage group III: 6, 42E, 47, 53, 54, 75, 84, 85; phage group V: 96; miscellaneous phages (M): 81, 95, 187, 812, 78; phage group IV: 42D). Only the human phages 3C, 71, 77, 83A and 94 with a low relevancy for typing bovine strains in accordance to Davidon (1961) and Vintov et al. (2003b) are missing. The elimination of phages 187 and 42D and the addition of phage 84, 85, 95, 94 and 96 is the difference between the old basic set (IPS) and the new human phage

set (HPS) (Subcommittee on Phage Typing, 1975). Eight phages are of bovine origin (phage group II: 116; phage group IV: 102, 107, 117, 108, 111; miscellaneous phages (M): 118, 119). All phages of the international bovine phage set were used (phage group I: 29, 52A; phage group II: 3A, 116; phage group III: 6, 42E, 47, 53, 75, 84; phage group IV: 102, 107, 117; miscellaneous phages (M): 78, 118, 119). The human phage 812 and bovine phages 108 and 111 were not part of international phage sets.

3.7 FTIR-Spectroscopy

3.7.1 Sample preparation

Sample preparation was performed according to the methods of Kümmerle et al. (1998). Cells were incubated at 30 °C for 24 h in tryptone soya agar (15 g tryptone, 5 g soya peptone, 5 g sodium chloride and 15 g agar per litre; Oxoid).

3.7.2 FTIR spectroscopy

All spectra were recorded and evaluated according to the methods of Kümmerle et al. (1998) using an HTS-XT Tensor 27 FT-IR spectrophotometer (Bruker). The quality and reproducibility of spectra were tested with help of using the digital quality test of Bruker (2001) and with help of cluster analyses (< 0,4 spectral distance by avarge linkage).

3.7.3 Selection of spectral windows on cluster analysis

The combination of spectral windows was performed according to Kümmerle et al. (1998). The best discrimination results were obtained with spectral windows (W) W1 2800-3000 (groups of fatty acids), W2 1200-1350 (fatty acids; protein; polysaccharide), and W5 700-900 (called fingerprint region). Alle weighting factors were 1 and repro-levels 30.

3.7.4 Cluster analysis

For the cluster analysis the average linkage algorithm was used**.** Usually one strain has a reproducibilty of a minimum of 0.4 spectral distance by using average linkage algorithm. The consistency of the clusters at average linkage was controlled with clusters using Wards algorithm. All isolates were first of all measured in triplicates. If subsequently samples neither were nor ordered in the same position of the clusters, then corresponding samples were removed from the analysis. While this was the most labour intensive part it proved to be at the

time the best method for typing different isolates using FTIR-Cluster analysis (Helm et al., 1992).

3.8 Macrorestriction analysis by PFGE

Macrorestriction analysis was performed following the protocol of the Robert Koch Institute, using the restriction enzyme *SmaI* (New England Biolaps, Beverly, MA) followed by pulse field gel electrophoresis (PFGE). *S. aureus* isolates were checked for purity and grown aerobically in brain heart infusion broth at 37 °C for 18 h to 24 h. The cells were harvested and resuspended in Pett IV (PIV) buffer (1 M NACL, 25 mM Tris-HCL, pH 8.0). The suspension was mixed with equal volume of 1.2% low melting point SeaKem Gold agarose (Cambrex Bio Sience, Rockland, ME). Plugs were incubated overnight at 37 °C in EC lysis buffer (6 mM Tris-HCL, 1 M NaCl, 0.1 M EDTA, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine) with 10 mg/ml RNAse, 10 mg/ml lysozyme and 5 mg/ml lysostaphin. The lysis buffer was then removed and each plug was incubated overnight at 50 °C in 1 ml of ESP buffer (0.5 M EDTA, 1% lauryl sarcosine, 1 mg/ml proteinase K). On the following day, the plugs were washed four times in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) at 54 °C for 30 to 60 min. The plugs were then stored in TE buffer at 4 °C.

Plugs were digested with 40 U *SmaI* (New England BioLaps) according to the manufacturer's instructions. Digested DNA was separated in a 1% SeaKem Gold agarose gel (High melting, Cambrex Bio Science) with a CHEF DR III (Bio-Rad Laboratories, Hercules, CA) pulsed-field electrophoresis system in 0.5 M Tris-borate-EDTA (1 M Tris, 0.01 M EDTA, 1 M boric acid). Running parameters were as following: 5 s to 15 s ramping for 7 h followed by a 15 s to 60 s ramping for 19 h; 6 V/cm; 120° angle; 14 °C.

Gels were stained with ethidium bromide (0.5 µg/ml) for 30 min and destained for 30 min to 60 min in fresh distilled water. The patterns were visualized using a UV transilluminator and then photographed. *Salmonella enterica subsp. enterica* serotype Braenderup H9812 digested with XbaI was used as molecular size marker.

DNA restriction bands were analyzed by using the Molecular Analyst Fingerprinting II software package, version 3.0 (Bio-Rad). Similarity coefficients were calculated and dendrograms were constructed using the Dice coefficient and the unweighted pair group with arithmetic averages with an optimization value of 1% band tolerance and a position tolerance of 1% respectively.

3.9 Selection of 12 herds for investigation regarding the control of *S. aureus* in the dairy chain

In the years 1996 to 2003 tank milk samples and raw milk products were submitted by raw milk processors located in southern Germany (federal districts of Bavaria, Baden-Württemberg, Hesse and North Rhine-Westphalia) to our laboratory (Dr. Oskar Farny Institut, 88231 Wangen, Germany) for bacteriological investigations of *S. aureus*. Information about the parameters accompanying the prevalence of *S. aureus* in the corresponding herds was collected over six years. Half of the number of farms sent in samples that exceeded the limit of 100 S. *aureus*/ml bulk tank milk or 1,000 *S. aureus*/g in raw milk cheese. In the farms whose samples exceeded the limits, the high *S. aureus* rate was controlled by consecutive sampling from each cow and dispatching the shedders.

In order to investigate influencing factors with respect to the shedding patterns, twelve herds out of those with a different *S. aureus* prevalence were selected. These were grouped in HCPS (high prevalence of coagulase-positive staphylococci: >10 % positive foremilk samples) and LCPS (low prevalence of coagulase-positive staphylococci: <10 % positive foremilk samples) herds (Roberson et al., 1994a). 105 cows belonged to the group of LCPS herds with 28 % in the first, 18 % in the second and 15% of cows in the third lactation and 39 % of cows had >3 lactations. 164 cows belonged to the group of HCPS herds with 26 % in the first, 24 % in second, 16% of cows in the third lactation and 34 % of cows had >3 lactations. Thus, the herds in both groups showed similar herd sizes (10 to 35 cows) and number of lactations during the sampling period. No differences could be detected in the herd specific parameters, such as distribution regarding breed of cows (Simmental, Holstein Frisian, Brown Swiss and regional ancient breeds), type of animal husbandry (pasture and housing), herd management, feeding and milking devices.

In the first sampling rounds only the foremilk on the udder quarter-level of all lactating cows was sampled for the presence of *S. aureus*. In the last sampling round in each herd different milk fractions (foremilk, main milk and strippings) were sampled. In the last sampling round, between March and October 2004 the somatic cell counts (SCC) of the main milk were recorded as well. All selected farms participated in a mastitis control program in 2004 and only cows that were considered as clinically healthy were sampled.

3.10 Defintion of IMI

A cow was considered to have an intramammary infection (IMI) when *S. aureus* was detectable after three consecutive sampling intervals in ≥ 100 cfu/ml foremilk of one udder quarter and/or ≥ 25 cfu/ml foremilk in composite quarter samples (Zadoks et al., 2001; Roberson et al., 1994a). The definition according to Zadoks et al. (2001) was used since most herds were screened by routine udder health service in a mastitis control program during our study, who investigated only 0.01 ml foremilk on the quarter level.

3.11 Sampling of the main milk

After the first sampling round in September 2003, it turned out that sampling the foremilk as usual from all lactating cows had not been sufficient to detect all *S. aureus* shedding cows contaminating the bulk milk. Sampling of the main milk was considered essential to obtain a more comprehensive picture. The main milk was collected using a Tru-Test auto sampler (Tru-Test Ltd., Auckland, New Zealand) or milking pail in case of small herds. The milking equipment, milking pail or Tru-Test auto sampler was cleaned with a CIP-system prior to sampling. In order to avoid *S. aureus* cross-contamination between individual cows, the entire milking equipment and Tru-Test auto sampler were flushed with 3-5 liters of drinking water at 70 °C according to Davidson and Slavin (1958) and Davidson (1963). Cows identified as high shedders within the mastitis control program were milked after the others. Sampling procedures, milking pail and the Tru-test auto sampler agreed in terms of milk yield of each *S. aureus* positive cow and the *S. aureus* count in the bulk milk. In a second and third sampling round which included the sampling of the main milk the bacteriological examinations for *S. aureus* revealed no contamination in 0.1 ml main milk samples when using the sampling procedures described above.

3.12 Sampling Procedure

Between March and October 2004 all lactating cows of the 12 farms (A – L) were sampled a fourth time for the presence of *S. aureus* in different milk fractions (foremilk, main milk and strippings). In addition, somatic cell counts (SCC) of the main milk as well as the *S. aureus* count in the bulk milk and in raw milk cheeses were recorded as above. All selected farms participated in a mastitis control program in 2004 and only those cows which were considered as clinically healthy were sampled. Their udders were cleaned with sterile paper towel before milking and the first milk beam of each quarter was discarded. Foremilk was collected within

one minute in order to guarantee a pure cisternal milk sample (Bruckmayer et al., 2004). In order to obtain a representative main milk sample 40 ml milk were taken. The milking equipment was flushed with hot water after milking each cow as described above, in order to avoid contamination via the milking machine. After machine milking the strippings were collected from the residual milk. In order to obtain a representative sample of the foremilk and the strippings 10 ml milk from each quarter were pooled.

3.13 Isolation of *S. aureus*

The isolation of single colonies on blood agar without plating as well the egg-yolk reaction on Baird parker, according to ISO/FDIS 6888 1/1, revealed to be a further critical step in accordance to IDF-Bulletin (2006), when monitoring during September 2003. Hence the isolation of *S. aureus* was then performed according to ISO/FDIS 6888 1/1 using Baird Parker medium without telluride (Oxoid, Ltd, Hampshire, UK) but with 5 % of sheep blood (Oxoid, Ltd, Hampshire, UK) and additionally 5 µg/ml sulfamethazine (Sigma-Aldrich, St. Louis, MO, USA) in order to suppress the growth of *Proteus*. This approach allowed the assessment of haemolysis, clumping factor and pigmentation on the agar plate using Baird parker medium.

S. aureus was isolated by plating 100 µl of each sample plus 100 µl of a tenfold dilution on the modified Baird Parker plates. The plates were incubated at 42 °C for 24 h. The *S. aureus* counts (cfu/ml) were calculated as the arithmetic mean of both counts. Using 100 µl instead of just 10 µl as usual, the dilution effect of non shedding quarters for composite milk samples is not relevant. Furthermore, the detection limit is assumed to be 40 cfu/ml instead of 100 cfu/ml for one shedding quarter, considering according to Godden et al. (2002) the dilution effect of milk from uninfected quarters. Presumptive *S. aureus* was detected by the spatula method. This method ensures that colonies are able to grow in regular distances from each other and that the individual phenotypic characteristics of each colony can be assessed. These typical colonies allowed the determination of colony morphology and the selection of single colonies for detection of the clumping factor. In order to obtain hot-cold lysis, the plates were incubated at 6 °C overnight and subsequently kept at room temperature for five days to control growth and pigment production. The identification of the bacteria was performed according to Kurzweil and Busse (1973). The clumping factor was determined on four morphologically indistinguishable colonies which were grouped to the genus Micrococcaceae by using rabbit plasma fibrinogen. Clumping factor negative colonies corresponding to the

Micrococcaceae were confirmed with the combined protein A-agglutination test. The isolates were all tested in a coagulase tube.

3.14 Determination of the milk yield, SCC, and fat, protein and urea contents

These routine investigations are part of milk recording schemes. The Tru-Test auto-sampler is used for the determination of milk yield of each cow. Somatic cell counts were carried out in the main milk of each cow using the Fossomatic 5000 (Foss Electric). The fat, protein, urea content were determined by FTIR technology (MilkoScan MS 6000 FT, FOSS). The tests were performed at Milchprüfring Baden-Württemberg e.V. (Association for Raw Milk testing in the Federal State of Baden-Wuerttemberg, Germany).

3.15 Statistical Analysis

Cows were grouped according to the number and type of *S. aureus* positive milk fractions. Outliers identified by Nalimov's method were removed prior to statistical calculation (Kaiser and Gottschalk, 1972). The distribution of SCC and CFU data within the groups was investigated using the Kolmogorov-Smirnov test for best fit and the SPSS 13.0 statistical software package (SPSS Inc., Chicago, IL, USA). The quality- Test of the spetra (*t*-test) comparison between groups and calculation of correlations between data sets were performed using the same software package.

4 Results and Discussion

4.1 Biodiversity of *S. aureus* isolates in bovine mastitis

4.1.1 Role of traditional elective criteria in epidemiological analysis

Election criteria are the first major source of errors during the process of culturing isolates for population analysis. They are the most important influence factor for the failure or success in the detection of *S. aureus* strains during bacteriological investigations. The development of media or new isolation methods is frequently connected with different election criteria respectively phenotypic characteristics. The preferences of different election criteria have to be discussed in more detail regarding epidemiological studies because of the performance of different methods especially in the dairy chain. Furthermore, the shift which has occurred in the bovine *S. aureus*-population over the past 40 years, or which was observed after sanitation programs, could possibly change the significance of traditional phenotypic characteristics (haemolysis, egg-yolk reaction, coagulase and clumping factor activity). These changes could be very important because of the close correlation in many cases between important phenotypes and certain phage types, respectively genotypes.

4.1.1.1 Problem and Goal

Three different media are used according to international dairy federation for the detection of *S. aureus* in the dairy chain with different elective criteria. The traditional elective criterion β-haemolysis and pigmentation is according to ISO 2006/408 the elective criterion on Blood agar (BA). The BA however is only used on the cow level for mastitis diagnostic. The egg - yolk reaction on Baird parker (BP) or the coagulase reaction on Baird parker with rabbit plasma fibrinogen supplement (BP-RPF) are the elective criteria according ISO 6888 1/1 and ISO 6888 1/2 for raw milk and raw milk products. The huge importance of these phenotypic characteristics on epidemiological questionings is confirmed through the similar discrimination index of biotypes (0.842) in relation to phage typing (0.795) or ribotyping (0.863) (Aarestrup et al., 1995). The importance of phenotypic characteristics for the selection in susceptibility testing of colonies was recently reported as one of possibilities in the failure of antibiotic therapy for *S. aureus* in the hospitals (Goerke et al., 2007). The high correlation between typing results of pulsed - field gel electrophoresis and SE - profiles with BA or BP-

RPF according to Loncarevic et al. (2005; Appendix: Table 45) was the reason for their assumption that different plating media suppress or favour different *S. aureus* strains.
The kind of haemolysis or the activity of the egg - yolk and coagulase reaction are not only high correlated to certain *S. aureus* biotypes and in discussion with zoonoses, they are further the crucial criteria during culturing these strains from the corresponing agar - plates. All elective criteria are additionally virulence factors and are suitable together with bovine and human phages to classify pathogenic *S. aureus* from different hosts (Hajek and Marsalek, 1973). The elective criteria are the main reason for the low correlation in bacteriological investigations of *S. aureus* on BP (according to ISO 6888 1/1 BP has a standard deviation (Vs_R) in $\log_{10}$ cfu/g: 3.24- 5.61!). Coagulase negative *S. aureus* are not that problem on BP-RPF in the dairy chain as it is observed for egg - yolk negative strains on BP (IISO 6888 1/2 reproducibility limit in $\log_{10}$ cfu/g: 0.27- 0.32, s_R $\log_{10}$ cfu/g: 1.91- 2.94). The variability of haemolysis is the critical point on BA (ISO 2006/408).
First of all a collection of strains is testet after typing for all typical phenotypic characteristics, which are in use. Only with this procedure it is possible to estimate the suitability and risk of failure of each method in detecting certain *S. aureus* biotypes. Deciding in this context is the correlation between the elective criteria respectively virulence factors, penicillin resistance, enterotoxin genes and an established typing method. Possible differences and influence factors by using different media for the isolation of *S. aureus* are discussed. Special regard was taken to the coagulase activity due to the importance of the identification of *S. aureus* (*S. aureus* is almost used in the literature as synynom for coagulase positive staphylococci.).

4.1.1.2 Results

4.1.1.2.1 Correlation between enterotoxigeneity, penicillin resistance and election criteria

All three collections showed a high percentage of egg-yolk negative and αβ, respectively β-producing isolates (Table 3). Collections A and C showed a similar distribution of egg-yolk positive and β- producing isolates, whereas collections B and C had a significantly higher percentage of isolates with weak or no haemolysis compared to collection A ($p= 0.057$). Only one isolate from collection B showed β-haemolysis. The difference in relation to isolates with αβ-haemolysis was significant ($p= 0.045$). The strains of phage type 119, 78, 96 and 116 in collections A, B and C, considering only strong phage reactions, were almost identical regarding the phenotypic characteristics with exception of single strains.

20 of 28 investigated penicillin sensitive isolates of herds with a high frequency of drug use belonged to the clumping factor negative phage types 119 or 96, and four isolates were only sensitive to phage 78. 71 %, respectively 73 %, of α- producing isolates in collections A and B belong to the phage type 78.

Table 3 Important phenotypes of collection A, B and C and types considering only phage reactions ≥ 1000 plaques (penicillin resistant (Pen+); clumping factor positive (CF+), egg-yolk positive (Lec+); haemolysis patterns: α, β, αβ, weak, no)

	Collections and Phage group IV (%)				Phage types (%)			
Phenotypes	Collection A Germany (n= 213)	Collection B Mastitis (n= 71)	Collection C Resistant (n= 68)	Group IV (n= 55)	96 (n= 37)	116 (n= 14)	78 (n= 52)	119 (n= 92)
Pen+	24	37	(100)	65	8[a]	100	6[b]	0
CF +	69	65	99	96	15	100	95	8
Lez +	53	36	55	44	93	83	9	4
Hemolysis:								
α	10	21	6	9	3	16	87	0
β	28	2	32	37	3	0	0	4
αβ	52	50	39	28	94	32	6	96
weak	6	17	14	19	0	16	7	0
no	4	10	9	7	0	36	0	0

[a] Penicillin resistant isolates were all clumping factor positive

[b] Only the penicillin resistant isolates had weak phage reactions with other phages ≥ 20 plaques

All 72 isolates of collection B reduced telluride, 33 % of the strains had a good visible orange pigmentation, and two strains were white without any pigmentation. However, the pigmentation on blood agar was clearly visible only after two days. Only 19 (26.4 %) of all 72 *S. aureus* showed weak or no haemolysis (Table 4), but their share of penicillin resistant isolates was 57.7 %. The isolates with weak and no haemolysis displayed high similarity for these phenotypic characteristics (r^2=0.98). Only 60 % of the egg-yolk positive isolates showed an, according to ISO 6888 1/1, clearly visible egg-yolk reaction after 24 h. 68 % of the penicillin resistant *S. aureus* were egg-yolk positive.

Six *S. aureus* isolates (8%) were haemolyse negative. The clumping factor negative isolates were all penicillin sensitive. 21 % showed α-haemolysis and 18 % displayed a weak haemolytic activity. 39 % of the isolates were according to PCR toxin positive with the

enterotoxin genes *sec, sed, seg, sei, she* and *sej* and six different profiles. With three exceptions, all isolates with toxine genes belong to the αβ-haemolytic biotypes (Table 4). The most frequent enterotoxins are G and I with 19 %. All nine isolates with enterotoxin C gene reacted also positive in the toxic shock syndrome gene.

Table 4 Correlation between haemolysis, egg-yolk reaction, penicillin resistance and different toxin genes in collection B (sea, seb, sec, sed, seg, sei, seh, sej and tst)

	ALL (n=72)		Kind of haemolysis			
			αβ (n=38)	α (n=15)	Weak (n=13)	No (n=6)
	SE - profile	(%)	(%)	(%)	(%)	(%)
Enterotoxin (*se*) negative	0	61.1	34.2	92.3	100.0	66.7
sec + *seg* + *sei* + *tst*	1	8.3	15.8	0.0	0.0	0.0
sec + *tst*	2	4.2	7.9	0.0	0.0	0.0
sed + *sej*	3	2.8	0.0	6.7	0.0	16.7
seg + *sei*	4	19.4	34.2	0.0	0.0	16.7
sej	5	2.8	5.3	0.0	0.0	0.0
seh	6	1.4	2.6	0.0	0.0	0.0
Egg yolk negative		63.9	71.1	73.3	46.1	33.3
Clumping factor negative		37.5	68.4	6.7	0.0	0.0
Coagulase negative		8.3	15.8	0.0	0.0	0.0
Penicillin resistant		36.1	21.1	13.3	76.9	83.3

4.1.1.2.2 Coagulase activity in the tube in comparison to BP-RPF

Only the use of broth with approximately 10^9 *S. aureus* cfu/ml guaranteed a reproducible coagulase activity on BP-RPF. A different number of cells in different conditions of colony forming units (cfu) induced obviously to a variable diameter of turbidity zones. Investigations of streaking single *S. aureus* colonies on BP-RPF resulted in single coagulase negative colonies from isolates with a weak activity in profiles 1, 2 and 3.

The coagulase activity in the tube is well correlated to the coagulase activity on BP-RPF (Table 5). Four strains showed variable results in the clumping factor and two in the coagulase reaction before and after freezing. The clumping factor positives were usually strong positive during the first two hours. One strain showed the clumping factor negative isolates the initial coagulase reaction in the tube after 4 h. 11 of 15 isolates of profile 2

showed the initial strong coagulase reaction after 24 h and had only ambigious weak results with not rising diameter after 48 h on BP-RPF.

Table 5 Coagulase activity by the coagulase tube and BP-RPF with respect to clumping factor (Clumping factor negative strains are in brackets; w = weak reaction; + = strong reaction; -= negative reaction)

	Coagulase tube (reading time in h)						Baird Parker with rabbit plasma (RPF) (colony diameter in mm)		
Profile	Isolates (n)	1h	2h	4h	6h	24h	Isolates (n)	24h	48h
0	6 (6)	-	-	-	-	-	5 (5)	-	-
1	2 (2)	-	-	-	w	+	2 (2)	9 weak	-
2†	15 (14)	-	-	w	w	+	11 (11)	10-12 weak	10-12 weak
3	1 (1)	-	-	w	+	+	2	11	11- 13
4	5 (3)	-	-	+	+	+	10 (5)	10-11	16-17
5	3	-	w	w	w	+	14 (5)	11-12	18-19
6	4	-	w	+	+	+	3	12-13	16-17
7	12 (1)	w	w	+	+	+	12	13-14	18-19
8	25	+	+	+	+	+	13	13-16	19-22

† Two isolates were 6 month before freezing in the coagulase tube positive, one negative

4.1.1.3 Discussion

The real challenge for bacteriological examinations with respect to *S. aureus* is not the quantification but the isolation of different types in a sample. The presence of different *S. aureus* types according to Loncarevic et al. (2005) in cheese is one possible explanation why some *S. aureus* collected from food poisoning outbreaks were not able to produce enterotoxins in subsequent investigations (Mochmann et al., 1976; Niskanen and Koiranen, 1977). The high correlation between colony morphological types on different media and phage types concerns the most frequent *S. aureus* types and is therefore one of the most crucial influencing factors for population analysis with respect to enterotoxigeneity and antibiotic resistance (Table 3, Table 4; Appendix: Table 20, Table 23-27, Table 45). The high correlation between phenotypes and phage types has in the past been the reason why these phenotypic characteristics were considered in epidemiologic questions (Blair and Williams, 1961; Meyer, 1966b; Gedek, 1972; Hummel et al., 1992). The same was as well observed in

recent years for bovine *S. aureus* using genotyping methods, but its great relevance for epidemiological questions and the necessity for standardised bacteriological examinations in the dairy chain have not been discussed up to now (Fitzgerald et al., 2000; Stephan et al., 2001; Loncarevic et al., 2005).

The high agreement between penicillin resistance and phage types, or egg-yolk reaction and β-haemolysis to bovine strains has been recognized 40 years ago between phage types and penicillin resistant or sensitive *S. aureus* strains (Seto and Wilson, 1958; Reid and Wilson, 1959; Pulverer, 1966; Wallmark and Thörne, 1958; Meyer, 1966b; Nyhan and Archer, 1967). The traditional use of BA for pathogenic bacteria as well as the investigation of only cows with mastitis are the reasons why *S. aureus* without or with only weak haemolysis and the egg-yolk reaction were not of interest for most of the investigators (Appendix: Table 27). Only Munch-Peterson (1965) took samples of each cow in a herd and determined clumping factor and coagulase reaction of all cultured staphylococci. Munch-Peterson is the single investigator who mentioned the high correlation between clumping factor and coagulase positive for non-haemolytic *S. aureus* in contrast to CNS. Furthermore, herds with a high prevalence of non-haemolytic *S. aureus* were according to Munch-Peterson the herds with lowest percentage of cows with a long duration of mastitis, respectively high SCC. The lower SCC of cows with non-haemolytic *S. aureus* is in accordance with the observation of Nakakwa (1960b), and it is probably the reason why Munch-Peterson (1972) classified non-haemolytic *S. aureus* in the group of minor pathogens with staphylococci, respectively micrococci. The heterogeneity regarding the haemolytic activity, respectively phenotypic characteristics, was on the other hand the reason why Munch-Peterson (1970) searched for a more suitable medium for the dectection of *S. aureus* in milk samples. He concluded that it is not possible to detect *S. aureus* with a good reproducibility in a mixture with CNS (Munch-Peterson, 1970).

The high prevalence of orange pigmented *S. aureus* has according to Albo-Elnaga and Kandler (1965a) in the past been a suitable election criterion for counting coagulase positive staphylococci on plate count agar. The loss of pigmentation however, caused by the black colour of the telluride reduction, is a problem of BP in general. This is especially a disadvantage on BP-RPF because of the low elective suitability of the coagulase activity. The egg-yolk reaction on BP is no specific reaction for *S. aureus*, which is the reason why each suspected colony-morphological type has to be confirmed via the clumping factor, or at least in the coagulase tube. The necessity of confirming all staphylococci in the coagulase tube on BP naturally induces a higher detection of different *S. aureus* types in comparison to BP-RPF.

The strong reduction of isolates in phage group IV is in accordance to Hummel et al. (1992) now obviously the main reason for the decreasing importance of pigmentation and β-haemolysis as traditional election criteria for *S. aureus* of bovine mastitis.

The suitability of β-haemolysis for the detection of clumping factor negative *S. aureus* strains on BA has been confirmed by the results of Devrise (1979), Fitzgerald et al. (2000) and Stephan et al. (2001). The low prevalence of only 32 % egg-yolk positive strains in collection B is the best evidence for the negative correlation between β-haemolysis and egg-yolk reaction for *S. aureus* of bovine mastitis (Meyer, 1966a; Gedek, 1972; Untermann et al., 1973; Mayer, 1975; Becker et al., 1987). High prevalence of strains with strong phage reactions for bovine phages, production of enterotoxin C, and β- confirms the pathogenic relevance and association to different phenotypic characteristics and are one explanation why strains of mastitis monitoring programs with more than 90 % β- producing strains have a low number of enterotoxin A producing *S. aureus* in comparison to the prevalence of strains producing enterotoxin C (Untermann et al., 1973; Mayer, 1975; Zschöck et al., 2005; Kumagai et al., 2007; Appendix: Table 24-26). The suitability of the egg-yolk reaction as election criteria for bovine strains did not increase in relation to decreasing numbers of bovine *S. aureus* in phage group IV, because of the high prevalence of isolates belonging to phage type 119 and 78. The high prevalence of the current two clumping factor negative *S. aureus* types induces on the one hand a still large number of strains producing β-, but on the other hand it causes problems regarding the coagulase activity on BP-RPF (Table 5).

The strong positive egg-yolk reaction without β-haemolysis and isolates with a high sensitivity for human phages are well known for bovine and human *S. aureus* (Gedek, 1972; Farrah et al., 1988). The strong egg-yolk reaction of human *S. aureus* in comparison to CNS was the reason why Baird and Parker developed the BP-medium for the isolation of human pathogenic staphylococci in hospitals (Baird-Parker, 1962). The high prevalence of penicillin resistant bovine strains without β-haemolysis but strong egg-yolk reaction is in accordance to Gedek (1972) and shows the better suitability of BP for the detection of antibiotic resistant strains in comparison to BA (Tables 3 and 4). According to Stephan et al. (2001) 18 % of all enterotoxin producing *S. aureus* have only weak haemolysis, but 80 % of these strains are egg-yolk positive (Appendix: Table 25). The most of them produced enterotxin A. One strain was the single isolate producing SEC but without *tst*. All *S. aureus* of bovine mastitis producing enterotoxin A show weak or only α-haemolysis and belong to the same or closely related genotypes using PFGE (Stephan et al., 2001). On the other hand, the strains of subclinical mastitis are more likely to produce enterotoxin A, D are frequently ß- negative but

egg-yolk positive, and have a high sensitivity for human phages in phage group III (Olson et al., 1970; Lombai et al., 1976, Appendix: Table 25). The high prevalence of about 60 % egg-yolk positive *S. aureus* in dairy products or bulk tank milk, frequently without ß-haemolysis, confirm the relevance of subclinical mastitis for *S. aureus* from raw milk or raw milk products (Appendix: Table 24).

The enumeration of *S. aureus* is more practicable and reproducible on BP-RPF through direct counting of coagulase positive colonies. Loncarevic et al. (2005) could detect in two cheeses enterotoxin C producing strains only on BA, whereas on BP-RPF no *S. aureus* were detectable. However, they discussed neither the role of different coagulase activity in the tube and BP-RPF, nor did they mention that only BP, not BA, is the other method for investigating raw milk products according to international standards (ISO-6888). The negative correlation between bulk tank milk samples and mastitis samples from Switzerland with respect to the distribution of SE-profile is strongly associated with election criteria on BPF-RPF, BP and BA (Stephan et al., 2001 and 2002; Appendix: Table 24, 25). The investigation of *S. aureus* from bulk tank milk or raw milk products using BP-RPF, according to Stephan et al. (2002), Loncarevic et al. (2005) and Morandi et al. (2007), has to be seen as one reason for the low prevalence of isolates with *sec* and *tst* in relation to *sea* and *sed*.

The instability of the coagulase reaction regarding bovine *S. aureus*, especially for enterotoxin C producing strains, has been nevertheless recognized (Mayer, 1975; IDF-Bulletin, 2006). However, nobody investigated the connections between the distribution of colonies with a low coagulase activity and ambiguous or wrong negative results on BP-RPF. The problem in this context is the fact that important *S. aureus* types, which are clumping factor negative, cause the high prevalence of strains with ambiguous or wrong negative results on BP-RPF (Table 5). Population analysis considering only clumping factor or coagulase positive strains can exclude therefore important *S. aureus* types, as shown when comparing the results of Zschöck et al. (2005) with the findings of Hummel et al. (1992) and the here presented results.

The coagulase tube is more sensitive than the coagulase reaction on BP-RPF, especially after 24 hours (Table 5). The clearest differences regarding the coagulase activity in the tube can be observed between the first two and four hours. This could be the reason why Blobel and Schließer (1979) recommended stopping to read the reaction in the coagulase tube after four hours in order to avoid incorrect negative results. The use of citrate in addition to the rabbit plasma has in the past been one reason for a fast detection of the coagulase activity, because of wrong results in the tube after a long incubation in the coagulase tube (Sperber and Tanini,

1975). The high prevalence and coagulase activity of bovine *S. aureus* in phage group IV is assumed to be the reason why a greater incubation time than four hours was not necessary. The recommendation of Stadthouders et al. (1976) using BP-RPF for the detection of *S. aureus* in dairy products is now over thirty years old. At this time the clumping factor negative types were not wide distributed in dairy herds of many countries. The low prevalence of clumping factor negative strains in the past is not only relevant for the identification of *S. aureus* with the positive coagulase reaction. It is also very important when discussing the use of agglutination tests for the rapid identification of *S. aureus* isolates.

The increasing number of clumping factor negative bovine strains was the reason why combined agglutination tests with additionally specific antibodies for protein A were used for the rapid detection of bovine *S. aureus* (Becker et al., 1987). All of the clumping factor negative *S. aureus* strains with *sec* and *tst* are according to Stephan et al. (2001), with one exception, negative in the "Staphaurex" reaction, which is a combined agglutination test. The combined agglutination test is established in the routine diagnosis of bovine mastitis, even through Becker (1987) and Stephan et al. (2001) showed, that these combined agglutination tests do not provide satisfying results for the fast detection of clumping factor negative bovine *S. aureus* strains.

It is therefore concluded that a standardized application of one media on both levels, the animal and product level, are necessary in the dairy chain. A modified Baird parker containing 5 % sheep blood with polymyxin B and/or sulfamethazine, instead of telluride for the assessment of the pigmentation, and clumping factor reaction would allow the determination of all important characteristics on one agar plate. This agar plate in combination to the spatula method would be probably more effective than the recommendation according to ISO/2006/408 of using a secondary agar. The egg-yolk reaction, clumping factor reaction and haemolysis with β-haemolysis for clumping factor negative *S. aureus*, would guarantee a fast detection of *S. aureus* and differentiation of the non-haemolytic and clumping factor negative *S. hyicus*. The consideration of all important election criteria of *S. aureus* is very signifant for the detection of as many different strains as possible without a preference to human or bovine biotypes. The quantification of *S. aureus* may be advantageous on new chromogenic media, but the diversity of phenotypic characteristics is not considered in this method.

4.1.1.4 Summary

72 *S. aureus* strains of bovine mastitis were phage typed and tested for the elective criteria of haemolysis, egg - yolk and coagulase reaction, penicillin resistance and SE genes (se) by a polymerase chain reaction (PCR). 60 % of the penicillin resistant *S. aureus* showed only a weak or no haemolysis. The difference in relation to isolates with αβ- haemolysis was significant (p= 0.045). *40 S. aureus* (56%) belonged to four different phage patterns with a strong negative correlation for the relevant elective criteria. The strains with a high sensitivity for phage 78 or 119 are with exception of one strain egg-yolk negative in contrast to the phage pattern 96 and 102/ 107/ 108/ 111. The phage pattern 119 and 96 showed all αβ-haemolysis with a weak coagulase activity and were the most important subpopulations with enterotoxin genes or coagulase negative isolates.

Therefore *S. aureus* should be investigated with one plating medium on the cow level, bulk milk and product level considering haemolysis, pigmentation and egg-yolk reaction.

Significance and impact of the study: The use of different plating and high prevalence of single virulent *S. aureus* biotypes are the reason, why the comparison between *S. aureus* monitoring programs in dairy herds and S. *aureus* of the bulk milk or milk products is difficult.

4.1.2 Methodological difficulties related to phage typing

Methodological difficulties related to phage typing are the second most relevant problem in the discussion of *S. aureus* in the dairy chain. Most knowledge about comparative long-term data regarding the biodiversity of bovine *S. aureus* is based on phage typing. Since 50 years and until today the phages of the old basic set have been continuously used. They belong to the most sensitive phages for certain *S. aureus* types in the bovine as well as in the international human phage set from 1974. Most phage types have usually a characteristic reproducible phage profile with strong and weak reactions for the same, or closely related phages. Furthermore, the strong host specificity of phages causes difficulties when typing strains from different hosts or ecological niches with only-human phages, or a phage set containing phages of different origins, as in case of the bovine set. This is the reason why strong and weak phage reactions together with the use of different phage dilutions and modifications in the phage sets are the real challenge when interpreting different results obtained with this method. The evaluation of the phage profile, considering all weak and strong phage reactions from the most widely distributed phage types by using international phages, is therefore the first priority, when investigating methodical difficulties of phage typing from an epidemiological point of view.

4.1.2.1 Problem and Goal

The first phage set contained only human phages and the old basic set and the new human phage set are still used for typing bovine staphylococci (Olson et al., 1970; Adesiun, 1996; Vintov et al., 2003). One reason using additionally human phages in the bovine phage set for typing bovine staphylococci was according to Davidson (1961a) the comparability of results with the human phage set. A satisfying sensitivity of *S. aureus* strains at RTD is however only observed, when isolates are typed with phages of the same habitat (Hajek and Howak, 1976). The comparison of phage typing results between different authors is therefore difficult because of the use of different phage sets and different concentrations of typing phages (Aarestrup et al., 1995a). Furthermore weak phage reactions are one of the distinct methodical problems in phage typing (Wenworth, 1963). According to Blair and Williams (1961) it is not necessary to specify the dilution at RTD, 100 × RTD or 1000 × RTD or the strength of phage reaction (50-100 plaques (++), 100 - 1000 (plaques), ≥1000 plaques (beginning of confluent lysis) in which ≥50 plaques was achieved. Phage reactions with 20 up to 50 plaques (+) are usually separately noted. Still Degre (1967) could show that the international instruction of

phage typing with respect to the use of different phage dilutions at RTD, 100×RTD and 1000× RTD is the deciding problem determining different phage types. Important parameters influencing the reproducibility and discriminatory efficiency are the second and third issues which have to be discussed. Very important in this context is the different degree of sensitivity of phages from bovine and human origin for the same isolates. This is on the one hand necessary for comparing results with different phage sets. On the other hand it is necessary to verify changing phenotypic characteristics in the dominating *S.aureus* populations during population analysis over decades. The third important questioning is the correct classification of *S. aureus* strains in the corresponding phage groups. The biggest difference between phage sets from different hosts is the number of phage groups or phages and the correct classification of phages in the corresponding phage group. The specificity of single phages is further high correlated to isolates from different origin. This is the reason, why the results of phage typing in collection A, B and C are compared with each other for each single phage.

4.1.2.2 Results

4.1.2.2.1 Effect of different phage sets and strength of phage reactions

Not exclusively the number of used phages, but to a much higher degree the strength of phage reactions and the origin of phages were the distinct parameters influencing the results of phage typing (Table 6). Using all phages or the bovine set and considering only phage reactions with confluent lysis (≥ 1000 plaques) the majority of isolates belong to bovine miscellaneous phages, or were sensitive to phages of the bovine phages set. The miscellaneous (M) phage 119 was the most frequent phage type of the respective phage group with 17 isolates using bovine phages or considering only phage reactions with confluent lysis. The high sensitivity of bovine phages was the reason why no strain was grouped in the human phage groups with the exception of phage 96. Phage 96 is however not part of the bovine and the basic phage set. The low sensitivity of human phages, respective low number of isolates with confluent lysis in the basic and human set, however induces a high percentage of non-typable isolates. Only with consideration of phage reactions ≥20 plaques (+) the majority of isolates were typable with human phages and belonged then to different human phage groups. Considering additionally phage reactions ≥20 plaques (+) of the bovine phages the number of isolates in different phage groups (NI) increased considerably, especially in case of the additional bovine phages 108 and 111.

Both human and bovine miscellaneous phages are not part of a certain phage group. This induced a high number of 28 phage group combinations, defined as phage profiles, when all 30 phage reactions of ≥20 plaques were considered (Table 7). The use of bovine phages was only essential for the phage profiles 26, 27 and 28. The high number of specific weak phage reactions showed the results of profiles 3, 4, 5, 6 and 15, profile 13 and 14 or the isolates of phage profiles 23 and 25 (Table 7). The isolates in phage profiles 23 and 25 showed all confluent lysis for the phages 102, 107, 108 and 111, but only 50-100 plaques for phage 81. The isolates of phage profiles 3, 4, 5, 6 and 15. showed all merely confluent lysis for phage 119, except of the isolate in profile 5, and many of them displayed weak reactions ≥20 plaques for the same phages in phage group I and IV. A similar situation was observed for isolates in the phage profiles 13 and 14 with confluent lysis only for the phage 96. The isolates of phage profiles 4 and 6 showed additionally weak reactions for phage 55 of phage group II, while the isolates of phage profile 4 were additionally sensitive for phage 96. (60%) of collection B belonged to four *S. aureus* strains considering only strong phage reactions ≥1000 plaques.

Table 6 Results of phage typing in collection B considering different phage sets and strength of phage reaction ≥ 20 plaques, respectively ≥ 1000 plaques

Important phage groups	All 30 phages (%) ≥ 20 / >1000	Bovine set (17 phages) (%) ≥ 20 / >1000	Basic set (16 phages) (%) ≥ 20 / >1000	Humane set (19 phages) (%) ≥ 20 / >1000
Human phages:				
I	0 / 0	0 / 0	28 / 0	22 / 1
II	0 / 0	0 / 0	0 / 0	0 / 0
III	0 / 0	0 / 0	11 / 7	12 / 8
81[†] (M)	0 / 0	(-/-)[‡]	5 / 0	5 / 0
96 (V)	0 / 8	(-/-)[‡]	(-/-)[‡]	0 / 10
Bovine phages:				
IV	11 / 17	14 / 13	42D[§]: 4 / 1	(-/-)[‡]
116 (II)	3 / 3	3 / 3	(-/-)[‡]	(-/-)[‡]
78 (M)	17 / 18	17 / 18	(-/-)[‡]	(-/-)[‡]
119 (M)	1 / 24	1 / 25	(-/-)[‡]	(-/-)[‡]
Different groups (NI)	67 / 20	61 / 20	24 / 7	29 / 8
Non- typeable (NT)	1 / 10	4 / 21	28 / 71	32 / 72
Phage types (n)	42 / 25	33 / 16	27 / 11	31 / 12

[†] These isolates belong according to Parker (1983) to the phage complex 80.

[‡] This phage group combination respectively phages belong not to this phage set.

[§] The phage 42D is the single human phage of the old basic set in phage group IV.

Table 7 Profile of phage reactions of ≥20 plaques in the corresponding human and bovine phage groups of the 72 isolates in collection B

	Human phages (except of phage 116)				Bovine phages (except of phage 42D and 78)	
Profile	Group I	Group II	Group III	M / V	Group IV	M
0	Non- typable (NT)					
1	52A/ 80	3A	6/ 47+/ 54+/ 75+/ 85+	**96**[†]	102+/ 107/ **117**/ 111	**118**/ 119+
2	80+	3A	6/ 42E/ 47/ **53**/ 84/ 85	81/ 95	42D/ 102/ **117**/ 108/ 111	**118**
3	29/ 52/ 52A/ 80	3A+/ 55+	6		102/ 117/ 111	**119**
4	29[‡]/ 52[‡]/ 52A/ 80	55		96	102/ 117	**119**
5	52A/ 80+	55+			102/ 117+	
6	29[‡]/ 52[‡]/ 52A/ 80	55+			102/ 117	**119**
7	29/ 52A		6+/ 42E/ **84**	81+	102/ **108**/ **111**	**78**/ **118**
8	79		6[‡]/ 42E[‡]/ 47[‡]/ 53/ 54[‡]/ 75[‡]	81[‡]	**102**/ **107**/ **108**/ **111**	**118**
9	29/ 52/ **52A**/ **80**		**6**/ **42E**/ **47**/ 53+/ **54**/ **84**	**81**/ **812**	117/ **108**/ **111**	**78**/ 119
10	52A/ **79**		**6**/ 42E/ **47**/ **53**/ **54**/ **75**/ **85**	**81**/ 812	**102**/ **117**/ **108**/ **111**	**118**
11	52/ 52A/ **79**		**6**/ **42E**/ **47**/ **53**/ **54**/ **75**/ **85**	**81**/ 812/ 95	**102**/ **117**/ **108**/ **111**	**118**
12	29[‡]/ 52[‡]/ 52A[‡]/ 79/ 80		6[‡]/ 42E[‡]/ 47[‡]/ 53/ 84[‡]/ 85[‡]	95	**42D**[§]/ 102/ **107**[§]/ **117**[§]/ **108**[b]/ 111	**118**
13	29+/ 80			**96**	102/ 107	
14	29[‡]/ 52[‡]/ 52A[‡]/ 79[‡]/ 80			**96**	102/ 107[‡]/ 117[‡]	118
15	29[‡]/ 52[‡]/ 52A/ 80				102/ 117[‡]	**119**
16	**29**				**42D**/ **102**/ **107**/ **108**/ **111**/ **117**	
17		**3A**	**6**/ **42E**/ **47**/ **53**/ **54**/ **75**/ **85**	**81**/ **95**/ **812**	**102**/ **108**/ **117**/ **111**	**118**
18		3A	6		117	**78**
19		**116**				
20		116	6/ 53		108	
21		116+	**6**/ 47/ **53**/ 84	81	102/ **117**	119
22			75+		102/ 117/, **108**/ 111	
23			**6**[§]/ 42E[‡]/ 47[‡]/ 54[‡]	**81**[§]	**102**/ **107**[§]/ **117**[§]/ **108**[§]/ **111**[§]	
24			53/ 84/ 85	81/ 95+	42D, 108	118
25				81	**102**/ **107**/ **108**/ **111**	
26					42D[‡,§]/ 102[‡,§]/ **107**[b]/ 108[‡,§]/ **111**[§]/ **117**[§]	
27						**78**
28						119

[†] The bold marked phage numbers indicate that the phage showed only confluent lysis in this profile.

[‡] Variable: single isolates showed <20 plaques or no reactions or 20-1000 plaques, but never confluent lysis

[§] Variable: single isolates showed no reaction or have confluent lysis

These isolates showed a high similarity regarding the election criteria on BP, BP-RPF or haemolysis on BA, respectively other characteristics, and belonged to a limited number of phage types considering weak phage reactions (≥ 20 plaques) or PT´s(Table 8). Different election criteria and phenotypic characteristics of single closely related strains however were confirmed through weak phage reactions and different results in the PFGE. Most of the strains in profile 15, and many isolates of profiles 4 and 6 considering only phage reactions ≥50 plaques, were grouped according to Parker(1983) to the 80-complex.

Table 8 Most frequent phage types considering only strong phage reactions (≥ 1000 plaques) and number of different phage types with weak phage reactions (≥ 20 plaques) in relation to number of different PT`s, elective criteria and other phenotypic characteristics

Result of phage typing				Election criteria (%)					Other phenotypic characteristics (%)			
Phage type (≥ 1000 plaques)	n=72 (%)	Number (n) phage types[1]	Number (n) PFGE types	Coa[2]	Haemolysis			Egg yolk reaction	Clumping factor positive	Production of pigments	Penicillin resistant	SE-profile[3]
					α	αβ	w					
119	17 (24)	7	5	0-4[a]	0	100	0	0	0	6	0	0-4
96	5 (7)	4	1	0	0	100	0	100	0	100	0	4
102,107, 108, 111	7 (10)	4	4	7, 8	0	71[b]	71	0	100	0	100	0, 5
78	13 (18)	2	6	4, 7, 8	89	8	12	16	92	16	8	0

[1] Number of phage types considering phage reactions ≥ 20 plaques

[2] Profile of the coagulase activity according to Table 3

[3] SE-profile according to Table 2

[a] The coagulase negative isolate had additionally a strong phage reaction for phage 96.

[b] our isolates showed a strong egg-yolk reaction after 24 h.

4.1.2.2.2 Specificity of single phages

The correlation and *P*-values or the comparisons of collection A, B and C with respect to the strength of phage reaction in corresponding phage groups is shown in Table 9. The phage group specificity and the sensitvity for penicillin resistant strains especially with strong reactions of ≥1000 plaques were the best indicators for the suitability of a single phage (Table 10). The lack of, or low prevalence of strains being sensitive only to human phages of phage group I were the best evidence for a low specificity of these phages for bovine isolates.

Table 9 Correlation (r2) and P-values between the comparisons of collection A, B and C for the phages in corresponding phage groups with respect to strength of the phage reaction (≥ 30 plaques / ≥ 1000 plaques (SCl+ CL))

Phages / Phage groups		Collection A+B	Collection A+C	Collection B+C
		≥ 20 / ≥ 1000	≥ 20 / ≥ 1000	≥ 20 / ≥ 1000
All 30 phages	P- values	0.232 / 0.409	0.018 / 0.301	0.020 / 0.393
	Correlation	0.89 / 0.92	-0.26 / 0.20	-0.07 / 0.19
21 human phages	P- values	0.236 / 0.407	0.001 / 0.018	0.001 / 0.020
	Correlation	0.79 / 0.63	-0.38 / 0.57	-0.02 / 0.53
9 bovine phages	P- values	0.867 / 0.802	0.358/ 0.093	0.353 / 0.095
	Correlation	0.87 / 0.78	0.56 / 0.26	0.46 / 0.13
I	P- values	0.596 / 0.578	0.845 / 1.000	0.518 / 0.666
	Correlation	0.86 / 0.92	-0.35 / 0.51	-0.09 / -0.45
II	P- values	0.478 / 0.148	0.618 / 0.530	0.233 / 0.157
	Correlation	-0.68 / 0.76	-0.11 / 0.50	-0.65 / -0.95
III	P- values	0.002 / 0.028	0.0001 / 0.013	0.002 / 0.042
	Correlation	0.60 / 0.58	0.64 / 0.76	0.26 / 0.09
81, 95, 96, 187, 812	P- values	0.546 / 0.824	0.048 / 0.410	0.053 / 0.354
	Correlation	0.65 / 0.95	0.30 / 0.31	0.45 / 0.59
IV	P- values	0.734 / 0.695	0.191 / 0.088	0.233 / 0.182
	Correlation	0.85 / 0.86	0.50 / 0.69	0.25 / 0.32
78, 118, 119	P- values	1.000 / 0.869	0.821 / 0.028	0.798 / 0.023
	Correlation	0.68 / 0.99	-0.96 / -0.63	-0.91 / -0.87

Table 10 All positive reactions (in %) with ≥ 20 plaques and only semiconfluent or confluent lysis (≥1000 plaques) for each bovine and human phages in collections A, B, C, with special regard to the sharing of weak and strong reactions of penicillin resistant isolates

Phage groups	Phages in corresponding phage groups	Collection A (%) (Germany) ≥ 20 / ≥ 1000		Collection B (%) (Mastitis) ≥ 20 / ≥ 1000		Collection C (%) (Resistant) ≥ 20 / ≥ 1000
		All (n= 213)	Resistant (All %)	All (n= 72)	Resistant (All %)	Resistant (n= 68)
I	29	21 / 2	23 / 100	7 / 1	40 / 0	19 / 6
	52	12 / 1	11 / 100	7 / 0	20 / 0	14 / 0
	52A	31 / 8	9/ 18	35 / 4	12 / 33	27 / 5
	79	5 / 1	11 / 0	10 / 3	33 / 50	27 / 3
	80	35 / 2	10 / 0	35 / 3	8 / 50	9 / 0
	All of phage group I:	*41 / 12*	*(5 / 3)*	*47 / 8*	*(6 / 3)*	*34 / 11*
II	3A	4 / 3	17/ 25	6 / 1	67 / 100	8 / 2
	55	10 / 1	6 / 0	4 / 0	0 / 0	6 / 2
	116[a]	6 / 3	50 / 100	4 / 3	67 / 100	11 / 8
	All of phage group II:	*18 / 5*	*(5 / 3)*	*14 / 4*	*(7 / 4)*	*20 / 9*
III	6	15 / 8	50 / 58	17 / 9	54 / 83	45 / 33
	42E	8 / 3	57 / 60	11 / 4	88 / 67	41 / 25
	47	6 / 4	58 / 57	11 / 6	88 / 75	33 / 17
	53	6 / 4	57 / 67	17 / 11	50 / 63	41 / 6
	54	5 / 3	56 / 67	10 / 6	83 / 75	42 / 14
	75	6 / 4	56 / 67	6 / 4	67 / 67	42 / 19
	84	6 / 2	43 / 100	10 / 3	71 / 100	26 / 3
	85	4 / 2	40 / 0	10 / 4	67 / 67	30 / 6
	All of phage group III:	*18 / 10*	*(9 / 5)*	*27 / 14*	*(17 / 13)*	*53 / 38*
M	81	8 / 5	64 / 75	18 / 7	92 / 80	42 / 36
	95	8 / 1	0 / 0	10 / 1	50 / 0	11 / 2
	96[b]	14 / 13	4 / 0	10 / 10	0 / 0	38 / 11
	187	0 / 0	- / -	0 / 0	- / -	23 / 0
	812	3 / 3	60 / 60	6 / 3	75 / 100	34 / 13
	All mixed humane phages:	*21 / 17*	*(7 / 4)*	*32 / 17*	*(17 / 6)*	*67 / 47*
IV	42D[c]	10 / 5	20 / 25	11 / 4	50 / 86	0 / 0
	102	55 / 22	23 /39	62 / 25	33 / 67	20 / 8
	107	25 / 19	43 /45	25 / 20	47 / 64	16 / 6
	117	42 / 22	23 /39	27 / 15	56 / 73	34 / 28
	108	23 / 22	46 /47	35 / 30	70 / 67	17 / 14
	111	30 / 24	39 /42	32 / 25	64 / 44	39 / 20
	All of phage group IV:	*73 / 35*	*(20 / 19)*	*73 / 38*	*(30 / 25)*	*58 / 47*
M	78	26 / 23	19 / 11	20 / 20	20 / 3	6 / 0
	118	16 / 8	22 / 40	20 / 15	45 / 45	14 / 3
	119	29 / 27	4 / 2	31 / 27	11 / 0	8 / 3
	All mixed bovine phages:	*65 / 58*	*(9 / 7)*	*65 / 55*	*(14 / 10)*	*19 / 6*

[a] The single bovine phage of the bovine set in the phage group II

[b] This phage belongs to 94/96 complex, phage group V in the human phage set.

[c] The single human phage in phage group IV of the old basic set

The phages of phage group I had the lowest suitability for typing penicillin resistant strains. Single penicillin resistant isolates in collection A and C however showed a high sensitivity to phage 29 (Table 10). The results of all three collections confirmed the low association of phage 80 to penicillin resistant isolates. Phage 80 was the human phage with highest sensitivity for isolates with strong phage reactions for phage 119. Phage 79 displayed low phage group specificity and belonged to mixed groups. Phage 79 reacted frequently together with phages of phage group III. Phages of phage group II showed the highest group specificity of all phages with a high association to penicillin resistant strains. The most important phage in this context was the single bovine phage 116 of phage group II. Predominately those isolates with a high sensitivity for phage 119 showed additionally weak reactions to phage 55, contrary to penicillin resistant isolates in collection C.

No other phages in one single phage group showed such a high sensitivity for penicillin resistant isolates as it was observed for the phages of phage group III. The specificity for penicillin resistant strains however was frequently observed only when weak phage reactions ≥20 plaques were considered in epidemiological questions, especially in collection C. Phage 6 was one of the phages contained in all three collections with the highest association to penicillin resistant isolates. The high sensitivity of phage 42E for penicillin resistant strains was only confirmed for isolates of collection C.

Phages 84 and 85 of the 83A-complex showed the lowest sensitivity of all phages of phage group III in collection C. However, all isolates with strong reactions for phage 84 in collections A and B were penicillin resistant. The results for human miscellaneous phages and phage 96 were very heterogeneous. The strains of phage types 95 and 96 in collections A and B were usually penicillin sensible. No isolates in these collections were sensitive for phage 187. Many antibiotic resistant isolates in collection C however were sensitive for these phages, but usually only with phage reactions of ≥ 20 plaques. The high specificity of phages 81 and 812 was confirmed by the results of all three collections. Phage 81 belonged together with phage 6 to the most sensitive human phages for penicillin resistant strains. Phage 812 showed the same specificity for penicillin strains, even if only for a smaller *S. aureus* subpopulation. The variation of in the farms whose samples exceeded the limits the high *S. aureus* rate was controlled by consecutive sampling from each cow and dispatching the shedders. Levels of strong phage reactions for each bovine phage of phage group IV was correlated with the prevalence of penicillin isolates in each collection. The phages of phage group IV had in collection C only a discriminative function: only 5 % of the strains in collection C were solely sensitive to phages of phage group IV.

Considering weak phage reactions of ≥ 20 of human phages the number of isolates in phage group IV decreased in collection A to approximately 25 %, in collection B to circa 33 % and in collection C to about 50 %. No strain in collection C was sensitive to phage 42D, the single human phage of the basic set in phage group IV. Only in collection A the level of strong phage reactions for each bovine phage was inbetween 19 and 24 %, whereas the level of strong reactions for bovine phages in collection B varied between 15% and 30 %, and for collection C between 6% and 28%. Phage 108 was in collections A and B the phage of phage group IV with highest association to antibiotic resistant strains. The prevalence of positive reactions for phage group IV was in collection C strongly correlated to the strength of phage reactions. Phage 117 showed the highest specificity and prevalence in collection C, considering the prevalence and relation to weak phage reactions, whereas phage 111 had the highest prevalence considering only weak phage reactions of ≥ 20 plaques. The penicillin resistant isolates of collection C showed the highest overall association to phage 117. An increasing association for penicillin resistant isolates to phages 117 and 111 was still observed in collection B. Phage 108 however was the most important phage for penicillin resistant isolates in collection A. This was also observed for penicllin resistant isolates on collection B, even though on a higher level.

The bovine miscellaneous phages displayed a significantly lower specificity for penicillin resistant strains. The most sensitive phage for penicillin resistant isolates in all three collections was phage 118.

4.1.2.2.3 Mixed phage groups with human and bovine phages

The good correlation between the distribution of positive reactions with all 30 phages in collection A and B is according to Table 11 mainly due to the high number of isolates in the same mixed phage groups. The strains showed not only the same strength of phage reactions in the corresponding phage group (Table 11). Furthermore the results of the phage profiles in Table 7 were almost completely confirmed through the stains with similar characteristics from collection A and and C. The isolates in all three collections with > 1000 plaques for phage 119 and 96 only showed weak reactions for the phages 52A, 55, 102, 107 and 118. Only few other strains of collection A with phage pattern I/119 showed strong phage reactions for phage 80. Not only in collection C the penicillin resistant strains belonged with a single exception to different phage patterns or other phage group combinations. The heterogeneity of isolates being sensitive for phages of phage group III and other human phages was confirmed through the sensitivity to different bovine miscellaneous phages, as well as the phenotypic

characteristics. Phages 187 and 96 showed weak reactions of ≥ 20 plaques to the phage group III. Phage 812 was only observed in collection C and was the reason for the high number of isolates in the mixed group of this collection with the human phage set (Table 11). The strains in collections B and C, which belonged to phage type 81 considering only human phages, were usually penicillin resistant and egg-yolk positive.

Table 11 *S. aureus* (in %) of collections A, B and C in phage groups of phages from the human and the bovine phage set with important phenotypic characteristics.
(abbreviations of phenotypes : Pen+= penicillin resistant; CF+ = clumping factor positive; Lez+= egg-yolk positive on BP; α/β/αβ-hemolysis on BA)

Human phage groups	Share	Phenotypes (%)						Phages of the HPS set (%)						Phages of the BPS (%)			
/ Collection	(%)	Pen+	CF+	Lec+	α	αβ	β	III	95	96	187	812	42D	IV	78	118	119
Group I																	
A (Germany)	16 (7)	7	7	0	0	100	0	0	0	2	0	0	0	100[b]	0	0	98[a]
B (Mastitis)	22 (3)	0	0	0	0	100	0	0	0	6	0	0	0	86[b]	0	0	100[a]
C (Resistant)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Group I+ II																	
A (Germany)	9 (0)	0	2	0	0	88	12	0	0	6[a]	0	0	0	100[b]	0	13	100[a]
B (Mastitis)	6 (0)	0	0	0	0	100	0	0	0	25[a]	0	0	0	100[b]	0	25	75[a]
C (Resistant)	7 (1)	100	100	50	0	20	60	100[a]	0	80[b]	60[b]	60[b]	0	80[a]	0	0	2
Group I 96																	
A (Germany)	8 (0)	0	19	84	0	100	0	0	0	100[a]	0	0	0	76[b]	12	65[b]	6
B (Mastitis)	7 (0)	0	0	100	0	100	0	0	0	100[a]	0	0	0	100[b]	0	40[b]	0
C (Resistant)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Group I + III																	
A (Germany)	8 (2)	50	79	64	0	79	7	100	23[b]	11[b]	0	22	16	97	25	56	4
B (Mastitis)	13 (4)	44	89	33	10	40	0	100	64[b]	0	0	31	23	100	22	78	26
C (Resistant)	28 (9)	100	100	56	0	44	56	100[a]	33	44[b]	44[b]	83	0	78	0	33	3
Group II+III																	
A (Germany)	2 (0)	0	50	50	50	0	50	100	0	50	0	0	25	50	50	50	50
B (Mastitis)	4 (0)	100	100	100	33	33	0	100	67	0	0	0	25	100	33	67	0
C (Resistant)	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Group III																	
A (Germany)	4 (3)	67	100	56	11	44	33	100	0	0	0	0	11	77[a]	33	22	11
B (Mastitis)	7 (10)	67	100	60	20	0	0	100	0	0	0	0	20	100[a]	0	40	20
C (Resistant)	20 (28)	100	100	46	6	50	44	100	0	46[b]	15[b]	22	0	54[a]	8	8	0
M 81[1]																	
A (Germany)	1 (1)	0	50	0	0	100	0	0	0	0	0	0	0	100[a]	100	50[a]	0
B (Mastitis)	6 (0)	100	100	100	75	25	0	0	0	0	0	0	0	100[a]	0	0	0
C (Resistant)	5 (2)	100	100	67	33	33	33	0	0	0	0	33	0	100[a]	0	0	0

[a] >90 % of these phages had only semiconfluent or confluent lysis (> 1000 plaques)

[b] >90 % of these phages had only ≥ 10 -< 1000 plaques

[1] They belong in the human phage set according to Parker (1983) to the phage-complex 80.

[2] (The allocation to mixed groups considering only ≥ 1000 plaques is shown in brackets.)

4.1.2.3 Discussion

The long history of phage typing is very important in understanding the shift and biodiversity of the *S. aureus* population in the more than 40 past years. Phage typing has been used since 1935 for typing *S. aureus*, and the most phages used at this time are still part of the human set (Burn and Lush, 1935; Smith, 1948a). Probably most, if not all staphylococci are lysogenic (Wenworth, 1963). Phage action however, can be blocked completely, with up to four being blocked on one strain (Frost and Bradshaw, 1980). The crucial problem of phage typing is therefore a too low number of specific phages, or different phages, in one phage set, and the performance of phage typing with different phage dilutions (Williams and Rippon, 1952; Degre, 1967). Phage typing is initially performed with the lowest phage dilution at RTD. When no phage reactions with ≥ 50 plaques are observed, according to Blair and Williams (1961) phage typing is repeated, but this time at 1000×RTD. It is not necessary to mention at which phage dilution ≥ 50 plaques were detected. However, almost all weak phage reactions with 10-49 plaques at RTD showed usually phage reactions >50 plaques at 100×RTD or 1000×RTD (Appendix: Table 30, 31).

The results of Davidson (1972) considering only phage reactions ≥ 50 plaques at RTD show the difficulties when 16 phages are selected from a set of 30 human and bovine phages for the creation of the international bovine phage set (Appendix: Table 35). Only the exclusion of the human phages 79, 80, 3C, 55, 71, 85, 187 from the international basic set was without crucial consequences, as shown by all collaborating laboratories from different countries. Exclusive the importance of phage 42D, 102, 107 and 117 in phage group IV was indisputable in almost every laboratory, whereas the relevancy of phage 78, and the bovine phages 116, 118 and 119 was very doubtful and only of relevance in single countries. The additional specific phages 108 and 111 in phage group IV were very important for the discrimination efficiency and typeability of single strains in collection A, B and C (Tables 7 and 10). The high sensitivity of many strains for the phages 108 and 111 was still observed from Frost (1967), Bonin and Blobel (1967) and Gedek (1972).

The relevance of the human phage 187 and non-relevance of phage 42D were only observed for penicillin resistant strains in collection C, whereas the specificity for penicillin resistant strains of the additional human phage 812 in collection C was confirmed by the results of collection A and B. This demonstrates, in agreement with Bajljosov and Grigorova (1968), the problem when eliminating single phages from the set of 30 phages. There exists however no reason for the reduction of a phage set to less than 30 phages. 30 phages can be easily

placed on one agar plate. The additional effort for breeding phages is not high. The phage dilutions we recieved from the Robert Koch Institute were sufficient enough for typing 4,000 strains. These phage suspensions however were not even the whole amount of a single enrichment for most phages.

Many strains are sensitive for phages of different phage groups. This is the reason for the high number of phage group combinations (Davidson, 1972; Tables 7 and 11; Appendix: Table 14). The intermediary phage types with the same sensitivtiy for phages of different phage groups are usually heterogeneous with respect to phenotypic characteristics in comparison to strains of phage type 78, 119 and 116 (Tables 3 and 11). The most important characteristic justifying the discrimination of strains which are only sensitive to human phages, is according to Meyer (1966b, 1967, Appendix: Table 22, 28) the lack in coagulation of bovine plasma and the presence of egg-yolk positive and penicillin resistant strains in mixed phage groups (Table 11). The coagulation of bovine plasma is therefore not only the distinct characteristic for isolates in phage group IV, but it is also the crucial characteristic for the high number of strains in mixed phage groups with sensitivity for human phages and phages of phage group IV.

According to the results of Meyer (1967) in particular a high number of strains which are sensitive for phages of phage group III are incorrectly classified when the bovine phages of phage group IV are not used in accordance to the here presented results (Tables 6 and 11, Appendix: Table 28). The high prevalence of isolates with ≥ 50 plaques at 100 or 1000×RTD for human phages is not realized when using the common approach of phage typing according to Blair and Williams (1961). Many isolates show according to Meyer (1967) and Bonin and Blobel (1967) with the IPS only phage reactions ≥ 50 plaques at 100 or 1000×RTD, whereas the same strains are classified at RTD in phage group IV when additionally bovine phages of phage group IV are used (Tables 7 and 11, Appendix: Table 28). Only the strength of phage reactions at 100×RTD provides information about the specificity of the used phage set. Therefore an incorrect classification into phage groups can only be avoided with the exclusive use of 100×RTD. Additionally, almost all strains, with exception of about 1% of isolates, which are sensitive for phages, are still typable at 100×RTD (Nakakawa, 1960; Bonin and Blobel, 1967; Gedek, 1972). Furthermore, the reproducibility of phage typing is at least as good at 100×RTD as it is at RTD and 1000×RTD (Degre, 1967; Appendix: Table 31). Therefore there exists no reason for the use of different phage dilutions.

The strong increasing number of phage types when considering phage reactions with 20-1000 plaques however is not only the best example for the methodical difficulties of phage typing (Tables 6 and 7). It is furthermore a distinct problem when comparing results of different

phage sets, or results of one phage set with different phage dilutions, respectively strength of phage reactions. The phage titre at 100×RTD is not only necessary for isolates without any phage reactions ≥20 plaques, the phage titre at 100×RTD allows furthermore the distinguishing between phage reactions of confluent lysis (≥ 1000 plaques) and weak, respectively inhibited lysis (≥20 plaques), which are not detectable at RTD (Nakagawa, 1960; Bonin and Blobel 1967). The evidence for the relevance of strong phage reactions, respectively high sensitivity of single phages, especially for phages 96 and 119, is the high agreement between single phage types considering only phage reactions ≥1000 plaques, results of PFGE and phenotypic characteristics (Table 9).

The comparison of the here presented results with Davidson (1972) , Gedek (1972) and Mackie et al. (1987) show the high correlation between strong phage reactions ≥ 1000 plaques at 100×RTD and ≥ 50 plaques at RTD. The high number of strains in collection A, B and C of phage types 78, 116 and 119 agrees with observations according to Davidson (1972). With exception of phage 3A are phages 78, 119, 116 according to Davidson (1972) the phages with highest prevalence of single phage reactions per strain with only ≥ 50 plaques at RTD (Appendix: Table 35). The high sensitivity of single bovine strains to phage 96 has been still discussed in the introduction.

The results of Meyer (1967) are the best example that not the prevalence of typable isolates, but much more the higher specificity of bovine phages at RTD is the main argument for the use of bovine phages (Appendix: Table 28). The high sensitivity with confluent lysis of the phage 119 at 100×RTD in comparison to the weaker phage reactions of the phage group I was the reason why Davidson reclassified the phage 119 from phage group I to the miscellaneous group (Gedek, 1972). Gedek (1972) and the here presented results were able to confirm the observations made by Davidson. The most sensitive phage of the IPS in phage group I is phage 80, which is in agreements with the findings to Davidson (1972), Gedek (1972), and Vintov et al. (2003b). Phage 80 had usually 100, and up to 1000 plaques for the strains having confluent lysis with phage 96 and 119. This explains why one or more strains belong according to Davidson (1972), Hajek and Howak (1978) and Vintov et al. (2003a) into mixed groups in phage group I/119, respectivley I/96. This is as well the reason for the low number of strains belonging to phage type 119 in collcection A (Table 11 and 13).

All bovine strains with a high sensitivity for phage 119 or phage 96 had 20-100 plaques for human phages of phage group I (Table 8: phage profiles 10, 11, 15). This is not only a proof for the close relationship between human phage 96 and the single bovine phage AC1 of phage group I, according to the results of Hajek and Howak (1978); it has furthermore to be

considered whether the reclassification of phage 119 according to Davidson has only been the consequence of the fact that phage 119 had been isolated from bovine mastitis milk. It is as well questionable to differentiate bovine strains which are sensitive to human phages of phage group I in phage types of the 80-complex and other phage types. Phage types of the 80-complex were known 40 years ago as the most important human pathogenic *S. aureus* causing hospitalism (Williams and Jevons, 1961). They are usually antibiotic resistant, sensitive for phage 81 and egg-yolk positive, but β-haemolysis negative (Görschel, 1968). All egg-yolk positive, penicillin resistant strains without β-haemolysis of phage 81 showed additionally stronger phage reactions for bovine phages of phage group IV (Table 12). The low specificity and no increasing number of typable strains with phages 52 and 80 in comparison to phage 119 were the reason why these phages have not become part of the bovine phage set (Davidson, 1972).

One of the most important reasons why Davidson (1961a) created the bovine phage set was the low fidelity regarding the classification of bovine strains to one phage type using only human phages. Degre (1967) could show that the number of phage reactions, usually of the same phage group, increases in the order RTD, 100×RTD and 1000×RTD to a high degree. All isolates which are typable at the lower phage dilution show phage reactions for the same phages at the higher phage titre, but unfortunately with a variation of more other phages (Degre, 1967; Appendix: Table 30). The high number of phage types which belong to phage group I or 80-complex are according to Olson et al. (1970), Aarestrup et al. (1997), Larsen et al. (2000) and Vintov et al. (2003b) the best example for a too low specificity of the human phages in this context. This demonstrates that weak phage reactions are the main problem of low reproducibility when using phage typing.

The low reproducibility is induced through closely related phages from usually the same phage group, together with a too low specificity of human phages. One problem in this context is the reproducible equivalent concentration of all phages at RTD (Blair and Williams, 1961). The immediate typing at 100×RTD does compensate small variations regarding the phage dilution at RTD in different sampling rounds. More phages with reactions ≥50 plaques at 100×RTD are advantageous for phage types with a high proportion of reactions with 10-100 plaques at RTD.

The diversity of phage types in phage profiles 13 and 14, or 4, 6 and 15, is only observed when phages 96 or 119 are not used. Some of these isolates show no, or only weak phage reactions with less than 20 or 50 plaques when excluding phages 96 and 119. These strains have to be subsequently phage typed again at 100 or 1000×RTD, which leads in the most

cases to different phage types with phage profiles including more phage reactions.The weak phage reactions however are the distinct difference to the clumping factor positive penicillin resistant strains of phage type 96 in collections A and C. The same was observed for the two penicillin resistant strains of phage type 78 considering only strong phage reactions in collection A and B (Tables 3 and 8). These were the single strains with strong reactions for phage type 78 and weak reactions of other phages (Table 3, Table 7: phage profile 18). Similar results were observed for strains with weak reactions for phage 81 and strong reaction for phages 102, 107, 108, and 111 (Table 7: phage profile 25, Table 11).

In summary, the good correlation between phenotypic characteristics and weak phage reactions of 20 to 100 plaques at 100×RTD usually for the same or close related phage patterns confirms the specificity of weak phage reactions, and justifies the consideration of these phage reactions for epidemiological investigations. The separate counting of strong phage reactions with confluent lysis at 100×RTD provides comparable results when considering only ≥50 plaques at RTD. A larger number of phages than in the bovine phage set, especially in phage group IV, is necessary for a better discrimination of penicillin resistant strains.

4.1.2.4 Summary

The high host specificity of phages and strains is not only a problem of typing, the host specificity plays as well an important role in the correct classification into phage groups of single phages and type strains used for typing. First of all this leads to difficulties when comparing results of phage tpying with host specific and not host specific phages. Unfortunately the set up priorities of results is the distribution of isolates in the common phage groups and the performance of phage typing initially with the dilution at RTD. These problems can be only solved by using immediately the dilution at 100×RTD and the presentation of results in phage profiles considering phage reactions ≥ 20 plaques respectively strong phage reactions ≥ 1000 plaques (SCl and Cl).

Especially bovine strains should only be grouped to phage group IV, when only strong phage reactions of bovine phages are detectable or the isolates show no reactions for other phages than phages of phage group IV. Weak reactions of phages of phage group III for strains with strong reactions for phages of phage group IV at RTD are not unusual (Meyer, 1967; Appendix: Table 28). The bovine phage 119 and his type- strain are the best example for problems when using host specific phages at different phage dilutions and the classification of

strains to corresponding phage groups. A similar situation is observed for the human phage 96 and his type - strain. Both phages have a high affinity to phages of phage group I when typing bovine strains. Unfortunately phage AC1 was not considered during the international creation of the BPS, although almost each laboratory had few strains showing only strong reactions for phage AC1 as it was observed for phage 78 (Appendix: Table 35). Phage 78 is the best example for mixed phage groups. Phage 78 is the only human phage, who is essential for the BPS, because of his high sensitivity for one important bovine clone in the dairy chain. The high sensitity of phage 78 to bovine strains was the reason, why this phage was further used instead of the bovine phage 115 (Davidson, 1961a). Therefore phage 78 is best evidence for the dilemma of phage typing with respect to the host specificity of phages and sensitivity of different strains. The same situation, however especially for human strains, is observed for phage 96. The type - strain of phage 96 is the single strain of all type - strains for phages in the HPS, which have intermediary results for methillicin resistance. This would explain the affinity of single antibiotic resistant and clumping factor positive strains for phage 96 from collection C (Table 3).

4.1.3 Relationship of the *S. aureus* population to international human and bovine phages and penicillin resistance during the past 40 years

A significant influence regarding the change of *S. aureus* populations in dairy herds has been the long period in which cows were treated with penicillin (Vintov et al., 2003a,b). Since 40 years phage typing is the only typing method for the quantification of a shift in the *S. aureus* population. Only the investigation of *S. aureus* collections obtained from different regions, respectively herds (collections A, B and C), provides a comprehensive overview about the actual *S. aureus* population in german dairy herds, and permits the assessment of the actual sensitivity of phages which have been in use since 40 years. Best evidence for the changing sensitivity to single phages is the separate analysis of typing results obtained by using only human phages, or a set with additional bovine phages, under consideration of the reaction strength. Of great concern in this particular context is the distribution of phage types in phage group IV. Special consideration of only antibiotic resistant isolates (collection C) reveals the discriminatory efficiency and relevance of single phages and phage groups. The good correlation between phenotypic characteristics and weak phage reactions of 20 to 100 plaques at 100×RTD usually for the same or close related phage patterns confirms the specificity of weak phage reactions, and justifies the consideration of these phage reactions for epidemiological investigations. The separate counting of strong phage reactions with confluent lysis at 100×RTD provides comparable results when considering only ≥50 plaques at RTD. A larger number of phages than in the bovine phage set, especially in phage group IV, is necessary for a better discrimination of penicillin resistant strains.

4.1.3.1 Problem and Goal

Only the results of phage typing in the last 40 years until this day give us deciding links how the *S. aureus* - population has chanced, because genotyping methods were not available. Additionally the sanitation programs of subclinical mastitis with special regard to bovine staphylococci started all over the countries at this time.

The randomised monitoring program with one strain per herd according to Davidson (1961) was used for the development of an international bovine phage set and is the base for solid data to compare the results of today with fourty years ago. German veterinarian universities in cooperation with Robert Koch institute were part of this project investigating *bovine S. aureus* from about 2000 german dairy herds (Meyer, 1966; Gedek, 1967a; Bonin and Blobel, 1967).

Special regard in phage typing with bovine phages had always the phage group IV. About 65 % of strains in all investigations in Germany fourty years ago belonged to phage group IV. These strains were according to Meyer (1966) pigmented, egg - yolk negative, penicillin sensitive, β- haemolytic, clumping factor positive and showed coagulation of bovine plasma. Unfortunately the results of Vintov et al. (2003), representing the biggest international scale screening for *S. aureus* from 10 countries during the last 10 years, was performed by phage typing, but only by using the international human phage set. The lack of bovine phages should be the reason, why the collection with the lowest penicillin resistance (2%) had according to Vintov et al. (2003) also the lowest percentage of non typeable isolates (58%), whereas all isolates of the collection with the highest penicillin resistance (71 %) were sensitive for human phages.

The aim of this chapter is to show the shift of the *S. aureus* populations during the last 40 years. All important human and bovine phages with additional international phages are used to compare the results of Germany in the sixties with special regard to phage group IV. The consideration of all phages and only human phages allow comparisons with results by using only human phages. A further aim was also to show the influence of the SCC and clinical mastitis with frequent use of penicillin on the *S. aureus* population analysis in the dairy chain. Therefore bovine strains of different origin in this context were compared with each other. (collection A: National monitoring based on randomized sampling; collection B: strains of herds with high somatic cell counts or cows with acute mastitis; collection C: *S. aureus* from herds with a high frequency of mastitis. In collection C, only antibiotic resistant isolates were considered, which facilitate differences in phage typing in accordance to Gedek and Kopp (1967b).

4.1.3.2 Results

4.1.3.2.1 S. aureus population in Germany during 1998-2003

The results of collection A representing strains from a national monitoring program agreed to a high degree with results obtained from collection B, whereas penicillin resistant strains in collection C from herds with a high frequency of drugs use showed considerable differences (Table 12). More than 90 % of all strains were typable in all collections with 30 phages considering ≥ 20 plaques. About 36 % of isolates in collection A and B were typable, and less than 66 % of strains showed strong phage reactions using only human phages.

Table 12 Results of phage typing in collections A, B and C considering only strong phage reactions with confluent lysis (>1000 plaques: SCL, CL) or all phage reactions ≥ 20 plaques with a mixed phage set (30 phages) in comparison to the human set comprising only 22 phages

Phage groups	Phage reactions mixed phage set (%)			Phage reactions human phage set (%)		
	Collection A Germany (n=213)	Collection B Mastitis (n=72)	Collection C Resistant (n=68)	Collection A Germany (n=213)	Collection B Mastitis (n=72)	Collection C Resistant (n=68)
	≥ 20 / >1000	≥ 20 / >1000	≥ 20 / >1000	≥ 20 / >1000	≥ 20 / >1000	≥ 20 / >1000
Phage group I	1 / 1	- / -	- / -	12 / 7	11 / 1	- / -
Phage group II	1/ 1	- / -	5 / 2	2 / 3	- / -	5 / 2
116[a]	3 / 3	3 / 3	9 / 6			
Phage group II all	4 / 4	3 / 3	11 / 8			
Phage group III	1 / 2	- / -	3 / 9	5 / 5	7 / 10	5 / 36
Miscellaneus humane:						
81[b]	-/-	- / -	- / -	1 / 1	6 / -	5 / 2
95	-/-	- / -	1 / 1	1 / 1	- / -	2 / 2
96[c]	2 / 8	- / 7	3 / 4	3 / 11	6 / 10	4 / 5
187	-/-	- / -	- / -	- / -	- / -	2 / -
812	-/-	- / -	- / -	- / -	- / -	- / -
Human phage groups[d]	8 / 15	3 / 10	18 / 22	29 / 28	41 / 23	27 / 49
Bovine phage group IV	15 / 17	11 / 17	5 / 13	Phage 42D of the old international basic set:		
42D[e]	- / -	1 / 1	- / -	3 / 4	4 / 1	- / -
Miscellaneus bovine:						
78	15 / 16	15 / 17	- / -			
118	- / -	- / -	- / 3			
119	1 / 18	1 / 24	- / -			
Bovine phage groups	31 / 51	29 / 58	5 / 16			
Mixed groups (NI)	57 / 24	67 / 19	68 / 43	37 / 4	24 / 7	50 / 18
Not typable (NT)	4 / 9	1 / 13	9 / 19	36 / 68	35 / 70	25 / 33
Phage types:						
Numbers	107 / 58	42 / 25	44 / 31	*53* / 35	28 / 11	33 / 21
Phage types per isolate	2.0 / 3.7	1.7 / 2.9	1.5 / 2.1	4.0 / 6.1	2.6 / 6.5	2.1 / 3.2

[a] The single bovine phage of the bovine phage set in the phage group II

[b] They belong in the human phage set to phage-komplex 80 (Parker, 1983).

[c] This phage belongs to the 94/96 complex, phage group V in the human phage set.

[d] Including the single bovine phage 116 of the phage group II

[e] The single human phage in the human basic phage set of phage group IV

Significant better results regarding strong phage reactions (67 %) and a higher prevalence of typable isolates were observed only in collection C (Table 12). Some strains of phage group IV (especially from collection C) are classified to mixed phage groups considering phage reactions with ≥ 20 plaques. The heterogeneity of phage group IV was only observed in all three collections when also considering phages 108 and 111.

4.1.3.2.2 Heterogeneity of phage group IV

The high variability regarding phenotypic characteristics of strains in phage group IV and their relevance in the past is the reason why the obtained results have to be discussed in more detail (Table 13). Strong phage reactions (> 1000 plaques) only were considered for classifying isolates in lyogroup IV. This allows for better comparison with the results in the past because the isolates 40 years ago were usually all typable at RTD. Only when the strains had no strong reactions weak reactions were used. The distribution of penicillin resistance isolates in phage group IV was 53 % in collection A and 67 % in collection B. The penicillin resistant isolates in phage group IV represented 32 % of all penicillin resistant isolates in collection A, 23 % in collection B and only 5 % of all penicillin resistant strains in collection C. The sensitivity for phage 42D of isolates belonging to phage group IV was very low, except for phage type number 1 in collection A (Table 13). The opposite was observed for the additional phages 108 and 111.

The number of phage types increased with the additional use of phage 108 and 111 from nine to 18. Six of 47 strains (13 %) in phage group IV could not be phage typed without using phage 108 and 111. 44 % of the isolates in collection A of phage group IV and 7.5 % of all strains in collection A belonged to phage types 1 and 2 (Table 13). 64.3 % of all 14 strains of phage groups IV being sensitive to phage 42D belonged to phage type number 1. All strains of this phage type were penicillin sensitive and homogeneous regarding haemolysis on BA.

Phage type 108 was the only phage type with single isolates in each collection. The isolates of phage type 108 were predominately egg-yolk positive and penicillin resistant. The other strains of phage group IV in collections B and C were usually classified in different phage types and no isolates could be classified to phage type number 1. The heterogeneity of these frequently penicillin resistant isolates was confirmed through the varying haemolysis and egg-yolk reaction.

Table 13 Phage types and phenotypes of isolates in phage group IV with the bovine set and all phages (numbers of phage types with all six bovine phages in brackets; Pen+= penicillin resistant; CF+ = clumping factor positive; Lez+= egg-yolk positive on BP; α/β/αβ-haemolysis or no or weak haemolysis on BA)

Number		Phage types (additional phages)	Collections (n)			Phenotypes (n)						
			A Germany (n= 36)	B Mastitis (n=15)	C Resistant (n=9)	*All (n= 47)*	Pen+ (n= 29)	Lez+ (n= 22)	α (n=3)	αβ (n= 20)	β (n= 13)	Other[a] (n= 11)
1	(1)	42D /102/107/ 117/ (108/ 111)	9			*9*	0	0	0	2	7	0
	(2)	42D /102 / 117/ (108/ 111)	1			*1*	0	1	0	0	0	1
2	(3)	42D /102 /107/ (108/ 111)		2		*2*	2	0	0	2	0	0
	(4)	42D /102 /107/ (111)	1			*1*	1	0	0	1	0	0
3	(5)	102/ 107/ (108/ 111)	7	6		*13*	13	10	2	2	0	9
	(6)	102/ 107/ (108)		1	1	*2*	2	1	0	0	1	1
	(7)	102 /107/ 117/ (108/ 111)	4	1		*5*	3	2	0	5	0	0
4	(8)	102 /107/ 117/ (111)	1	1		*2*	2	1	0	1	1	0
	(9)	102 /107/ 117/ (108)	1			*1*	1	0	0	1	0	0
	(10)	102	3	1		*4*	1	1	0	2	1	1
5	(11)	102/ (108)			2	*2*	2	0	0	2	0	0
	(12)	102/ (108/ 111)	1			*1*	1	1	0	1	0	0
6	(13)	117	2		1	*3*	3	3	0	2	0	1
	(14)	117/ (111)	2			*2*	2	2	0	0	1	1
7	(15)	107/ (108)	1			*1*	1	1	1	0	0	0
	(16)	107/ (108/111)			1	*1*	1	0	0	0	1	0
8	(17)	42D		1		*1*	0	0	0	0	1	0
9	(18)	102 /117/ (111)	1			*1*	0	0	0	1	0	0
10	(19)	107/117/ (111)			1	*1*	1	1	0	0	1	0
NT	(20)	(108)	1	3	2	*6*	5	5	1	3	0	2
NT	(21)	(108/111)			1	*1*	1	0	0	0	0	1
NT	(22)	(111)	1			*1*	1	1	0	0	1	0

[a] Strains with weak or no haemolysis

4.1.3.3 Discussion

For the creation of the international bovine phage set Davidson (1961a) developed the randomised monitoring program of dairy herds investigating only a single strain per herd. This forms the basis for solid data for the comparison of present day results with those obtained 40 years ago. German academic veterinary departments in cooperation with the Robert Koch Institute were part of the here presented project investigating *bovine S. aureus* from approximately 1,000 German dairy herds (Meyer, 1966b; Kopp, 1967; Bonin and Blobel, 1967; Gedek, 1972). Additionally, the world wide use of BA, respectively haemolysis as election criteria for *S. aureus* isolates from bovine mastitis guarantees a representative selection of the *S. aureus* population in the sampling periode over the past 50 years. Differences in epidemiological investigations are according to Vintov et al. (2003a,b) mainly caused by the different frequency of using drugs in dairy herds. However, lack of standardized monitoring programs, as well the use of the same phage set, have been distinct deficiencies in many epidemiological investigations in the past. The collaborative approach for the development of the bovine phage set is one example regarding these problems (Appendix: Table 31-34).

The resistance to penicillin was always in the focus when investigating the bovine *S. aureus* population in dairy herds. The prevalence of penicillin sensitive isolates of phage type 78, respectively phage group I, for clinical mastitis has already been observed by Edwards and Rippon (1957), but this phage type includes only single isolates together with the penicillin resistant strains of phage group III, in comparison to the usually penicillin sensitive phage type 42D. The risk of acute bovine *S. aureus* mastitis increases according to Gudding (1980) with sensitivity for penicillin, production of α-, phage type 78, no production of antibodies, the time since calving, and young cows. The great importance of penicillin sensitive bovine strains causing bovine mastitis despite of the frequent use of drugs should be seen as the main reason for the little increase in prevalence of penicillin resistant *S. aureus* in many resistance monitoring programs during the past 50 years. This is also one specific reason for the high distribution of single clonal *S. aureus* complexes in monitoring programs, due to the great importance of single phage types as observed for phage patterns with strong phage reactions for phages 78, 119, 96 and phage group IV.

The significant higher number of non-haemolytic strains, or strains with weak haemolysis in collection B and C, in comparison to collection A, indicates that the isolation of *S. aureus* on BA leads to an undervaluation of certain phage types, for example phage type 116. The high

number of egg-yolk negative and β-haemolytic isolates in collection A, B and C shows that the shift in the current bovine *S. aureus* population is caused by biotypes with typical bovine characteristics. Frequently, these bovine strains are typable only by using additional bovine phages, which however belong in many cases not to the current international bovine phage set. Phage typing using only the HPS , according to Vintov et al. (2003a,b), depends on the sensitivity of isolates from phages of phage group I and III, and provides no information about the distribution of the bovine phage types, particularly in phage group IV and for phage types 78 and 116 (Appendix: Table 36). However, the best evidence for a shift in the bovine *S. aureus* population of Germany, without consideration of the low prevalence of isolates in phage group IV, is the high number of strains belonging to phage types 119, 78, 116 and phage type 96, respectively AC1 (Table 12). In different countries these phage types were usually only detected in low numbers (Davidson, 1972, Appendix: Table 34). 35 % and 51 % of strains in collection A and B, and all penicillin sensitive strains in collection C showed strong phage reactions only for phages 119, 78, 116 and 96. In Germany for example, less than 2 % of strains were sensitive to these phages and it was considered not to include phages 119, 78, 116 and AC1 in the bovine phage set (Davidson, 1961a; Bonin and Blobel, 1967; Gedek, 1972; Davidson, 1972).

The strong reduction of *S. aureus subspecies bovis* in phage group IV to approximately 20 % agrees according to Hummel et al. (1992) with the here presented results when considering only strong phage reactions in collection A (Table 12). 44.9 % of bovine strains from raw milk in Germany were in the early 1960s according to Seelemann et al. (1963) only sensitive to phage 42D when using only the international basic set. Phage 42D is according to Davidson (1961a, 1972), Bonin and Blobel (1967), Meyer (1967), and Gedek (1972) the most important phage of the IPS for typing bovine staphylococci. Only 2 % of all British isolates were still sensitive for phage 42D during the early 1980s, whereas during the 1960s 20 % of all bovine strains showed phage reactions ≥ 50 plaques at RTD for this phage (Caroll and Francis, 1985).

Adesiyun (1995) used phage 42D from the IPS because he believed that this phage has a high sensitivity for typing bovine strains. The low sensitivity of phage 42D (4 %) in phage group IV of collection A, and no positive reaction of phage 42D in collection C however is the best evidence for a strong decrease of isolates in phage group IV due to the frequent use of drugs (Tables 12 and 13). The low prevalence of sensitive bovine strains for phage 42D is the reason why the only use of phage 42D cannot be recommended anymore. However, phage 42D, the single human phage of phage group IV in the old IPS, is one of the most important

phages in the BPS when discussing the shift in bovine *S. aureus* population over the past 40 years; phage 42D is according to Gedek (1972) furthermore the most important phage in phage group IV regarding the discriminatory efficiency for many isolates belonging to this phage group. Despite the strong reduction of phage group IV, still at least 13 % of all strains in collection C belong to phage group IV or closely related phage types, when considering weak phage reactions.

The high prevalence of *S. aureus* of phage group IV in dairy herds, as according to Hummel et al. (1992) it used to be the case in ancient Mongolian herds, should have its reason in the adaptation of this *S. aureus* type to long IMI. Gedek (1972) was able to show that these strains can persist in the mammary gland of cows for more than one lactation period. The distinct characteristic of bovine *S. aureus* mastitis with a long duration is the persisting colonisation of the epithelium cells in the udder cistern (Sordillo et al., 1989). The coagulation of bovine plasma of *S. aureus suspecies bovis* in phage group IV is the virulence factor, which is the most relevant evolutionary adaptation process of bovine mastitis in this context (Appendix: Table 22). The strong decrease of phage group IV combined with an increasing penicillin resistance in national monitoring programs from about 6 % to now 53 % is according to Meyer (1966a) and Gedek (1972) the consequence of frequent use of penicillin. This is in agreement with the findings of Østeras et al. (1999) who were able to show that the increasing penicillin resistant of bovine *S. aureus* strains is associated to the preventive application of antibiotics during the dry off period. The low prevalence of isolates in phage group IV of collection C however indicates that its strong reduction in herds with frequent use of drugs is also caused by antibiotic treatment during the lactation period.

Vintov et al. (2003a) observed a shift in the *bovine S. aureus* population during the 1950s and 1990s using only the human phage set, which was mainly due to the only use of penicillin for the treatment of bovine mastitis (Appendix: Table 36). In accordance to Sabolic et al. (1980) and Krabisch et al. (1999) a penicillin resistance of 40 % has to be the consequence of frequent use of drugs in combination with older cows in dairy herds. Randomly selected herds showed about half the number of penicillin resistant *S. aureus* strains in comparison to herds with a frequent use of antibiotics. The isolates from the 1950s represent herds with apply of penicillin to eradicate *Str. agalactiae* a penicillin resistance of 11.6 %, whereas the isolates from random selected herds in the 1960s have according to Vintov et al. (2003a) only 5 % penicillin resistant strains. Krabisch et al. (1999) found in randomly selected herds 23.8 % penicillin resistant *S. aureus* strains in comparison to dairy herds with a high occurrence of mastitis with 47.8 % penicillin resistant *S. aureus* strains.

The low average of the BMSCC in German dairy herds of approximately 171,000 SCC/ml, according to IDF-Bulletin (2001), is one explanation for the considerable lower level of penicillin resistant strains (approximately 25%) in national monitoring programs. According to Sobiraj et al. (1997) a penicillin resistance of about 40 % in 63 collections from veterinary practices from all German districts,with low variability, agrees with the results of Tenhangen et al. (2006), as well as with our results from collection B, and confirms the connection to herds with high BMSCC. The distribution of 26 % penicillin resistant staphylococci from cows in the 1st lactation, and the 39 % penicillin resistance of older cows according to Rataja-Schulz et al. (2004), further demonstrates the great importance of the number of lactations in resistance monitoring programs. An up to 40 % prevalence of penicillin resistant *S. aureus* strains is therefore explainable with a high number of samples obtained from older cows. With exception of the USA, all countries with a penicillin resistance over 30 % have according to IDF-Bulletin (2001) an average of three, four or more number of lactations (Vintov et al., 2003b; Appendix: Table 36).

According to Davidson (1972) and Vintov et al. (2003b), the countries which displayed a high prevalence of isolates in phage group IV in the 1960s have 40 years later a high prevalence of penicillin sensitive *S. aureus* isolates in phage group I (Appendix: Table 36). This could be partially explained with a larger number of younger cows. Obviously, the teat canal of younger cows is fast colonized with clumping factor negative *S. aureus* types (phage types 119, 96) or *S. aureus types*, such as phage type 78, wich have possibly a higher persistence in deeper udder cistern. However, the high prevalence of *S. aureus* belonging to phage group I, complex-80, respectively phage type 119 in the bovine phage set, is much more the result of systematically segregating, treating or culling of cows with subclinical mastitis. This leads finally to the elimination of the dominating strain in each herd. According to Davidson (1972) the results of the Danish laboratory in comparison to the Danish strains of Vintov et al. (2003a), are the best example from the 1950s in this context (Appendix: Table 36 and 33, collaborating laboratory 3).

In the 1960s, according to Davidson (1972) the observed high prevalence of strains (43 %) belonging to phage group II, and a prevalence of 16 % of isolates belonging to phage group I, respectively phage type 119, in Danish dairy herds, did not confirm the results from the 1950s of Vintov et al. (2003a). The prevalence of only 12.9 % strains in phage group II and 25.6 % of isolates in phage group I belonging to the phage pattern group I/119 observed in the 1950s agree more with results from the 1990s (with less than 6.0 % of strains in phage group II, and about 20 % of strains in pattern group I/119; Appendix: Table 36). The high similarity

regarding the same level of the two most relevant subpopulations in the collections of the 1950s and the 1990s can only be explained with a frequent use of anibiotics treating clinical mastitis or the culling of older cows with high SCC. The results obtained from the Danish herds confirm not only the fast reduction of the dominating *S. aureus* population over a period of few years. These results also show that the frequent use of drugs in herds with high prevalence of *Str. agalactiae* during the 1950s had a similar effect as the treatment of cows with high SCC. The first effect of antibiotcal treatment is the reduction of the dominating subpopulation without an increased in prevalence of antibiotic resistant strains (Appendix: Tables 36, 37).

Additionally to the low sensitivity of phage 42D, the heterogeneity of isolates in phage group IV is one more important observation when discussing the shift in the dairy chain over the past 40 years (Table 12, Appendix: Table 34). High prevalence of penicillin resistant, egg-yolk positive and no β- producing strains in phage group IV is the opposite to what was observed for the isolates in this phage group 40 years ago (Meyer, 1966b; Gedek, 1972, Table 13). In the past, between 11% and 24 % of the bovine population belonged to the penicillin sensitive phage type 1 (Table 13). These are according to Meyer (1966b) and Gedek (1972) the only strains with the typical characteristics of *S. aureus subspecies bovis*. Only in collection A at a very low level of only 3% is phage type 1, the common type 40 years ago, still detectable. The mulitresistant strains cultured from bulk milk in Austria belong all to phage group IV, possibly a consequence of a frequent use of drugs in small herds because of low BMSCC limits (Appendix, Table: 23). According to Vintov et al. (2003b) the great concern regarding bovine phages of phage group IV was in many countries confirmed by the wide distribution of non-typable penicillin resistant strains with the HPS. Similarly, most penicillin resistant typable bovine strains from Tansania were only sensitive to bovine phages of phage group IV (results not shown).

40 years ago, most strains of phage group IV were sensitive to all phages of phage group IV (Meyer, 1966b; Bonin and Blobel, 1967; Giesecke et al., 1972; Gedek, 1972). Today, most *S. aureus* isolates in phage group IV are predominately sensitive only to single phages (Table 13). The results obtained by Giesecke et al. (1972) show the low sensitivity of phages from phage group IV for penicillin resistant strains, and confirm the great importance of the actual *S. aureus* population for the creation of a phage set (Appendix: Table 29). 72.9 % of all strains in phage group IV were still typable at RTD (Giesecke et al., 1972). However, the minority of isolates in phage group IV, which were only typable at 1000×RTD, included 66.1 % of all penicillin resistant isolates of phage group IV (Giesecke et al., 1972). Until the early

1960s the high prevalence of non-typable isolates with human phages were usually penicillin sensitive, often showing a high sensitivity for phage 42D (Gedek, 1972). When Smith (1948a,b) and Davison (1961a) searched new specific phages in mastitis milk for the typing of bovine strains, most new suitable phages belonged to phage group IV. The bovine phages of phage group IV showed usually a higher sensitivity in comparison to the human phage 42D. The low specivicity of phage 42D should be the reason why only the bovine phages are sensitive for penicillin resistant strains of phage group IV (Gedek, 1972; Gisecke et al., 1972; Abbar et al., 1986). The increased discriminatory efficiency by using phage 42D justifies the use of human phages, despite their low sensitivity for many bovine strains. The lower sensitivity of bovine phages in phage group IV for penicillin resistant strain in comparison to 40 years ago is a further argument for the necessity of typing all isolates at 100×RTD.

The high prevalence of mixed phage groups comprising human phage groups and the bovine phages of phage group IV was discussed in chapter 4.1.2. The results of Vintov et al. (2003a) indicate as well an increasing prevalence of bovine strains in human mixed groups (phage group I, phage group III and phage 95) with the HPS during a longer period covering several decades (Appendix: Table 36). According to Lombai et al. (1976) the intermediate bovine-human, often penicillin resistant phenotypes which are sensitive to different human and bovine phages, are predominately isolated from cows with only elevated SCC but not clinical signs, contrary to isolates from acute mastitis. The isolates contained in collection C are the best evidence for the high prevalence of penicillin resistant strains in mixed groups (Table 11). 43 % of *S. aureus* strains in collection C belong with the combined set to mixed groups and a high number of different phage types when only strong phage reactions are considered. Furthermore, collection C has in relation to the number of isolates the highest number of different phage types. The sensitivity for many bovine phages of the BPS is strong reduced and many strains are sensitive only to single phages from one phage group. The frequent use of antibiotics further leads to a high prevalence of intermediary types in phage group III and IV so that the phage groups III and IV are no longer valid anymore.

The increasing number of individual phage patterns (“one patient-one strain”) and cross lysis by phages of phage groups I, II and III, such that this grouping was no longer epidemiologically valid, has been still observed in cases of human *S. aureus* from hospitals (Zierdt et al., 1980). The frequent use of antibiotics in the hospital 40 years ago was obviously the reason for the shift in the population to many *S. aureus* strains belonging to mixed groups or having a sensitivity for phages of different phage groups.

4.1.3.4 Summary

The shift of the *S. aureus* subpopulation associated with penicillin can be only proven with international bovine phages, which are used since more than fourty years. The treatment of mastitis with penicillin in the last 40 years reduced in Germany the dominating subpopulation of phage group IV from about 65 % to now < 20 %. The penicillin resistance of the *S. aureus* in phage group IV increased form < 10 % to > 50 %. This reduction was compensated through the increase of the penicillin sensitive phage types 119 and 78 to about 25 % respectively 17 %. The isolates in phage group IV and phage type 78 were not typeable (NT) with the human phage set. The isolates of phage type 119 lead to the high numbers of strains with weak phage reactions in phage group I or I and II when using only human phages. These phage types are not only the most important strains in acute mastitis. They are also responsible for the still low level of penicillin resistant strains in the dairy chain. The increasing number of penicillin resistant isolates in dairy herds with high somatic cell counts or frequency of mastitis resulted in a significant increase of phages in phage group III and phage 81 ($P < 0.003$) usually together in mixed groups with phages of phage group IV. Important variables, inducing the variability of typing results in the bovine *S. aureus* population, are discussed.

4.1.4 Comparability and discriminatory efficiency of various typing methods

In combination with the isolation of microorganisms typing methods are the most important influencing factor when investigating the biodiversity of bacteria populations. The suitability of typing methods can only be determined when comparing different methods with each other (Tenover et al., 1994). Suitable typing methods for the determination of different pathogens in epidemiologic investigations have to be able to efficiently discriminate between outbreak strains and isolates from other sources. This characteristic is the basis for an adequate assessment of the quality of hygienic measurements. A further criterion for the suitability of typing methods is their agreement with respect to antibiogram typing, virulence factors and biotyping. The more the typing method is able to keeps systematic information through clustering of strains with the same crucial characteristics, the more relevant criteria in the course of a disease can be assessed.

4.1.4.1 Problem and Goal

Different genotyping methods are available since now about 20 years and are used for typing bovine strains. Difficulties comparing genotyping methods and the relevancy of biotyping for bovine *S. aureus* were soon discussed (Lange et al., 1999). In previous studies good accordance between different genotyping techniques and phage typing in characterisation of bovine *S. aureus* has been found (Schlichting et al., 1993; Aarestrup et al., 1995b), and for large scale screening phage typing has previously proved valuable for both human and bovine strains (Larsen et al., 2000). None of these workers however compared genotyping methods with the common human and bovine phage sets using bovine strains. Some phage types of bovine strains with the human phage set belonged according to Aarestrup et al. (1995) to different ribotypes. Additionally the Fournier transformed infrarot spectroscopy (FTIR) was used in accordance to Helm et al. (1992). This is the single method, which want to consider all different biochemical components of the whole cell in their mathematical calculations. In my knowledge nobody has compared for bovine strains typing results obtained through genotyping (PFGE), FTIR - cluster analysis and phage typing using human and bovine phages together with all important phenotypic characteristics. Such comparison is necessary in order to asses the reliability of typing methods and the most likely important information which is missed out on when using only phage typing.

The aim of this chapter is to investigate the comparability of traditional and modern typing methods. Finally, this approach is the only possible way to confirm the shift in the *S. aureus* population over the past 40 years with different phenotypic characteristics of bovine isolates. Very important in this context is phage typing with different phage sets using bovine and human phages and the strength of phage reactions determining different phage types. These results are compared with pulsed field gel electrophoresis (PFGE), enterotoxine profiles and FTIR- cluster analysis to each other. The biochemical characteristics are determinated in accordance to Lange et al. (1997). The high correlation of penicillin resistance and clumping factor reaction to certain genotypes according to Fitzgerald et al. (2000) was the reason, why these characteristics were additionally used for typing. Lack of comparability between different typing methods or typing methods and phenotypic characteristics are discussed in consideration to the latest findings.

4.1.4.2 Results

4.1.4.2.1 Biochemical characteristics (Biotyping)

All 72 isolates of collection B were confirmed as *S. aureus* according to the API ID 32 staph with at least 80 % probability. The biochemical characterization yielded in 14 different biochemical profiles (Table 14). Few strains showed only weak reactions (w) for the biochemical characteristics. 54 % of the isolates were contained in profile 1, whereas the last six profiles were represented by a single isolate.

The isolates showed only positive, but variable reactions for Urea (Ure), Arginin (Adh), Esculin (ESC), Trehalose (Tre), Manitol (Man), Nitrat (Nit), Voges-proskauer reaction (VP), β-Galacasidose (β-Gal), Turanose (Tur), Raffinose (Raf); Celobiose (Cel) and Ribose (Rib).

Table 14 Biochemical profile in the API ID 32 staph of the 72 *S. aureus* isolates

(-= negative reaction; + = positive reaction; w= weak positive reaction)

Profile	Number of isolates	Biochemical reactions of ID 32 Staph											
		Ure	Adh	ESC	Tre	Man	Nit	VP	ß-Gal	Tur	Raf	Cel	Rib
1	39	-	+/ w	-	+	+	+	+	-	+/ w	-	-	-
2	4	-	+/ w	-	+	+	+	+	-	-	-	-	-
3	5	-	+	-	+	+	+	-	-	+	-	-	-
4	5	-	+	-	-	+	+	+	-	+/ w	-	-	-
5	6	-	-	-	+	+	+	+/ w	-	+	-	-	-
6	2	+/w	+	-	+	+	+/ w	+/ w	-	+	-	-	-
7	2	-	+/ w	+	+	+	-	+	-	+	+	w	+
8	2	-	+	-	+	-	+	+	-	-	-	-	-
9	1	-	w	+	+	+	+	+	-	-	-	-	-
10	1	-	w	-	+	+	-	+	-	+	-	-	-
11	1	-	-	-	+	+	+	+	+	+	-	-	-
12	1	-	-	-	-	+	+	+	-	+	-	-	-
13	1	-	+	-	+	-	+	+	-	+	-	-	-
14	1	-	+	-	+	+	-	+	-	+	-	-	+

4.1.4.2.2 FTIR-analysis and Puls-field-Gel-Electrophoreses (PFGE)

The FTIR-cluster analysis consists of 28 profiles considering the reproducibility of the method (Figure 1). The *S. aureus* isolates in clusters 6, 8, 11 and 24 can always be grouped in different subclusters which are distinguishable from each other, but showed a low validity for a single cluster in different measurements.

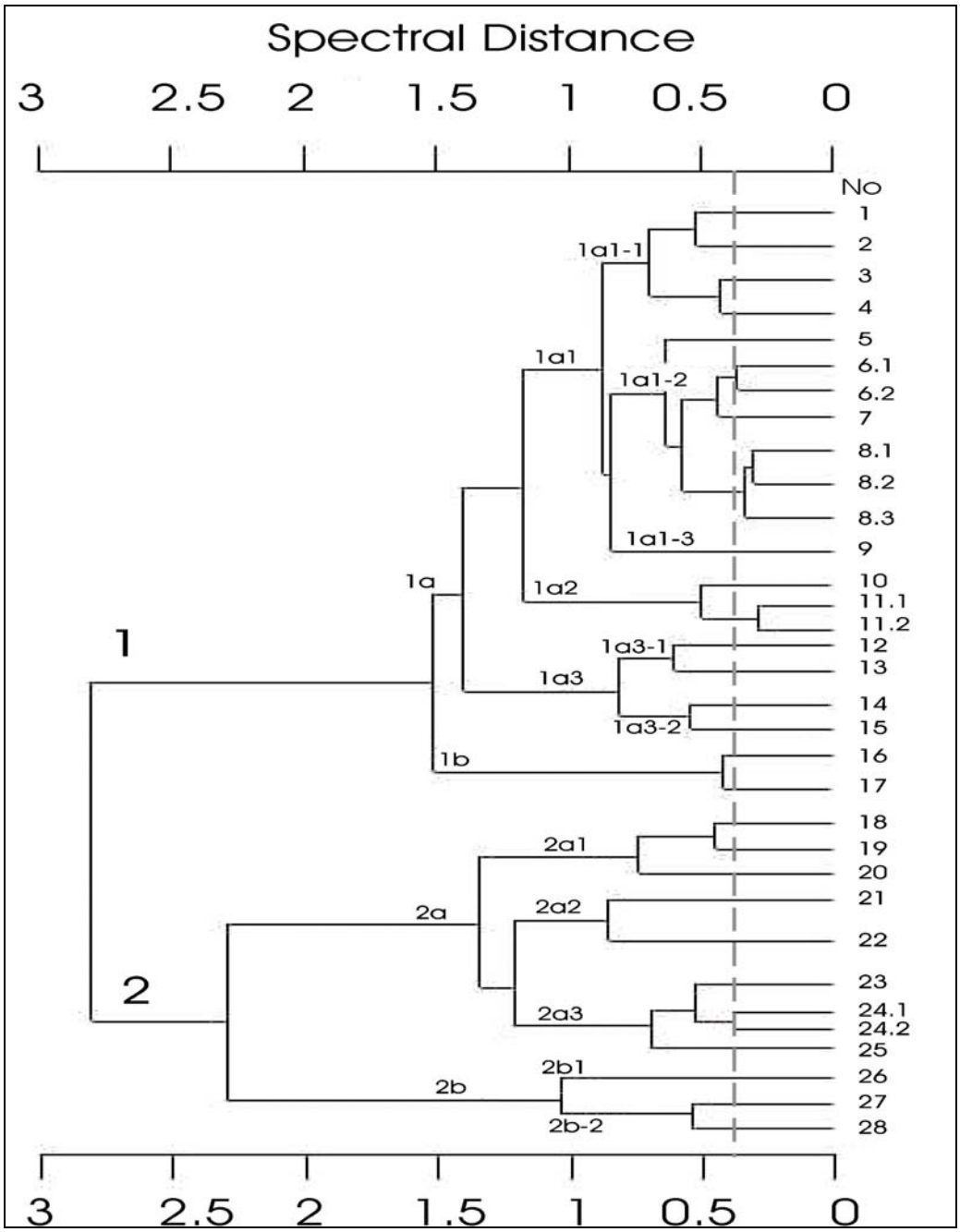


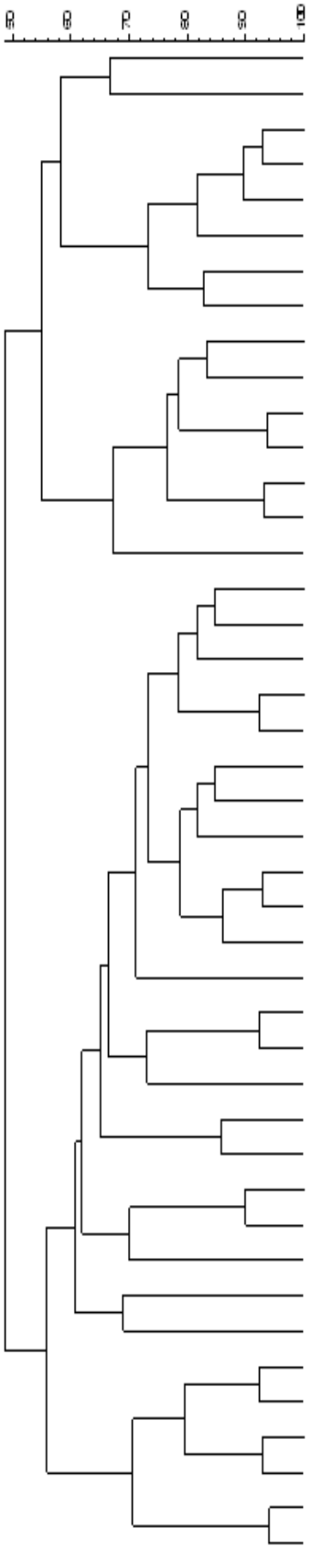
Figure 1 FTIR-Dendrogramm obtained from cluster analysis (Average Linkage method; Frequences ranges with weights and reproducibility level: 700-900 cm^{-1} /1/30, 1200-1350 cm^{-1} /1/30, 2800-3000 cm^{-1} /1/30), the vertical line shows the reproducibility of the method)

All the measurements from these isolates with a spectral distance over 0.4 belonged to clusters 1a1, 1a2 or 2a with a spectral distance difference of 1.4 at maximum (Figure 1). Only single measurements of the isolates in subcluster 11.1 were grouped to the cluster 22. One isolate in subcluster 11.2 showed no stability in clusters 1a1 and 1a2. All isolates of the subclusters 8.1, 8.2, 8.3 and some isolates of the subcluster 11.2 had different results in phenotypic characteristics before and after freezing.

69 of 72 isolates showed 43 different macrorestrictions profiles by using PFGE (Figure 2). PFGE of three strains was accompanied with methodological difficulties. These three isolates were not added to the cluster analysis because of a possible wrong calculation. The best correlation considering all characteristics was found by a similarity cut-off with 65 %. This however was still quite difficult. Except of single outliers, some strains showed still considerable differences regarding phenotypic characteritics and at 65 % simililarity the difference between some clusters and subclusters was very small. The adition of single strains influenced therefore the grouping in corresponding clusters.

When using a 65 % similarity cut-off, the strains were grouped into seven clusters with the best correlation to the phage profile, especially for strong phage reactions (Figure 2). Cluster one (PFGE-profile 1-2) harbouring with exception of one strain all isolates with a high sensitivity for phage 96. Cluster two (PFGE-profile 3-8) contains all penicillin sensitive isolates with a high sensitivity for phage 78. All isolates in cluster three (PFGE-profile 9-15) showed, with one exception, all a high senstivity for phage 119. The other one (PFGE-profile 13) with a high sensitivity for phage 116 was positive for *seg* and *sei* like the most other strains in this cluster. Cluster four (PFGE-profile 16-32) contains the most isolates, which were all sensitive for phages of phage group III and IV. Two of three isolates in cluster five (PFGE-profile 33-35) were additionally positive for *sed* and *sej*.

One of the two penicillin resistant strains in cluster six (PFGE-profile 36-37) harbouring the single strain with only a weak reaction for phage 119. All isolates in cluster seven (PFGE-profile 38-43) were penicillin resistant, *se* negative and were lysed all by human and bovine phages, which belong to no phage group. The strains in PFGE-profile 42-43 were in comparison to the other isolates in cluster seven egg yolk negative and belong probably to a different subcluster.



PFGE-profile (n)	Phage pattern* (weak)	Enterotoxin profile (se)	Penicillin resistance
1(2)	119	C/tst;C GI/tst	sensitive
2(5)	96	GI	sensitive
3(8)	78	negative	sensitive
4(1)	78	negative	sensitive
5(1)	78	negative	sensitive
6(1)	78	negative	sensitive
7(1)	78	negative	sensitive
8(1)	78	negative	sensitive
9(1)	119	GI	sensitive
10(2)	119	GI	sensitive
11(2)	119	C/tst;GI	sensitive
12(9)	119	neg;GI;C GI/tst;C/tst	sensitive
13(1)	116	GI	resistant
14(2)	119	C GI/tst;C/tst	sensitive
15(2)	119	GI	sensitive
16(1)	IV	negative	resistant
17(1)		negative	sensitive
18(1)	IV	negative	resistant
19(1)		negative	sensitive
20(1)		negative	sensitive
21(1)	IV	J	resistant
22(1)	IV	J	resistant
23(1)	IV	negative	sensitive
24(1)	IV	negative	resistant
25(1)	IV	negative	resistant
26(1)		negative	resistant
27(1)	IV (w)	negative	sensitive
28(1)	IV (w)	negative	resistant
29(1)	NT	negative	resistant
30(4)	IV	negative	resistant
31(1)		negative	sensitive
32(1)		H	sensitive
33(1)		DJ	resistant
34(1)		DJ	sensitive
35(1)		negative	resistant
36(1)	119 (w)	negative	resistant
37(1)	116	negative	resistant
38(2)		negative	resistant
39(1)		negative	resistant
40(1)		negative	resistant
41(1)	78	negative	resistant
42(1)		negative	resistant
43(1)		negative	resistant

Figure 2 Dendogramm of PFGE shown (Dice Opt: 3 %; Tol 1.0 %- 1.0 %)

4.1.4.2.3 Correlation between the sum of phenotypic characteristics and different typing methods

The results in Figure 2 and Table 8 show the great importance in considering the enterotoxigeneity, Penicillin resistence and strong phage reactions. The necessity in considering other characteristics as well is shown in Table 15. The variability of election criteria, and enterotoxigeneity, Penicillin resistence and clumping factor activity in the phage profiles was according to Table 15 usually correlated with different *S. aureus* types, independently of the typing method used. The respective correlation between the election criteria, or phenotypic characteristics, and the phage group profile and phage groups was only high for the the four phage types 119, 96, 102/107/108/111 and 78 according to Table 4 and 9. Except of single isolates the majority of all other strains belonged to single genotypes in accordance to the combinations of all other characteristics or methods. Especially the penicillin resistant isolates were sensitive for phages of phage group IV and were separated by PFGE in a high number of genotypes with various combinations of charateristics.

All isolates which showed only strong reactions for phage 96, 78 and 119 belonged with single exceptions all to the FTIR-cluster 2a (Table 7, Figure 1). Only two strains of phage type 78 were associated with the FTIR-cluster 1a. All isolates with a high sensitivity for phages of phage group IV were grouped with FTIR to the big cluster 1 (Figure 1). This completely agreed with PFGE-dendrogram when considering a similarity index of 60 %. Considering only strong reactions together with strains in the phage profiles 5 and 27 (Table 7), all isolates of phage type 96 and 119 belonged to the big cluster with PFGE-profiles 1- 15 (Figure 2)**.** The detailed analysis according to Table 15 revealed a high correlation between the phage types in certain phage profile to the FTIR-cluster and PFGE-cluster analysis shown in Figure 1 and 2. The high agreement between PFGE-profiles, FTIR-profiles and phage-profiles justifed grouping of phage profiles according to the common phage groups together with the consideration of weak phage reactions.

All isolates in phage group profiles 1-16 were sensitive to phages of phage group I. They contained 82 % of the αβ-haemolytic isolates and, with exception of one isolate, were all clumping factor negative strains. 34 *S. aureus* strains (47%) in phage profiles 17-28 according to Table 7 were not sensitive to phages of phage group I/ 96, or showed only weak phage reactions for phage 119, with exception of two strains. These phage profiles contained 81 % of all penicillin resistant isolates and were the only profiles harbouring strains with strong egg-yolk reactions. Except of one strain they belonged as well all to the PFGE-profiles from

Table 15 Isolates (n) ranked according to the PFGE-Dendogram in Figure 2 and correlation between different phage sets, other typing methods, election criteria and se- Profile, Penicillin resistence and clumping factor activity (other characteristics) (+ = > 90% positive, CF+; -= < 90% negative; CF-; v= 10-90 % positive)

PFGE profile (Figure 2) (n)	Phage Profile (Table 7)	Phage types each phage set				FTIR profile (Figure 1)	API profile (Table 14)	Election criteria			Other characteristics		
		All:	BPS:	IPS:	HPS:			CoA-Profile (Table 5)	BA[1]	BP[2]	SE-Profile (Table 4)	Pen[3]	CF
1 (2)	4, 15	1,2	**1**	1, 2	1, 2	24.1/2	2, 8	1, 2	αβ	-	1, 2	-	-
2 (5)	13-14	3-6	**2-4**	3, **4**	3- 5	20	4	0	αβ[b]	+	4	-	-
3 (8)	27	**7**	**5**	NT	NT	1, 11.1, 22	11, 1	4, 7, 8	α	-	0	-	+
4 (1)	27	**7**	**5**	NT	NT	22	1	8	α	-	0	-	+
5 (1)	27	**7**	**5**	NT	NT	22	1	7	α	-	0	-	+
6 (1)	27	**7**	**5**	NT	NT	22	1	7	α	-	0	-	+
7 (1)	27	**7**	**5**	NT	NT	11.1	1	8	α	-	0	-	+
8 (1)	27	**7**	**5**	NT	NT	22	1	8	w	-	0	-	+
9 (1)	15	**8**	**6**	**1**	**1**	24.2	5	2	αβ	-	4	-	-
10 (2)	16	9	7	5	6	21	1, 5	2	αβ	-	4	-	-
11 (2)	4	**2**	**1**	**2**	**2**	18, 23	1, 2	1, 2	αβ	-	2	-	-
12 (9)	4-6, 15	**1, 2,** 10-14	**1, 3**	**1,2, 4,** 6	**1,2,** 7- 11	18, 23, 24.1/2, 25	1, 2, 10 13, 14	0-4	αβ, β	-	0-4	-	-
13 (1)	19	**15**	**8**	NT	NT	3	6	7	α	+[c]	4	+	+
14 (2)	6	**1, 2**	**1**	1, 2	1, 2	18, 24.1	8, 1	2	αβ	-	1,2	-	-
15 (2)	6, 15	**1, 2**	**1**	**1, 2**	**1, 2**	24.2	1	2	αβ	-	4	-	-
16 (1)	26	**16**	NT	NT	NT	8.1	14	6	αβ	+	0	+	w
17 (1)	16	17	9	7	12	11.2	1	7	αβ	-	0	-	-
18 (1)	26	18	10	NT	NT	8.3	1	8	αβ	+	0	+	+
19 (1)	12	**19**	**11**	8	13	26	1	8	k	-	0	-	+
20 (1)	12	**19**	**11**	8	13	26	1	8	w	-	0	-	+
21 (1)	26	**20**	**12**	**9**	NT	10	7	8	αβ	-	5	+	+
22 (1)	26	**20**	**12**	**9**	NT	10	7	8	αβ	-	5	+	+
23 (1)	26	21	13	NT	NT	11.2	1	8	αβ	-	0	-	+
24 (1)	23	22	14	10	15	12	2	8	αβ	+	0	+	+
25 (1)	26	23	15	NT	NT	2	12	2	k	-	0	+	+
26 (1)	9	24	16	11	16	15	1	5	w	-	0	+	+

Table 15 continued:

PFGE profile (Figure 2) (n)	Phage Profile (Table 7)	Phage types each phage set				FTIR profile (Figure 1)	API profile (Table 14)	Election criteria			Other characteristics		
		All:	BPS:	IPS:	HPS:			CoA-Profile (Table 5)	BA[1]	BP[2]	SE-Profile (Table 4)	Pen[3]	CF
27 (1)	26	25	17	**8**	NT	8.2	1	5	αβ	-	0	-	+
28 (1)	26	**16**	NT	NT	NT	8.3	1	4	αβ	-	0	+	w
29 (1)	0	NT	NT	NT	NT	16	5	6	w	-	0	+	+
30 (4)	23, 25	26, 27	18	12,13	17, 18	3	3	8	k, w	+[c]	0	+	+
31 (1)	10	28	19	14	19	11.2	1	8	k	+[c]	0	-	+
32 (1)	8	29	20	15	20	11.2	1	7	α	+	6	-	+
33 (1)	21	30	21	16	21	7	11	6	k[b]	-	3	+	+
34 (1)	20	31	22	17	22	8.1	1	7	α	+	3	-	+
35 (1)	7	32	23	18	23	14	5	5	αβ[b]	-	0	+	+
36 (1)	28	33	24	NT	NT	4	9	7	k	+[c]	0	+	+
37 (1)	19	**15**	**8**	NT	NT	5	3	8	w[b]	+[c]	0	+	+
38 (1)	2	34	25	19	24	6	1	8	w	+[c]	0	+	+
39 (1)	17	35	26	20	25	27	1	7	w[b]	+[c]	0	+	+
40 (1)	23	36	27	21	26	6	1	7	α[b]	+[c]	0	+	+
41 (1)	18	37	28	22	27	6	6	8	w[b]	+	0	+	+
42 (1)	11	38	29	23	28	11.2	1	8	k	-	0	+	+
43 (1)	12	39	30	24	29	5	1	8	αβ[b]	-	0	-	+
Not included	22	40	31	25	30	17	5	7	α	+[c]	0	-	+
	1	41	32	26	31	19	1	4	αβ	+	0	-	-
	11	42	33	27	32	28	1	6	w	+	0	+	+

[1] Type of haemolysis on Blood agar

[2] Egg-yolk reactions on Baird Parker

[3] Production of penicillinase (Penicillin resistance)

[a] The phage types marlked with bold letters are indistinguishable with corresponding phage set.

[b] Colony with pigmentation on blood agar

[c] Strong egg-yolk reactions after 24 h on Baird Parker

30 to 41 (Table 15). Phage sets containing a limited number of bovine phages and no phages from bovine origin (IPS in Table 15) had a lower discriminatory efficiency in comparison to the consideration of all phages. Smaller phage sets however had a lower percentage of weak reactions from isolates belonging to one clonal complex.

The classification of isolates in phage profiles according to the common phage groups together with the consideration of each single phage and the strength of phage reactions allowed an easier determination of closely related strains especially when using all 30 phages (Table 15). This is especially confirmed by the isolates in the PFGE-profiles 2, 12 and 30. Frequently, the clonal relationship of these isolates is only observed when considering first of all only phage reactions with semiconfluent- and confluent lysis. Additionally, it shows the high comparability with typing results at RTD considering only ≥ 50 plaques according to the international standards. On the other hand the consideration of weak reactions is confirmed through the PFGE- profiles 10, 30 and 49.

The 12 isolates in PFGE- profile 3-8 and phage profile 27 (Table 7) being sensitive only to the bovine phage 78, were the most homogeneous *S. aureus* type. With one exception, all isolates of phage type 78 were contained in the FTIR-cluster 11.2 and 22. The special position of one isolate belonging to phage type 78 was not confirmed by PFGE. Not only was this strain the single clumping factor negative isolate, but also the single strain belonging to FTIR-profile 1 and API-profile 11. On the other hand the isolate in PFGE- profile 37 (Table 7: phage profile 18) was the only strain with a strong reaction only for phage 78 and weak reactions of ≥20 plaques for four other phages, which was confirmed through the resistance test, FTIR-profile (Figure 1; Profile 6), as well the PFGE-type (Figure 2: Profile 41).

The 17 strains (24 %) in the phage type profiles 4, 5, 6 and 15 (Table 7 and 15) were all clumping factor negative and penicillin sensitive and with two exceptions belonged to one PFGE- cluster (Figure 2: PFGE-profile 9-15). The majority of strains (8 isolates) belonged to phage profile 15. The SE-profiles, API-profiles, FTIR-profiles and weak phage reactions varied highly for these 17 isolates. The variability for weak phage reactions of ≥ 20-100 plaques of the phages 52, 55 and 96 using the IPS or HPS or all 30 phages resulted in five or seven more different phage types in comparison to the only consideration of the phages from the bovine set. The other clumping factor negative strains with a strong reaction for phage 119 in phage profile 3 belonged to a different PT and the FTIR-profile 21 confirming the weak reactions of phages 3A, 6 and 111.

Two strains belonging phage profile 5 and 28 (Tables 7; Table 15: PFGE-profile 12 and 36) highlight the importance in considering weak phage reactions. The high reproducibility of weak reactions for the phages 52A, 80, 102 and 117 confirmed the close affinity of the *sec* and *tst* positive strain in phage profile 5 to the phage profiles 4, 6, and 15. The strain in phage profile 5 was also the only isolate with a test value of 1.6 in the vidas-system (Table 4). The additional consideration of phenotypic characteristics and results of PFGE provide a further

evidence that the strain in phage profile 5 belongs to the clonal complex of isolates with only strong reactions for phage 119, whereas the strains in phage profile 28 with only a weak reaction for phage 119 had completely different characteristics. Consequently, this isolate was therefore classified in a different cluster by FTIR and PFGE as well.

The five isolates (7%) of phage type 96 in phage profiles 13 and 14, when considering only strong phage reactions, belonged according to Table 15 all to the same SE-profile (4), API-profile (4), FTIR-cluster (20) and PT (2). Two strains with a strong reaction for phage 119 only and a high similarity regarding phenotypic characteristics represented the PFGE-profile 1 (Figure 2). The strains in phage profiles 13 and 14 were only distinguishable under consideration of weak phage reactions, but not through PFGE, or FTIR-analysis or phenotypic characteristics. They were all penicillin sensitive, CF and coagulase negative in the tube and on BP-RPF, showed all αβ- haemolysis and were egg-yolk positive.

The isolates with a strong reaction for phages of phage group IV showed a high diversity for all tested characteristics except of enterotoxigeneity. All PT`s in the PFGE-profile 16-30 with a high sensitivity for phages of phage group IV except of PFGE-profile 30 contain only one strain and in addition are frequently sensitive for phages belonging to other phage groups (Tables 7 and 15). The PFGE-profile 30 contained four of seven strains of phage type 102/107/108/111, when considering only strong phage reactions. Three of four strains in PFGE-profile 30 belonged to phage profile 23 (Table 7). All four isolates belonged to the same API-profile (3) and were classifed to the same FTIR-profile (3) (Figure 1, Tables 15 and 16). The other three isolates of phage type 102/107/108/111, when considering only strong phage reactions, belonged to the phage profile 26, the phage group IV. These strains were all penicillin resistant and CF positive. The two αβ - producing strains of phage type 102/107/108/111 (phage profile 26) were the single isolates of API profile 7, SE-profile 5 and belonged as single strains to FTIR-profile 10 and the PFGE-profiles 21 and 22. The other five isolates with no, or only weak haemolysis, showed weak phage reactions with 20-100 plaques for the phages in phage group III. This explained the high association to characteristic, which were in the past usually only observed for isolates belonging to phage group III.

4.1.4.3 Discussion

The predominance of strains belonging to single clonal complexes provides the base for epidemiological questions. One of the best "model bacteria" in this context is *S. aureus*. Independent from the typing method used, the high prevalence of single virulent types is the distinctive characteristic for pathogenic bacteria when investigating *S. aureus* isolates from different outbreaks or different herds. This is the main reason why different typing methods frequently show comparable results (Figure 2, Table 15). The good correlation between PFGE and FTIR-cluster analysis confirms the high prevalence of single *S. aureus* types and shows the suitability of traditional typing methods using bovine and human phages at 100×RTD. A low correlation between phenotypic characteristics and phage typing or genotyping is generally the best evidence for no clonal relationships between investigated strains, and could indicate methodical problems related to the corresponding methods. The results including prevalence and phenotypic characteristics, agree completely with the findings of Fitzgerald et al. (2000), Stephan et al. (2001, Appendix: Table 26) and Hennekinne et al. (2003, Appendix: Table 41). Also *S. aureus* with *sec* and *tst* belong to the same, or closely related genotypes (Table 15; Fitzgerald et al., 2000; Stephan et al., 2001). The results of PFGE and FTIR confirm the homogenity of phage type 78, 96 and 119 and the heterogeneity of isolates belonging to phage group IV (Figures 1 and 2; Tables 7 and 16). The phage group IV is not valid anymore. Gonano et al. (2009) also observed in cluster D a high prevalence of penicillin resistant strains producing no enterotoxins (Appendix: Table 43). All phage typed isolates in cluster D belonged to phage group IV or had a high sensitivity to phages of phage group IV in accordance to Figure 2 (results not shown). Many strains in phage group IV still show αβ-haemolysis with a negative egg-yolk reaction (Tables 7 and 15). The heterogeneity with respect to the sensitivity for penicllin resistance is the only characteristic, which was correlated with the high number of different PFGE-profiles for these strains. This is evidence that the frequent use of penicillin is the crucial influencing factor with respect to the shift of the *S. aureus* population to a higher number of different subtypes since 40 years ago.

Low standardisation and different interpretations are according to Zangerl (1999a) important problems of the egg-yolk reaction on BP and the haemolysis on BA. According to Brun et al. (1990) single weak reactions in the API ID 32 Staph cause problems when identifying single staphylococci species. Weak reactions or doubtful results are however more a methodical problem when the API-Profile is used for biotyping (Table 15). The 32 biochemical reactions from API ID 32 Staph have only too low discriminatory efficiency in comparison to phage

typing or genotyping. Differences regarding the activity of coagulase, protein A and clumping factor reaction are not only very effective in detecting *S. aureus*, they are also used for the classification of *S. aureus* or provide useful informations about certain types (Blobel and Schließer, 1979). Streaking of sufficient bacterial material in one line and determination after 2h and 48h is not only the most crucial step for a standardised determination of the coagulase activity on BP-RPF, it is also important for the standardised determination of egg-yolk reaction, haemolysis and pigmentation (Table 15). The large number of antibiotics in often different concentrations, with exception to penicillin, is a distinct problem when comparing antibiograms of S. *aureus* during population analysis. The sensitivity, respectively resistance to penicillin has been reported to be an important election criteria for clonal relationships in case of *S. aureus* strains (Lange et al., 1997; Fitzgerald et al., 2000; Stephan et al., 2001).

According to Vintov et al. (2003b) and Jørgensen (2005) the high numbers of penicillin sensitive bovine strains found in Norway are in agreement with the findings of Niskanen and Koiranen (1977) and Gudding (1980) who observed a high prevalence of penicillin sensitive strains belonging to phage type 78. This explains the low prevalence of typable isolates from Norway with the HPS (Vintov et al., 2003b) and the low number of bovine strains, which according to Jørgensen (2005) are classified to different PFGE–clusters or MLST-types. The high prevalence of 25 % penicillin sensitive strains in phage group I, according to Vintov et al. (2003b), is an indicator for the great importance of the clumping factor negative phage pattern I/119 as well. However, Jørgensen (2005) did not determine the clumping factor reaction and thus no data are available for the classification and distribution analysis of the *sec*/*tst* variable *S. aureus* type in the corresponding PFGE-cluster or MLST-types. Only the investigation and correlation of phenotypic characteristics indicates that isolates with phage pattern I/119 are the same strains belonging to the same genotypes, as reported by Matsunaga et al. (1993), Fitzgerald et al. (2000) and Stephan et al. (2001). It is the reason why biotyping, respectively the sum of all virulence factors for most frequent clones, has to be considered in population analysis. This is very important for population analysis based on genotyping because of the lack of information about other relevant characteristics.

The advantage of phage typing is its fast and easy performance in large scale screenings involving a large number of samples (Vintov et al., 2003b). According to Zadoks et al. (2002) one of the reasons why PFGE replaced phage typing as the gold standard for *S. aureus* typing is the lower discriminatory efficiency and high number of non-typable strains of phage typing in comparison to PFGE. Zadoks et al. (2002) did not mention that the main reason for using PFGE instead of phage typing is the high number of non-typable isolates for multiresistant

strains in hospitals, and that this is not a problem in case of typing of bovine strains. In publication of Zadoks et al. (2002) however, phage typing was performed with only 18 non-international phages, and no data about these phages was made available (Zadoks et al., 2002). Additionally, no crucial differences were observed when comparing phage typing with PFGE typing according to Zadoks et al. (2002), except in case of one type. Furthermore, no information was published about the used concentration of phages (RTD), the grouping of phages in corresponding phage groups, and the phenotypic characteristics of single *S. aureus* types. According to Aarestrup et al. (1995 and 1997), the use of only human phages can lead to unsatisfying results with respect to missing agreements between important genotypes, phage types or biotypes. However Aarestrup et al. (1995 and 1997) used only the HPS.

According to Novick (2003), the enterotoxins SEA, SEB,SEC and SED belong to mobile elements explaining the low suitability of enterotoxigeneity for epidemiologic investigations. The high variability of detectable enterotoxin genes is according to Loncarevic et al. (2005) and Morandi et al. (2007) mainly a phenomenon observed in bovine, not caprine raw milk. The detection of *sec* and *tst* is only a save criterion for one *S. aureus* type in caprine milk (Jørgensen, 2005). The caprine strains with *sec* and *tst* belong usually to phage type 78, respectively phage type 78/740, when using the caprine phage 740 (De Buyser et al., 1987). SEC however is not a suitable criterion for epidemiological investigations because its wide distribution in the ovine, bovine and human *S. aureus* population. SED, sei/seg, *seh* and *sej* however have a more distribution in bovine milk (Jørgensen et al., 2005; Zschöck et al., 2005; Zecconi et al., 2006). Especially the significance of *sej* for subclinical mastitis according to Zecconi et al. (2006) explains the high prevalence of *S. aureus* strains with *sej* according to Zschöck et al., (2005). The high similarity of bovine isolates with *sej* or *sed/sej* is confirmed through phage typing and PFGE shown in Figure 2 and Table 15. The classification of the strain with *seh* to a separate phage profile or branche in the PFGE and sensitivity for penicillin agree with the results according to Jørgensen (2005).

The easy and fast identification respectively screening of mastitis pathogens, as well the classification of certain *S. aureus* types in control programs, with a single bacteriological examination is only possible with the new FITR-technology. All other methods were only developed for either identification, or typing of cultured isolates. The comparison of different results of FTIR-cluster analysis however is impossible. The high sensitivity of strains regarding the chemical composition of the used agar medium is one of the most important problems with this method when comparing results derived from different investigations (Helm et al., 1992). Only the FTIR-technology takes the whole bacterial cell into

consideration. Therefore, the FTIR-technology is able to detect differences of regulatory mechanism in bacterial strains. On the one hand this explains the homogeneity but strong differences in a single FTIR-cluster (cluster 2a in Figure 1 and PFGE profile 1-15 in Table 15). On the other hand this is a crucial problem regarding solid determination of single bacterial clones by FTIR. Strains belonging to different clonal complexes are classified to the same FTIR- profile and vice versa.

Especially in big scale screenings with a high diversity of isolates, the reproducibility of the method for certain strains and the relativity of the calculations are a problem. In this case the spectral distance or validitiy for heterogenous clusters increased, whereas for closely related isolates with a high similarity the spectral distance or validitiy decreased. Especially for close related isolates is the reproducibility of the method the distinct limiting parameter. The discrimination of phage type 78, 96 and 119 and strains with a high sensitivity for phages of phage group IV confirms the suitability of the FITR–technology for resistance monitoring programs. Rebuffo-Scheer et al. (2007) could identify almost all strains from different *Listeria monocytogenes* serotypes by the FTIR identity-test, through optimization of the data base, spectral window, weights and reproducibility levels or by using aritifical neural networks. This should be possible for important bovine *S. aureus* subtypes as well. The heterogeneity of bacteria populations however can be only estimated by using FTIR-cluster analysis. The FTIR-cluster analysis is further the sole method by using the FTIR-technology when so many as possible different strains during monitoring programs wants to be elected.

Fifteen years ago genotyping methods based on restriction enzymes predominated. These methods result in highly reproducible information about clonal relationships, but not about phylogenetic relationships in comparison to the new genotyping methods MLST or *spa*-typing based on sequencing. Very important for the interpretation of typing results shown in Table 15 are recently published results according to Ikawaty et al. (2009).

Ikawaty et al. (2009) classified 90 % of isolates to three *spa*-types (t524, t543 and t529) and 92 % of isolates were represented through five MLST-types (71, 97, 151, 479 and 504 in Appendix: Figure 28). Ikawaty et al. (2009) distinguished by PFGE between seven branches at the 50 % similarity level. Only three clusters were valid when comparing results of *spa*-typing, MLST- typing and PFGE and were high correlated with the three *spa*- types t524, t543 and t529 (Appendix: Figure 28). These results have a high agreement with the results shown in Table 15. Using a 60 % similarity 5 branches were distinguished by PFGE (Figure 2) between seven branches at the 50 % similarity level. Six bovine phages of the BPS (116, 102, 117, 78, 118 and 119) would lead to an adequate classification of 83 % of all isolates in

the dendogramm shown in Figure 2 considering the 60 % similiarity in the PFGE-dendrogram. The high homogeneity between results of PFGE, *spa*-typing and MLST-typing according to Ikawaty et al. (2009) agrees with the results for phage type 78, 96 and 119 shown in Figure 2 and Jørgensen et al. (2005) (Appendix: Figure 25: PFGE-cluster 25- 41). However each strain in the branche containing all strains of *spa*-type t524 belong to a single PT (Ikawaty et al., 2009; Appendix: Figure 28). The high variability is confirmed through MLST-typing. With one exception each pulsotpye in this branche was represented only through a single strain. This is in accordance to the results shown in Figure 2 (PFGE-profile 16-43) and Jørgensen et al. (2005) (Appendix: Figure 25: PFGE cluster 8- 24).

In addition to Ikawaty et al. (2009), Jørgensen et al. (2005) had difficulties in classifying single MLST types (Appendix: Figures 27 and 28). *S. aureus* subtypes with a low agreement even between typing mehods based on sequencing are not only a challenge for the correct classification in PFGE. They are heterogenious as well with respect to phenotypic characteristics and sensitive to phages of different phage groups. According to Jørgensen et al. (2005), the MLST-type 30 and 97 belonged to the same PFGE-cluster but not to the same clonal complex. According to sequencing, the MLST type 30 was classified to the same clonal complex as MLST type 484, but the MLST type 484 was grouped by PFGE to cluster 51 (Appendix: Figure 25, Table 42). Additionally, MLST types 30 and 484 were the single *sea* positive isolates and were therefore from great concern for questions with respect to food poisioning outbreaks.

The *spa*-type t543 and MLST type 479, which according to Ikawaty et al. (2009) belong without any exception to the next big branche, indicate methodical difficulties in PFGE leading to misgrouping of isolate S0398 (Appendix: Figure 28). The dark and light fragments in this makrorestriction profile confirm this hypothesis. Considering only the clear visible respectively darker fragments this profile would be grouped to the next branche containing all other strains of *spa* type t543. In addition, isolate S0398 is one of two strains belonging to seven branches at a 50 % similarity level according to Ikawaty et al. (2009). Many variables, such as the concentration of DNA in the agarose plugs, the amount of agarose in the gel, the electrophoresis voltage, the gel temperature, the buffer strength and variation in the preparation of gel plugs, contribute to intra- and intergel variation and complicate comparisons of fragment patterns on multiple gels and in PFGE libraries (Birren and Lai, 1993; Chung et al., 2000). Comparisons of fragment patterns present on multiple gels from large sets of isolates are technically difficult (van Belkuum et al, 1998; Chung et al., 2000). These methodical difficulties do not have according to Peles et al. (2007) that importance

when investigating isolates of one outbreak or strains from single herds. A limited number of gels together with a low heterogeneity of strains facilitate the performance of PFGE in a high extend. The problems increase for strains from a higher number of different herds with only slight variations of one or more fragments in PFGE, as it is observed for many isolates of *spa*-type t524 or MLST-type 97 (Ikawaty et al., 2009; Appendix: Figure 28).

Mutations in the whole genome of bacteria alone could not explain, why high number PT`s (PT´s) belong to one cluster or *spa*- and MLST type with a high similiarity regarding all investigated characteristics. Using the old IPS Olson et al. (1970) observed still the instability regarding the manitol fermentation of entertoxin C variable strains, which were only sensitive to phages of phage group I (such as the isolates in phage profile 4, 5, 15; Tables 8, 15). No other *S. aureus* types with the same genotype, such as the PT 12, showed this degree of heterogeneity regarding all phenotypic characteristics and phage reactions. The strain *with sec* and *tst* in phage profile 5 (Table 7 and 15) with no phage reaction >20 plaques for phage 119 belonged to a separate, but related FTIR-cluster (Figure 1: Profile 23), and had as single SEC producing isolate a test-value of 1.6 in the *VIDAS* system (Table 3).

A high proportion of staphylococci, if not all, contain prophages which can inhibit the lysis of phages used for typing (Davidson, 1961a; Wenworth, 1963). Isolates from a single herd show high reproducibility for phage reactions even for single plaques. When phage typing *S. aureus* from one herd or outbreak, all phage reactions are considered (Witte, 2004). Phage reactions <50 plaques are a problem only when comparing strains from different herds. The only explanation is the strong influence of a different combination of prophages in the corresponding herds respectively isolates from different environmental conditions or locations. Only prophages in closely related chromosomal regions of one clonal *S. aureus* type would explain why small clusters frequently contain few strains with identical characteristics but with a low similarity in PFGE-dendrograms or FTIR- clusters. The existence of small separated PFGE or FTIR- clusters almost for all different types indicates a goal-directed adaption through prophages reasoned by specific environmental conditions.

However the relevance of prophages for population analysis -with exception for phage typing- is usually not dicussed. The high prevalence of close related PT`s belonging with MLST to one clone has to be the result of the high reproducibility of restriction enzymes detecting small DNA-fragments of different non-lysogenic prophages. This should be the reason why discriminative methods based on restriction enzymes according to Jørgensen et al. (2005), Gonano et al. (2009) and Ikawaty et al. (2009) have various clusters with a high degree of the same characteristics but only few identical genotypes (see Figure 2; Appendix: Figure 25).

This assertion is supported by the observation made by Frost and Bradshaw (1980): that the number of different phage types of a single strain is usually caused through prophages belonging to the same phage group. Frost and Bradshaw (1980) found a wide distribution of prophages for strains in phage group IV. It would explain the results of phage typing for the isolates in PFGE-profile 16, 18, 25, and 28, which are confirmed by FTIR- cluster anaylsis (Figure 2 and Table 15). They belonged all to different phage types in phage group IV, showed no β-haemolysis or were egg-yolk positive. The great importance of prophages is confirmed by the single not -typable strain in PFGE profile 29, who had a high similarity to the isolate in PFGE profile 28, showed no typical haemolysis and was egg yolk positive (Figure 2).

Through modern molecularbiological methods and research conducted in the past decade we obtain a more comprehensive picture about the importance of prophages regulating phenotypic characteristics or virulence factors, respectively the enterotoxigeneity of bacteria. Very important for understanding difficulties in epidemiological investigations are mobile elements such as *S. aureus* pathogenic islands (SAPI), plasmids or prophages (Novick, 2003). Fitzgerald et al. (2001) could show that the same bovine genotype with different combinations of toxins is linked to mobile SAPI encoding genes *tst* and *sec*. The authors concluded that the presence of SAPI is involved in the modulation of bovine immune response. Evidence for this hypothesis are the results of Kurioshi et al. (2003). Mairques et al. (2007) recently demonstrated that SAPI can be induced to replicate different staphylococcal phages, which cause the variation of single clones with or without production of enterotoxin C. It is also reported that bacteriophages of *S. aureus* mediate the simultaneous double or trible lysogenic conversion of enterotoxin A, staphylokinase and β-haemolysin (Coleman et al., 1989; Caroll et al., 1993). Expression of β- haemolysis or egg-yolk reaction can be lost by following lysogenization, whereas the capacity to express staphylokinase and enterotoxin A can be acquired after lysogenization (Kumagai et al., 2007).

The high correlation between the detection of *sec*/*tst* genes and enterotoxin production for the same clonal *S. aureus* type shows that the regulation of SAPI leads not only to negative results with methods which detect only the production of enterotoxins, but as well to negative PCR results. These results and the missing sensitivity for phage 119 can be explained with the presence of single prophages regulating the immune response in the SAPI-region, which according to Fitzergald et al. (2001), Stephan et al. (2001) and Gonano et al. (2009) leads not only in genotyping methods to a varying number of intermediate clumping factor negative *S. aureus* types, independently of the method used. It would further explain the missing

correlation between SE-profiles, the instability in the FTIR-cluster analysis, phage typing and the variability of the *S. aureus* clone in phage profile 4 and 5 (PFGE-profile 12) or differences in physiological characteristics and genotyping (Figures 1 and 2, Tables 8 and 16).

4.1.4.4 Summary

The egg-yolk reaction, clumping factor, haemolysis and enterotoxins are all virulence factors and therefore directly involved in the adaption process and coevolution of *S. aureus* to corresponding environmental conditions. Only single *S. aureus* types belong to one clone such as the strains in PFGE-profile 2, 3 and 30 (Figure 2, Table 15), which have a high agreement regarding phenotypic characteristics. The most of all other *S. aureus* types have a high variation for different characteristics inspite of the fact that some of them belong to one clonal complex. The interdependence between phenotypic characteristics and prophages is an important reason for the great diversity of phenotypic characteristics, phage types but also genotypes. Especially genotpying methods using restriction enzyms are problematic in this context. This leads to fundamental difficulties in the correct classification of single isolates and consequently in long time population analysis. In additon it justifies the performance of phage typing for epidemiological investigations over longer periods, because even the loss of sensitivity of single phages is an evidence for the shift in the *S. aureus* poulation itself. The clear definition of single *S. aureus* types together with phenotypic characteristics and antibiotic resistance is the base for a long time population analysis especially in medical epidemiological questionings. Phage types are not only high correlated with phenotypic characteristics, phage typing and sequencing are further the sole methods, which classify *S. aureus* isolates to well defined types in comparison to other genotyping methods.

4.2 Methodological difficulties in the control of *S. aureus* mastitis in dairy herds and their effects on population analysis in the dairy chain

The biodiversity of *S. aureus* in the dairy chain is highly correlated with the control of *S. aureus* mastitis in dairy herds. The interdependence of prevalence and biodiversity of this pathogen in the dairy chain is the reason why all distinct parameters and methodological difficulties in the control of *S. aureus* in dairy herds have to be discussed together. Only the comparison of two groups of herds, herds with a low (LCPS) and a high *S. aureus* prevalence (HCPS), provides a realistic measure for the control possibilities and for the change of the *S. aureus* population in the dairy chain**.** A detailed description of the selection of dairy herds, sampling and isolation methods is given in chapters 3.9 to 3.14.

4.2.1 Grouping in HCPS and LCPS herds and methodological difficulties in defining S. *aureus* prevalence of dairy herds

One methodological problem is the high number of possibilities for defining the *S. aureus* prevalence in dairy herds. The *S. aureus* prevalence is determined on the cow level or on the quarter level only, by using foremilk or stripping samples with or without consecutive sampling, at one time-point or over longer periods. Unsolved problems are further methodological difficulties of bacteriological examinations together with the volumes of milk, which are investigated. Environmental conditions, as well as the feeding and age of cows are also important influence factors when investigating the *S. aureus* prevalence in dairy herds. Therefore, a realistic assessment of the results can be achieved only when as many parameters as possible are involved in the discussion about the prevalence of *S. aureus* in dairy herds. Futher it is necessary not to consider exclusively bacteriological results or methodological deficiencies when you want to discuss all the problems in determining the prevalence of udder pathogens in dairy herds.

4.2.1.1 Problem and Goal

The main diagnostic problem of mastitis is the high degree of bacteriological negative samples which varies from 5 to 50 % (Pearson and Mackie, 1979; Kurzhals et al., 1985; Matilla et al., 1985; Zorah et al., 1993). Dinsmore et al. (1991) suggested that repeated sampling might be necessary since many sampling and culturing methods were not able to detect all infected cows at each survey. According to the definition of Zadoks et al. (2001) two consecutive sampling intervals are necessary for quarters shedding less than 1000 *S. aureus* cfu/ml to diagnose an intramammary infection. The test volume of foremilk and the definition of *S. aureus* prevalence in the herd is still an unsolved problem. The positive outcomes of *S. aureus* quarter milk samples increase up to 145 % depending on the herd, by investigating centrifuged quarter milk samples of the foremilk with the lower detection limit of 1 CFU / 10 ml instead of 10 µl foremilk (Zecconi et al., 1997). Furthermore herds with a low *S. aureus* prevalence have according to Zecconi et al. (2003) less than 20 % *S. aureus* shedding cows in 10 µl foremilk, according to Roberson et al. (1994) less than 10 % *S. aureus* shedding cows in 50 µl foremilk.

The possibility of higher *S. aureus* counts in the main milk in spite of low or negative foremilk was still realized, but not investigated in more detail (Abo-Elnaga and Kandler, 1965; Vries, 1976). Therefore, a more detailed survey was conducted. According to the classification of Roberson et al. (1994), the farms were grouped into HCPS (high prevalence of coagulase-positive *S. aureus*) and LCPS (low prevalence of coagulase-positive *S. aureus*) farms. The prevalence of *S. aureus* in these dairy farms was determined using a more sensitive diagnostic approach, and the influence of the diagnostic procedure (sample volume and type of milk fraction analyzed) on the prevalence was investigated.

4.2.1.2 Results

Grouping the herds of HCPS and LCPS herds according to Roberson et al. (1994) separated the farms into those that exceed the limit of 100 cfu *S. aureus*/ml raw milk and 1,000 cfu *S. aureus*/g raw milk products, and those which did not exceed this limit. The HCPS herds showed a significantly higher number of raw milk and cheese samples exceeded the limits than LCPS herds ($p=0.012$). No significant difference was observed for the SCC in the bulk tank milk (arithmetic mean value: < 300,000/ml) and total bacteria count of the bulk tank milk (95% of the samples < 10,000/ml). In a single farm (farm A) 84.5 % of all cows had an IMI

and showed significantly higher counts of *S. aureus*, but lower SCC than those from the other five HCPS herds (P <0.0005). The most shedding patterns of cows with *S. aureus* count in herd A were outliers according to statistical data; therefore this herd was excluded from the following calculations. Herd G, with usually low *S. aureus* counts, had at this time two old *S. aureus* shedding cows and was grouped by definition to the HCPS herds. The remaining eleven herds with in total 238 cows were grouped as following: six into HCPS herds with 133 cows and five into LCPS herds with 105 cows.

The results for the experiments of sampling the main milk during the first three sampling rounds are shown in Table 16. The correlation between the *S. aureus* count in the bulk milk and the calculated *S. aureus* count from each milk yield of *S. aureus* shedding cows per herd was high (r^2=0.95). The theoretical main milk contamination risk from subsequent cows before milking *S. aureus* shedding cows was very low. This was particularly observed in the LCPS herds (J, K, L), where the low prevalence of *S. aureus* shedding cows were in these herds only detectable when sampling the main milk.

The results in the HCPS herds (A, C, E, F) show the high agreement of consecutive main milk samples when compared to foremilk and stripping samples during the same sampling interval. 85 of 109 *S. aureus* positive tested cows (78 %) could be confirmed with consecutive sampling, or *S. aureus* positive foremilk and stripping samples. 91 % of positive main milk samples are confirmed when considering the third sampling interval in Herd F to be an outlier and excluding it from the other results.

The evaluation of all data from the third sampling interval in comparison to the first two sampling intervals in herd F revealed an increasing urea content in most of the milk samples during the second and third sampling, which was correlated with an increasing SCC in most cases (see Appendix: Table 18). The composition of the fodder during the second and third sampling was different due to the change of the fodder to one with too high levels of digestible protein and too low energy content. This change caused induced a metabolic disorder in many cows. The theoretical contamination in the main milk of non-confirmed *S. aureus* shedding of cows could be excluded through the bacteriological investigations, with exception of six cows. An *S. aureus* shedding cow had either higher *S. aureus* count from the cow milked before, or a considerably lower bacteria count of the contaminating flora compared to the cow milked before, indicating an effective cleaning of the milking equipment between the milking of the two cows.

Table 16 Number of *S. aureus* positive cows in the main milk with confirmed or theoretically contaminated *S. aureus* samples in comparison to consecutive sampling intervals or foremilk and stripping samples

	Consecutive sampling main milk: 1., 2., 3.						**Sampling different milk fractions:** Foremilk + main milk + strippings				
	Herd L			**Herd F**			**Herd K**	**Herd J**	**Herd E**	**Herd A**	**Herd C**
	1.	2.	3.	1.	2.	3.					
Number of cows	39	38	40	36	33	36	22	19	35	38	15
S.aureus positve	1	3	2	10	9	27*	2	4	13	35	9
Confirmed[1]	1	0	1	8	7	16	0	3	11	35	8
Bulk milk: *S.aureus* cfu/ml	< 10	50	60	180	110	480	<10	80	110	2,100	350
Herd average single cows[2]: *S.aureus* cfu/ml	11	45	40	251	142	454	7	72	233	2,566	310
Theoretically contaminated[3]	-	-	-	2	-	16	-	-	4	31	6
Theoretically contaminated without confirmed cows				1		6			1	1	1

[1] The cows were confirmed through positive consecutive main milk, foremilk and/or stripping samples.

[2] This *S. aureus* count is calculated with the *S. aureus* count and milk yield of each single cow.

[3] A sample of a cow was considered as theoretically contaminated in the main milk when the cows were milked after the milking of a *S. aureus* shedding cow.

* In this sampling interval the feeding had no equivalent protein and fat ratio so that many cows showed additionally an elevated SCC (see Appendix Table18).

114 (47.9 %) of 238 cows were *S. aureus* positive in the fourth sampling round. 21.8 % of cows shed *S. aureus* only in the main milk and/or the strippings. Only 37 cows (15.5 %) shed *S. aureus* in foremilk, main milk and strippings. 25 cows (10.5 %) shed *S. aureus* in two milk fractions and 52 cows (21.8 %) in only one milk fraction. 26.1 % of 238 cows shed *S. aureus* in the foremilk, 37.8 % in the main milk, and 30.3 % in the strippings. 25 of the 114 shedding cows belonged to LCPS herds with only 6.7 % *S. aureus* positive cows in the foremilk, whereas the prevalence of *S. aureus* positive foremilk samples in the HCPS herds (B-G) was 39.9 %. For detailed information regarding the cows shedding *S. aureus* in one, two and three milk fractions see Table 17. No cow of the LCPS herds sampled positive both in the foremilk and strippings. 185 of the 238 cows in the fourth sampling round were *S. aureus* positive

sampled at the three consecutive samplings before. None of the cows that shed *S. aureus* only in the foremilk**,** or in the foremilk and main milk during the fourth sampling round, had tested positive during the earlier three sampling rounds.

The opposite was observed for the cows with three positive milk fractions. These cows shed *S. aureus* in all three earlier sampling rounds. Some cows shedding *S. aureus* in two milk fractions also tested positive during the first three sampling rounds before, whereas the cows with a single positive milk fraction showed predominately spontaneously infections. Only four cows with a positive strippings and one cow with shedding of *S. aureus* only in the main milk were detected in one of the three earlier sampling rounds, but without showing positive results at consecutive sampling intervals.

Table 17 Prevalence of all cows (n= 238) shedding *S. aureus* in foremilk, main milk or strippings in one, two or all three milk fractions

	(n)	Prevalence (%)	Persistently *S.aureus* shedders[1] (%)
One positive milk fraction	52	21.8 (7.1)[†]	9.6
Foremilk	14	5.9 (1.7)	0
Main milk	21	8.8 (2.9)	4.8
Strippings	17	7.1 (2.5)	23.5
Two positive milk fractions	25	10.5 (2.1)	12.0[‡]
Foremilk + Main milk	6	2.5 (0.8)	0.0
Main milk + Strippings	14	5.9 (1.7)	14.3[‡]
Foremilk + Strippings	5	2.1 (0)	20.0[‡]
Three positive milk fractions			
Foremilk + Main milk + Strippings	37	15.5 (0.8)	100.0[‡]

[1]Prevalence of cows which were *S. aureus* positive in one of the three previous sampling rounds

[†]Corresponding prevalence (%) of LCPS herds in brackets

[‡]These cows were *S. aureus* positive in all three previous sampling rounds.

A detailed analysis revealed a complex relationship between the number of lactations and the prevalence of the pathogen in the herd on one hand, and the number and type of milk fractions on the other hand (Figure 3).

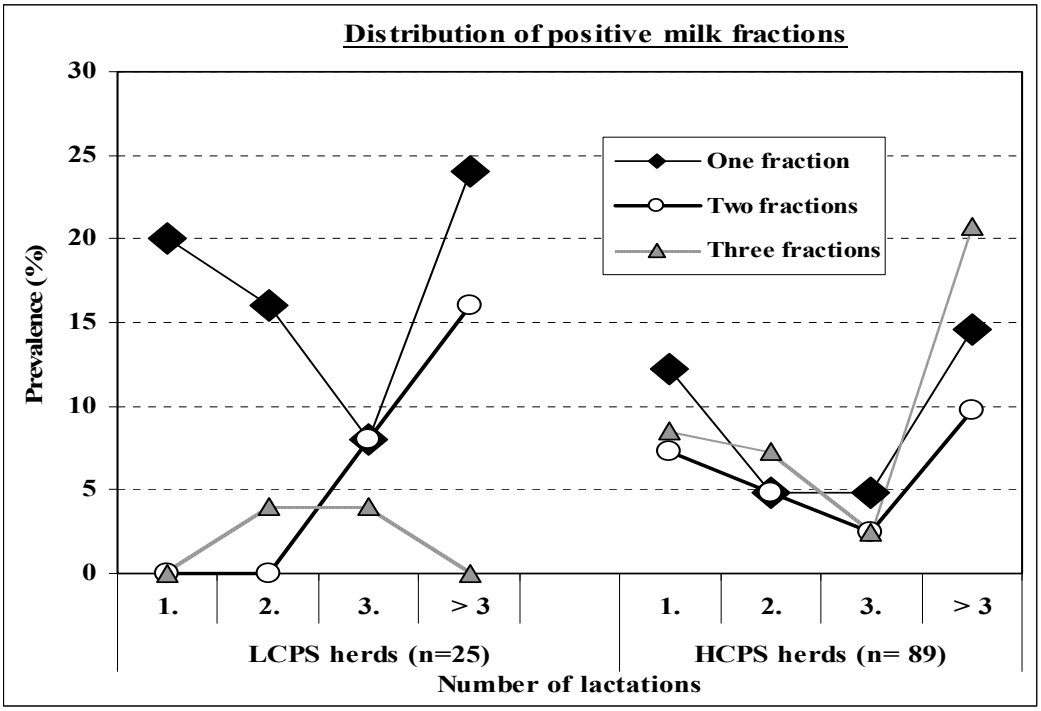


Figure 3 Distribution of *S. aureus* shedding cows in one, two and three positive milk fractions in LCPS and HCPS herds in relation to the number of lactations.

A significant difference between the HCPS herds (B-G) with 133 cows and five LCPS herds (H-L) with 105 cows was observed for all positive milk fraction combinations and for the percentage of cows with three positive milk fractions (Table 18). 39.5 % of the cows in the HCPS herds and 1.9 % of the cows in the LCPS herds shed *S. aureus* in all three milk fractions. In the LCPS herds fewer cows (7.4 %) had two positive milk fractions compared to HCPS herds (17.8 %), but this difference was only slightly above the limit of significance. The percentage of cows with one positive fraction in HCPS herds (16.8 %) was very similar to that in LCPS herds (18.1 %). In the HCPS herds the percentage of cows with stripping or main milk as the single positive milk fraction was significantly different for groups of cows with ≤ 2 lactations and > 2 lactations. In the LPCS herds the percentage of cows with main milk and strippings as two positive milk fractions was significantly different for these two groups. A significant difference between the six HCPS and five LCPS herds (n=105) was observed for all positive milk fraction combinations (Table 18).

Table 18 Comparison of number and types of positive milk fractions of LCPS and HCPS herds in relation to number of lactations, and in relation to the groups of cows with ≤ 2 and > 2 lactations of LCPS and HCPS herds.

	LCPS herds / HCPS herds (P- values)	**≤ 2 lactations / > 2 lactations**	
		LCPS herds (P- values)	**HCPS herds** (P- values)
All fraction combinations:	0.004	0.566	0.836
One positive milk fraction:	0.255	0.543	0.936
Foremilk	0.265	0.837	0.685
Main milk	0.769	0.826	0.044
Strippings	0.027	0.065	0.020
Two positive milk fractions:	0.057	0.127	0.205
Foremilk + Main milk	0.930[a]	0.184	0.165
Main milk + Strippings	0.069	0.021	0.662
Foremilk + Strippings	0.073	xxx[†]	0.306
Three positive milk fractions:			
Foremilk + Main milk + Strippings	0.034	0.919	0.965

[†] No cow in the LCPS herds shed *S. aureus* in foremilk and strippings.

Apart from the reduction of positive samples there was no difference considering only one, two or three positive milk fractions in comparison to the LCPS herds. A detailed analysis revealed the significant differences for cows shedding *S. aureus* only in the strippings or main milk, respectively to cows shedding *S. aureus* in main milk and strippings (Figure 4, Table 18). In the LPCS herds the percentage of cows with main milk and strippings as two positive milk fractions and only in the strippings was significantly different for these groups. In the HCPS herds the percentage of cows with stripping or main milk as the single positive milk fraction was significantly different for groups of cows with ≤ 2 lactations and > 2 lactations. Cows shedding *S. aureus* only in the foremilk were in the LCPS herds only detectable in the group of primipareous cows and cows with > 3 lactations. Many cows in the HCPS herds shedding *S. aureus* only in the foremilk belonged as well to primipareous or older cows.

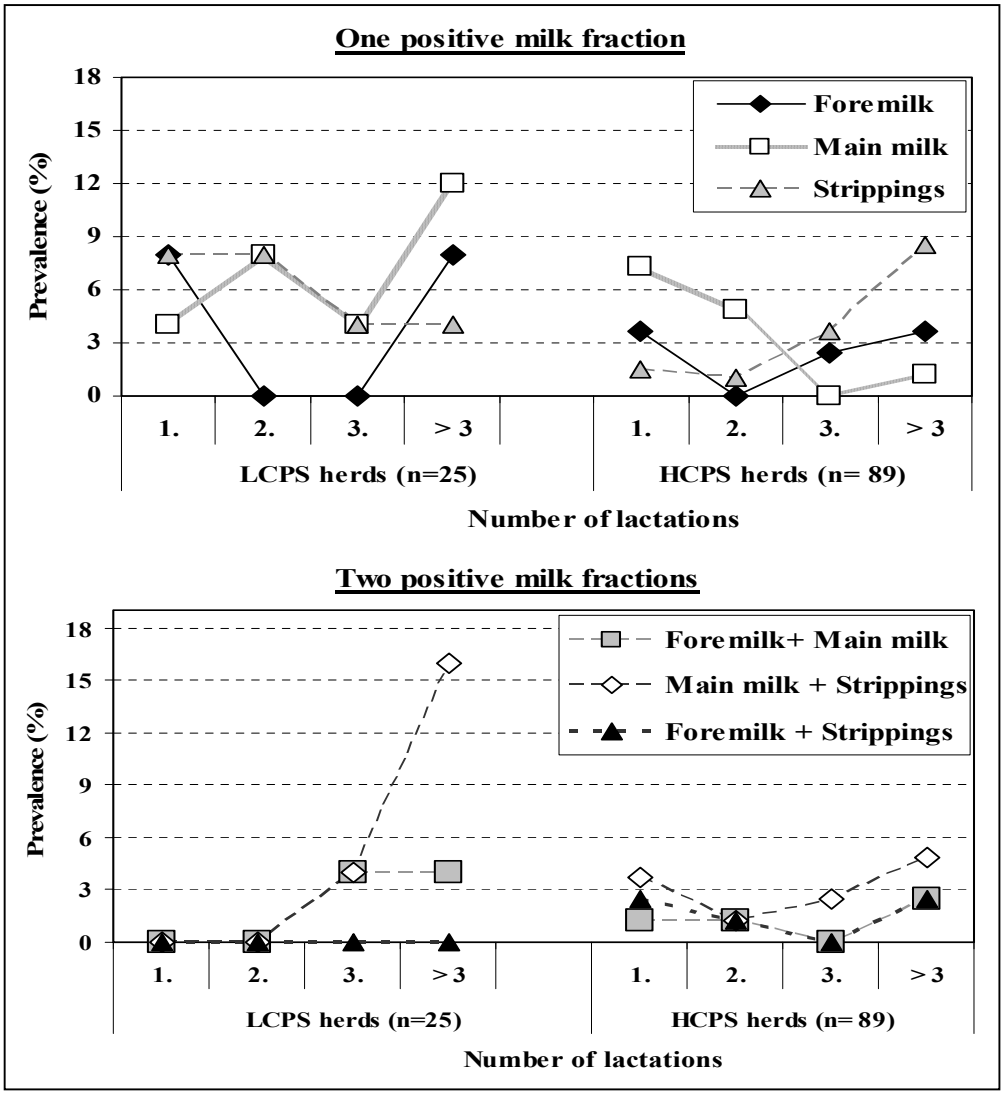


Figure 4 Shedding patterns of cows with only one or two different positive milk fractions in relation to the number of lactations in detail

4.2.1.3 Discussion

S. aureus is ubiquitous in the natural environment of dairy herds. Significant differences between LCPS and HCPS herds have only been observed on the bodily parts, the udder and in particular the teats of cows, not for environmental samples (Davidson, 1963; Roberson et al., 1994a,b). Methodological difficulties regarding sampling are the crucial problem in the definition of udder pathogen prevalence in dairy herds. Zecconi et al. (2003) define LCPS herds having less than 20 % *S. aureus* shedding cows, while according to Roberson et al. (1994a) LCPS herds have less than 10 % *S. aureus* positive cows at one timepoint of sampling. Furthermore, the definition of IMI and the volume of milk used for bacteriological

examinations are unsolved problems in the analysis of bovine mastitis (Zecconi et al., 1997). Pathogens in endemic states are another problematic factor in defining the *S. aureus* distribution in dairy herds (Zadoks et al., 2001). Probably this is the main reason why Zadoks et al. (2001) give no detailed information about the *S. aureus* prevalence during their own examinations. The occurrence of higher bacteria or *S. aureus* counts in the main milk compared to the foremilk of cows has been observed already, but it has not been investigated in greater detail (Abo-Elnaga and Kandler, 1965b; Vries, 1976). While in these cases the bacterium is found in the bulk milk, the cows actually remain healthy.

The sudden increase from approximately three to about 40 new *S. aureus* infections per 100 cow during one month in a herd which according to Zecconi et al. (2003) is classified as LCPS is the best example for the illustration of the problems encountered with the definition of the *S. aureus* prevalence in dairy herds, which is as well supported by the here presented data in case of herd F (Table 17) or herd I (Figure 14). The high number of cows shedding *S. aureus* in herd F during the third sampling round was explainable with a change in feeding with a too high protein but too low energy content, resulting in a larger number of cows with increased urea content and SCC (Wendt et al., 1998) (Appendix: Table 17).

The bacteriological examinations of foremilk, main milk and strippings of the same cow together with the consideration of the chronological milking order of the cows show the low relevance of the presented methodology of sampling the main milk. The significant large number of young cows with only positive main milk samples in the HCPS herds (Figures 3**,** 12) cannot be explained with accidental contamination, since both younger and older cows have been milked in herring-bone milking parlours indiscriminately after each another**.** Similar to observations of Davidson and Slavin (1958), it turned out that the high dilution effect brought about by three litres of cold water usually was satisfactory when the milking equipment and Tru-Test auto sampler was contaminated even with 10^4 *S. aureus* CFU/ml. The remaining ≤10 *S. aureus* CFU are again diluted with at least 40 ml milk of the following cow. A false positive result analysing only 0.1 ml of milk of a sample with less than one CFU/ml is statistical not relevant. The disinfection of the milking machine using hot water with 70 °C for 20 sec kills about 10,000 *S. aureus* CFU/ml. Despite this fact cows identified within the mastitis control program as heavy shedders were milked after the others.

Our data agree very well with the classification of Roberson et al. (1994): *S. aureus* counts of cows with three positive milk fractions were rather high, and the percentages of these cows in HCPS farms (farm A, 39.5 %) and LCPS farms (1.9 %) were similar to the distributions given for our foremilk samples (39 % in case of HCPS farms versus 3.9 % in case of LCPS farms).

These cows were identified as chronical shedders and they had a persistent mastitis over a long period. However, according to Munch-Petersen and Gardiner (1965), when monitoring seven herds four times a week, three herds with a minimum of less than 12 % had a maximum level between 24 and 45 % *S. aureus* shedding cows at one time-point of sampling. Two of five herds (A and E) grouped by Zecconi et al. (2003) during the control program to LCPS herds had the highest rate of new infections of up to 40 of 100 cows per month (Appendix: Figure 16). Similar high new infection rates in single months were observed by Zadoks et al. (2001). The limit of 20 % of cows with IMI, when sampling only 10 µl of foremilk, is clearly exceeded. When only the common approach for the monitoring of dairy herds is used the investigation of 10 µl of foremilk cannot provide the proof that no bovine *S. aureus* is to be found in the bulk milk since this approach causes inaccurate estimations of *S. aureus* prevalence in the herd.

The common approach of using only foremilk samples is according to Brolund (1985) a crucial problem in the diagnostic program of subclinical mastitis. The conventional bacteriological method based on international standards resulted in 43.1 % ± 20.8 % false positives, which have been mainly due to teat canal infections stimulating mastitis (Giesecke and Viljoen, 1974). The prevalence of *S. aureus* positive quarter samples of 0.05 ml instead of 0.01 ml foremilk increases only to about 8 % (Griffin et al., 1977). Since the data presented by Zecconi et al. (1997) give no detailed information about the *S. aureus* count in the 10-ml volume of centrifuged quarter milk samples, a cow sampled negative in a 0.01-ml volume of foremilk could be *S. aureus* positive in a volume of 0.1 ml, 1 ml or even 10 ml foremilk**.** According to Sears et al. (1991) the investigation of 0.01 ml milk in a herd with a large number of chronical shedders and high SCC is a possible explanation for the large number of positive foremilk samples in comparison to stripping samples. The here presented results suggest that the critical limit is between 1 CFU and 0.01 CFU/ml foremilk. In particular this was observed in case of strippings, which is in accordance with Godden et al. (2002) (Appendix: Figure 14).

In one herd all cows were according to Zecconi et al. (1997) rated negative using 0.01 ml foremilk, but 44.4% of the cows were *S. aureus* positive when using centrifuged quarter samples with a single colony in 10 ml foremilk. However, the fact that no more than five colonies were found in centrifuged 10 ml foremilk quarter samples, while the analysis of 0.01 ml of foremilk according to Zecconi et al. (1997) resulted in 18.5 % positive samples, indicates methodological problems suggesting inefficient detection rates for *S. aureus*.

It has to be analysed whether the use of centrifuges leads to an efficient concentration of bacteria, or whether the strongly increasing number of contaminating flora leads to difficulties in the isolation of single *S. aureus* colonies, which can be almost not detected without using suitable selective enrichments. According to Heeschen et al. (1968), the detection rate increased up to 40 % when investigating 0.1 ml foremilk samples with a selective enrichment instead using blood agar only. According to the same authors, the *S. aureus* prevalence observed by Zecconi et al. (1967) would increase to 46.9 %. A prevalence of 46.9 % *S. aureus* positive samples is almost identical with the results presented here when excluding farm A (47.9%), and is still similar when the results of farm A are included (53.9%).

In the literature the prevalence of *S. aureus* is furthermore associated with IMI or new IMI. The majority of new infections are according to Zecconi et al. (2003) observed during the 1st and 2nd lactation. The low numbers of older cows with new IMI, except of the 1st month after calving, indicate that either many older cows are chronic *S. aureus* shedders, or that they had in the past an IMI and their teat canals are still being colonized with *S. aureus* (Appendix**:** Figure 17). When cows with IMI would be added to the number of new infections during each sampling interval in the control programs, the prevalence of *S. aureus* positive cows would increase to a large extent.

Methodological difficulties are a further problem defining IMI. The recovery of a single *S. aureus* colony was considered by Roberson et al. (1994a) and Zecconi et al. (2003) as a positive result indicating an IMI, whereas according to Griffin et al. (1977) quarters have only IMIs when they shed udder pathogens in consecutive sampling intervals. In the here presented work only 67.9 % of all positive foremilk samples were confirmed through consecutive sampling, in contrast to Griffin et al. (1977) who were able to confirm 93.2 % of all *S. aureus* positive quarter foremilk samples with this technique. Furthermore, quarters with less than five colonies in 0.05 ml of foremilk, with 0.01 ml being sufficient for the detection of negative samples, have a prevalence of 65.4% confirmed udder infections (Griffin et al., 1977). According to Zadoks et al. (2001) consecutive sampling for quarters with ≥ 1000 *S. aureus* cfu/ml is not necessary for the confirmation of an IMI. According to Griffin et al. (1977) and Godden et al. (2002) and in agreement with the here presented results, the high prevalence of positive foremilk samples with about 66 % of quarters shedding ≥1000 *S. aureus* cfu/ml is the reason why consecutive sampling in many cases is not necessary when following the definition of Zadoks et al. (2001) for IMI.

A further methodological problem concerning the detection of udder pathogens on quarter level relates to consecutive sampling for determination of IMI. The examinations of Zadoks et

al. (2001) and Zecconi et al. (2003) are usually based on 0.01 ml of foremilk of each single quarter, whereas Hoblet (1990) and Roberson et al. (1994) used composite quarter samples for bacteriological examinations. Composite quarter samples are according to Hoblet et al. (1988) more practicable, but consider udder infections only on the cow level and are not in agreement with IDF-Bulletin (2006), which recommends the analysis of 0.01 ml foremilk from each individual quarter. The variability regarding *S. aureus* shedding quarters in consecutive sampling has been observed since quarter samples are investigated (Schalm et al., 1953; Parisi and Baldwin, 1963). The prevalence of *S. aureus* increases according to Zecconi et al. (1997) on cow level only up to 33.5%, in comparison to 130.0 % on quarter level, when using centrifuged quarter samples with a detection limit of 1 CFU / 10ml. The varying number of shedding quarters is the reason why Zecconi et al. (1997) defined a cow as persistently positive when one quarter out of two consecutive samples tested positive for *S. aureus*. The analysis of only composite quarter samples is then the only consequential step.

The number of *S. aureus* shedding cows with three or more lactations is very important when investigating the *S. aureus* prevalence in dairy herds. The levels of *S. aureus* shedding cows with one and two lactations in comparison to cows three or more lactations are significant (Figure 3, 4). These results are confirmed by the observations of Blackburn (1968) and Brolund (1985) (Appendix: Figure 2 and 20). Differences regarding the shedding patterns for *S. aureus* between HCPS and LCPS herds are mainly caused by the various infection status of cows. The presence of chronical shedders in HCPS herds leads to a larger number of animals with different shedding patterns in the foremilk, main milk or strippings compared to LCPS herds. The shedding cycle during milking is the main reason for problems with the determination of prevalence of S. *aureus*, particularly in HCPS herds. Number and type of positive milk fractions have to be discussed in greater detail because they are not only of great importance for the understanding of pathogenesis of bovine mastitis, they are furthermore highly correlated to the shedding rates, respectively duration of infections, which are crucial parameters regarding the dynamics of mastitis from contagious udder pathogens.

4.2.1.4 Summary

The percentage of *S .aureus* positive cows is less suitable for defining the *S. aureus* prevalence in one herd. Methodical difficulties concerning sampling and culturing as well as the test volume of milk samples do not alow safe statements about the number of infected quarters of one cow and particularly about the whole situation in the herd. The biggest variability in shedding udder pathogens is observed on the quarter level. The sense of investigating on the quarter level is questionable, because single cows shed *S. aureus* only in the main milk, too. Therefore differences regarding the shedding patterns during milking give probably a more realistic view about the situation in dairy herds than the number of all positive tested cows. However the dynamic of mastitis is too fast so that consecutive sampling is necessary. The age of cows, respectively the number of lactation periods and the risc of infection during the lactation period are the crucial influencing factors in this context. Herds with many *S. aureus* infected cows have usually more than 10 % cows shedding *S. aureus* in foremilk, mainmilk and strippings. Herds with a high number of positive samples (HCPS-herds) have usually ≥ 10 % primiparous and multiparous cows shedding *S. aureus* in three samples (foremilk, mainmilk and strippings). The opposite was observed for herds with a low number of positive cows (LCPS-herds). More than 10 % of the primiparous and multiparous cows shed *S. aureus* only in one milk fraction (foremilk, mainmilk or strippings). The cows with three positive milk fractions were all chronically shedders and were with single exceptions all positive in 0.01 ml formilk.

4.2.2 Detection of different *S. aureus* types at one timepoint of sampling - a critical step for investigations in the dairy chain

Having illustrated the methodological difficulties related to the determination of *S. aureus* prevalence in dairy herds it is justified to scrutinize investigations of the biodiversity of this pathogen in more detail. When bacteriological examinations cannot guarantee that a cow is shedding *S. aureus* in low volumes, it is consequently a much greater problem to verify all different *S. aureus* types within one sample. Only the comparison of monitoring programs investigating one milk fraction at consecutive sampling times, with one sampling interval in the dairy herd considering all milk fractions with as many as possible different bacteria colonies, can give an idea about the distribution of different strains. This is in particular the case in herds with a high prevalence of *S. aureus* in the dairy chain. How many strains are still present at one point in time of sampling, respectively remain undetected in case of common bacteriological investigations is of utmost importance for the understanding of dynamics in *S. aureus* populations as result of treatment with antibiotics or other sanitation programs over longer time periods.

4.2.2.1 Problem and Goal

The common contagious *S. aureus* are repeatedly detected in contrast to *S. aureus* with a high similarity of environmental udder pathogens, which seems to circumvent the control procedures (Sommerhäuser et al., 2003). In low prevalence situations increase the relevancy of the shedding cycle and time point of sampling (ISO 2006/408). Vries (1976) could show that in comparison to chronically shedders the bacteria count between 1-150 cfu/ml from quarters of subclinical mastitis with 150,000 up to 400,000 somatic cell counts (SCC) /ml can be the highest in the main milk.

During a control program Sommerhäuser et al. (2003) could detect in four of seven herds different strains with up to seven genotypes. The high number of different detectable *S. aureus* types in one herd is shown by the results of a single contributory laboratory according to Davidson (1972; Appendix: Table 32 and 34). One collaborator detected at least 13 types when sampling individual cows for three years (Collaborator 15 in appendix Table 34).

Additionally to the varying shedding cycle of one quarter the number of *S. aureus* positive quarter milk samples increases up to 145 % when investigating centrifuged quarter milk samples of foremilk, in which a lower detection limit (1 CFU/10 ml) is possible (Zecconi et al., 1997). It is therefore questionable if many *S. aureus* types are not detected at one time point of sampling using the common approach of 0.01ml foremilk. Therefore the crucial methodical question has to be, especially when considering the results of the chapter before (4.2.1), how many strains can be detected at one timepoint of sampling? Is always a long time study needed for the detection of more than single *S. aureus* strains in one herd?

Furthermore Sommerhäuser et al. (2003) could detect in two quarters of one cow five different *S. aureus* genotypes with other types at each sampling time. This is the best example for the insecurity of susceptibility testing regarding the antibiotic therapy. The non-observance of phenotypic characteristics in testing colonies is one of the reasons for deficient antibiotic therapy for *S. aureus* in the hospitals (Goerke et al., 2007). The elective criteria on Blood agar and Baird Parker medium are reported to be not safe enough to characterize bovine *S. aureus* (ISO 2006/408). Furthermore plating of 0.1ml milk in stead of streaking with an eye of only 0.01 ml should allow a more sensitive detection of different *S. aureus* types. One deciding advantage of this procedure is the easier detection of different colony morphological *S. aureus* types on the agar plate.

This chapter wants to show that the conventional sampling procedure and bacteriological diagnostic in relation to the pathogenesis of mastitis is the distinct problem for susceptibility testing of antibiotics, enterotoxin production and common population analysis of *S. aureus* in the dairy chain. Ten herds of farms producing raw milk cheese were investigated and grouped into HCPS (high prevalence of coagulase-positive *S. aureus*) and LCPS (low prevalence of coagulase-positive *S. aureus*) farms according to the classification of Roberson et al. (1994). *S. aureus* strains of mastitis, bulk tank milk and raw milk cheeses from single herds and a monitoring program using the common approach with conventional Blood agar or Baird Parker were collected observing the shift of *S. aureus* populations during a period of two years. One time the foremilk, main milk and stripping in 0.1 ml was investigated with a modified Baird Parker medium to detect so many different *S. aureus* strains as possible. All different colony morphological types were phage typed and tested for antibiotic resistance and enterotoxin production.

4.2.2.2 Results

4.2.2.2.1 Correlation between phage types and phenotypic characteristics

30 different phage types were observed among 547 representative *S. aureus* strains isolated from bovine mastitis, bulk milk and cheese samples in ten of the twelve herds (Table 19). The most isolates belonged to phage types 15, 17, 22, 27 and 28, which were detectable in different herds. The strains of different phage types showed a good correlation to other important characteristics (Table 20). In 11 of the 30 different phage profiles inclusive non-typable strains were isolates with *sea, seb, sec or sed* detectable. The most frequent entertoxin was *sea*, with six positive strains in one of the corresponding phage profiles.

With exception of one isolate, all strains testet positive in the Vidas system as well as in the enterotoxin *sea, seb, sec* or *sed* gene PCR. No strains with only *seg, sei* or *sej* produced enterotoxins according to the Vidas system. 20 % of strains showed α–haemolysis, 38 % αβ–haemolysis and 33 % respectively 9 % had only a weak or no haemolysis. 80 % of the strains were egg-yolk positive, 2 % clumping factor negative and 4 % were clumping factor positive but coagulase negative. Antibiogram typing detected four types. Phage type 55+ in herd F was the only phage type which varied regarding the penicillin resistance (Table 19: phage profile 18). 60 % of the strains were sensitive to all antibiotics tested. 33 % were penicillin resistant. 4 % were penicillin and erythromycin resistant and 2 % were penicillin and streptomycin resistant. The egg-yolk reaction, penicillin resistance and enterotoxin production of *sea* and *sed* is correlated to the phage 29 in phage group I, phages of phage group III and the bovine phage 117 (Table 19: phage profile 1, 7, 9, 11, 25 and 26). Phage 85 showed not only a high correlation to *sea* and *sed*, this phage was also associated to multi resistant strains with resistance to erythromycin or streptomycin (phage type 9, 10, 26). The strains with a high sensitivity to phage type 96 had a high correlation to s*eb* and/or *seg* and *sei*, were sensitive to all antibiotics tested and showed all αβ-haemolysis respectively egg-yolk reaction (Table 19: phage profile 19, 28).

Table 19 Phage reactions of the different phage patterns in detail

Phage profile (Herd)	Human phages (except of phage 116)				Bovine phages (except of phage 42D)	
	Group I	Group II	Group III	M / V	Group IV	M
0 (A,C, D, F, I , J)	Non- typable (NT)					
1 (F)	**29**/ 52/ 79					
2 (D)	29/ 52/ 52A/ 80	**3A/ 55/ 116**	6/ 42E/ 47/ **53**/ 54/ 75/ **84**/ 85	81/ **96**	42D/ **117/ 108/ 111**	78, 119
3 (D)	79	**3A/ 55/ 116**	**53**/ 54			
4 (L)[1]	29/ 52/ 52A/ 80	55	6/ 42 E	81	102/ 117	**119**
5 (A)	52A	**3A**	42E/ 47/ 54/ 84/ 85	81/ 95	102	
6 (L)	29/ 52/ 79/ 80		6/ **85**	81/ 812		
7 (A)	52A/ 79		53			
8 (A)	52A/ 79		53/ 54	95/ 812		
9 (I)	79		**6/ 42E/ 47/ 53/ 54/ 75/ 85**	**81**	**117**/ 111	78
10 (D)	52A/ 79		6/ **53**/ **85**	81	107	118
11 (B)	**29/ 80**		42E/ 47/ 53	**81**/ 95	42D/ 102/ 111	78/ 118/ 119
12 (D)	**29**/ 52/ 52A/ 80		**6/ 42E/ 47/ 53/ 54/ 75**/ 84/ 85	81/ 96/ 187/ 812	42D/ **117/108/ 111**	78, 118
13 (F)	29/ 52/ 79/ 80		**6**/ 42E/ 47/ 54/ 75/ 84/ 85	81/ 95/ 812	102/ 107/ **117**/ 108/ 111	118
14 (D)	29/ 52/ 52A		42E/ 84/ 85	95	102/ **117**/ 108 / 111	**118**
15 (A, C, F, H)	29/ 52/ 52 A/ 79/ 80			**96**	102/ 117	118
16 (I, L)	29/ 52/ 52A/ 80				102/ 117	**119**
17 (C,D, L)		**3A/ 55**/ 116				
18 (G)		55				
19 (C)		116	6/ 75/ 85	81/ 96 / 187	**102**/ 107/ 111	119
20 (B, F)			**53**			
21 (B)			**6**/ 42E/ **47**/ **53**/ **54**/ **75**/ **85**	**81**		
22 (B, J)			6/ 42E/ 47/ 53/ 75	81	42D/ **102/ 107/ 117/ 108/ 111**	78 / 118/ 119
23 (B)			6/ 42E/ 47/ **53**/ **85**	81	102/ 107/ 117/ 111	118/ 119
24 (D)			6/ 42E/ 54/ 75		**117**/ 111	
25 (A)			42E/ 53/ 54	812	102	
26 (F)			**6**/ 42E/ 47/ / 54/ 75/ **85**	812	**117**	
27 (B, D, H, L)				95		
28 (D, F)				96		
29 (B)					111	
30 (I)						**78**

† Bold marked phage numbers indicate phage reactions ≥ 1000 plaques with confluent lysis

[1] This strain was isolated from a cow during the 1st lactation. This cow was the daughter of a cow shedding the strain of phage profile 16.

Table 20 Prevalence of the phage types in the herds and correlation to important bovine phenotypic characteristics and enterotoxin genes (*sea-seh*)

Phage Profile (n herds)	Important data for mastitis			Other important characteristics			
	Dominating strain	IMI	Primiparous cows	Antibiogramm	Enterotoxin *(se)* profile	Haemolysis	Egg yolk
0 (6)	-	variable	-	Sensible; Pen	neg; seg/sei; sea	w	+
1 (1)	-	-	-	Pen	sea/ seg	αβ	+
2 (1)	+	+	+	sensible	neg	αβ	+
3 (1)	-	-	-	Pen	neg	w	+
4 (1)	-	-	´+	sensible	neg	αβ	-
5 (1)	-	+	-	Pen	neg	αβ	+
6 (1)		Only cheese		Pen	seg/ sei	α	+
7 (1)	-	+	+	sensible	sea	w	+
8 (1)	+	+	+	sensible	neg	w	+
9 (1)	-	+	-	Pen / Eryt	sed	αβ	+
10 (1)	-	+	+	Pen/ Stre	neg	α	-
11 (1)	-	-	+	Pen	sea/ seg	k	+
12 (1)	-	-	+	Pen	neg	α	+
13 (1)	-	+	-	Pen	neg	αβ	+
14 (1)	-	-	+	sensible	neg	α	-
15 (4)	+	+	+	sensible	seg/ sei; neg	αβ	+
16 (1)[a]	-	+	-	sensible	sec/tst ; neg	αβ	-
17 (3)	+	+	+	sensible	seg / sei; neg	variable	+
18 (1)	+	+	+	Sensible; Pen	neg	α	+
19 (1)	-	+	-	Pen	seb/ seg	w	+
20 (1)	-	-	-	Pen	neg	α	+
21 (1)	-	-	+	sensible	seg / sei	k	+
22 (2)	+	+	-	Pen	sec; neg	k; w	+
23 (1)	-	-	+	Pen	sej	k	+
24 (1)	-	+	+	sensible	neg	α	-
25 (1)	-	+	-	sensible	sea; neg	w	+
26 (1)	-	-	-	Pen / Eryt	sea	αβ	+
27 (4)	-	-	+	sensible	seg/ sei; neg	w	+
28 (2)	-	+	-	sensible	seb/ seg/ sei	αβ	+
29 (1)	-	-	+	sensible	neg	α	-
30 (1)	+	-	-	sensible	neg	α	-

[a] This is the single clumping factor negative phage type

4.2.2.2.2 Results of phage typing in relation to the number of S. aureus types per herd

Five phage types (15, 16, 17, 27, and 28) were isolated more than one time from different herds (Table 19). Considering these strains as different phage types per investigated herd and adding the 30 phage types, in total 41 different *S. aureus* phage types were detected in the ten dairy herds. The distribution of all cultured strains in relation to the 41 different phage types is shown in Figure 5.

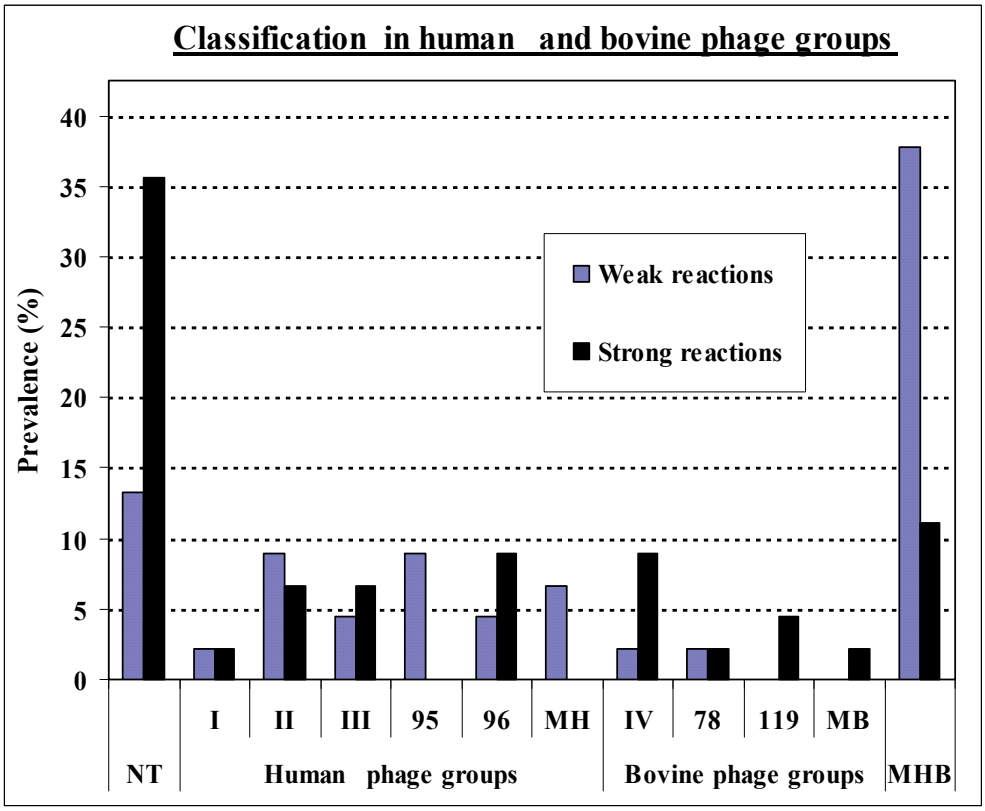


Figure 5 Classification of the different phage patterns in the corresponding phage groups considering only confluent lysis (≥1000 plaques) or weak phage reactions of ≥ 20 plaques (NT: not typable; MH: phages of mixed human group; MB: mixed bovine group; MHB: mixed human and bovine group)

The most sensitive phages were phages 85, 96 and the bovine phage 117. Only single isolates of the 30 phage types could be classified in bovine phage groups (Figure 5). 13 % of the strains were non-typable (NT). The amount of phage types with confluent lysis (strong phage reactions ≥ 1000 plaques) was 64 %. Considering only strong phage reactions 18 % were sensitive exclusively to bovine phages. 38 % of strains were sensitive to human and bovine phages (MHB) and 5 % were only sensitive to bovine phages considering weak phage reactions. 49 % of strains had phage reactions ≥ 20 plaques only for human phages.

4.2.2.2.3 Distribution of phage types in the dairy chain in relation to the *S. aureus* prevalence in the herd

The distribution and number of detected phage types is strongly associated with the prevalence of *S. aureus* in the herd (Table 21). The high number of different strains however is only observed with optimized bacteriological diagnostics that consider as many different phenotypic characteristics as possible, and with the investigation of different milk fractions. *S. aureus* CFU/g could be found in the bulk milk and therefore in the raw milk cheeses from the corresponding farms (Table 21: last sampling round in each herd after October 2003). Only with consecutive sampling of the foremilk these phage types were detected occasionally.

The opposite was observed in the HCPS herds. More cows in the HCPS herds shed the dominating *S. aureus* types in the bulk milk (50-1,200 CFU/ml), which were found in raw milk cheese in increasing numbers (4,800-120,000 CFU/g). Each new consecutive sampling round in the other HCPS herds revealed a new shedder in the herd, usually with the dominating strains. Culling or treating single cows in the LCPS herds lead to a reduction of *S. aureus* in many cases to below the detection limit in 0.1 ml bulk milk, and therefore 0.001 ml from the cheeses, until new strains colonized the udder. The measurements in the HCPS herds were performed with and low success. Only herd G was similar to the LCPS herds with two older and one younger *S. aureus* shedding cow with phage type 18. Phage type 15 and 17 in herd C and phage type 16 and 30 in herd I (Table 20, Table 21) were the reasons for culling or treating cows with penicillin, because the *S. aureus* count in the corresponding cheese was greater than 10,000 CFU/g. Treatment of the *S. aureus* shedders with penicillin induced the appearance of penicillin resistant and enterotoxin producing strains in herd C and I and the new phage types 9 and 19 were detected in the bulk milk and respective cheese. Only with consecutive sampling in subsequent sampling rounds could this phage type be detected in a single cow in the foremilk. When sampling 0.01 ml foremilk from a quarter of all lactating cow herds in September 2003, no *S. aureus* was detected in the most LCPS herds, but 10-40 *S. aureus* CFU/ml and 150-1000.

The number of different phage types increased particularly in case of the HCPS-herds to a high extent when using the sampling procedure of different milk fractions and plating 0.1 ml of milk on the modified Baird parker agar after September 2003. Cows milked before primiparous cows shedded *S. aureus* only in the main milk and in low numbers, had usually different phage types. It turned out that at least two cows in each HCPS herd shed two different phage types with different antibiogram types. Single cows in herd F, B, C, D and A had three or more phage types. Many of the primiparous cows with low shedding rates and

Table 21 Prevalence of phage types in the dairy chain of each herd and correlation to important bovine phenotypic characteristics and enterotoxin genes (*sea-seh*) by using the conventional sampling technique (until October 2003) or modified sampling technique at only one time with great effort (after October 2003)

Sampling (until and after October 2003) and measurements	Distribiution phage profiles (see Table 20)									
	HCPS herds						LCPS herds			
	A	B	C	D	F	G	H	I	J	L
Until October 2003:										
Milk + Products	25	22	15, 17	2	13, 15	18	15	16, 30	-	16
Monitoring foremilk 3 times each herd (0.01 ml)	5, 7, 8, 25	22	15, 17	2, 12	13, 15 26	18	15, -	9, -	22, -	16
Dominating strain	8, 25	22	17	2	15	18	-	-	-	-
Bulk milk + Cheese	8, 25	22, 20	15, 17	2	13, 15	18	15	9	22	16
Sum (phage types)	(4)	(2)	(2)	(2)	(3)	(1)	(1)	(2)	(1)	(1)
Culling / treating shedders	+	+	+	+			+	+‡		+
After October 2003:										
One Sampling all milk fractions (plating, 0.1 ml)	7, 8, 15, 25	11, 21, 22, 23, 27, 29	17, 19	2, 3, 10, 12, 14, 24, 27, 28	1, 13, 15, 26	18	27	9, 30	22†	4, 17
S. aureus IMI	7, 8, 15, 25	22	17, 19	2, 12 14, 24	13, 15, 26	18	-	9§	22	-
Dominating strain	8, 25	22	17	2	15	18	-	-	-	-
Environmental mastitis	25	11, 21, 23, 24, 29	19	3, 10, 12, 27, 28	1, 20	18	27	30	22	4, 6, 17
Bulk milk	8, 25	22	17, 19	2, 12	13, 15	18	-	9§, 30	22	17
Cheese	8, 25	22	17, 19	2, 12, 24	13, 15, 28	18	27	9§, 30	22	6, 17, 27
Sum (phage profile)	(4)	(7)	(2)	(8)	(6)	(1)	(1)	(2)	(1)	(4)

† This untypical bovine strain was not detected in laboratories investigating 0.01 ml foremilk over a period of one year.

‡ Cows were treated with penicillin or culled between June and July 2003.

§ The cow shedding phage type 9 in August 2004 was dry in the period during which all milk fractions were sampled; phage type 9 was detectable after calving of this cow in the bulk milk in October 2004.

lower SCC had a high diversity of phage types causing no IMI. Especially the phage types 4, 7, 24, 29 (Table 20, Table 21) were detectable on different cows and different milk fractions. One primiparous cow in herd D shed 5 different phage types. The most detectable phage types in the HCPS herds were always the dominating phage types, which were usually the same strains as in the previous sampling rounds. Culling or treating high shedding cows in HCPS herds did not reduce significantly the *S. aureus* count in the bulk milk or cheese. The lower level of the dominating strains on the plate however facilitated the isolation of other strains. A high number of different phage types were observed in the strippings and foremilk of cows shedding $\leq 10^2$ *S. aureus* CFU/ml.

S. aureus classified as environmental udder pathogens were usually only isolated in the bulk milk or in cheese samples from LCPS herds in low concentrations. Only once there was 1.2×10^3 CFU/ml detected in the bulk milk and 9.4×10^5 CFU/g in the corresponding cheese. All older cows in herd I on August 2004, as it will be discussed below, shed 2.3-3.9 $\times10^3$ *S. aureus* CFU/ml in the main milk only with phage type 30**.** Cows having an IMI with phage type 30 and phage type 16 were the reason for the control program in summer 2003. These strains have then not been isolated in the bulk milk or cheese until August 2004. Phage type 9 in herd I was detected again in October 2004 when the *S. aureus* count decreased to $<10^2$ CFU/ml in the bulk milk.

4.2.2.3 Discussion

The detection of all *S. aureus* shedders, even those with low SCC, is only a challenge in single farms producing certified milk, or raw milk products. Otherwise only the shedding cycle of cows with elevated SCC is of veterinarian interest since it is causing high SCC in the bulk milk (Sears et al., 1991; Godden et al., 2002). The common approach investigating 0.01 ml foremilk samples is not only identically with the detection of chronic shedders causing elevated SCC, respectively IMI, according to Zadoks et al. (2001). Single colonies of udder pathogens in 0.01 ml foremilk samples are furthermore the basis for susceptibility testing regarding antibiotic therapy or the detection of cows shedding *S. aureus* in the bulk milk.

The here presented results which were generated in September 2003 using the common approach investigating only foremilk samples (Table 21) agree almost completely with the findings of Larsen et al. (2000), considering the number of herds in relation to the number of different *S. aureus* types (Appendix: Table 41). Larsen et al. (2000) found 16 ribotypes, respectively 11 different phage types using only the human phage set, compared to the here

presented results with 14 phage types during the first sampling intervals in 2003. The large number of different *S. aureus* types at one point in time of sampling, were only observed during the last sampling round. Only with plating of the milk from different milk fractions, instead of streaking with an eye and an agar plate, while considering so many phenotypic characteristics as possible, more different types were detected in one sampling round. This proved that most *S. aureus* types are still present but undergo undetected when the common approach to sampling and bacteriological examination is used.

Roberson et al. (1994a) investigated composite quarter samples using a volume of 0.05 ml of milk, which is at least the same volume of 0.01 ml milk as used for conventional investigations at quarter level. According to Hoblet et al. (1988) and Roberson et al. (1994) the lack of sensitivity of composite quarter samples can therefore not be explained with the milk volume used. These results further indicate that the large number of different bacteria species, when investigating composite quarter samples, cause difficulties in bacteriological examinations. Still, the presence of different bacteria species in one quarter is according to Brolund (1985) and Øesteras et al. (1991) a challenge in the consideration of each major pathogen, and can induce errors in susceptibility testing (Appendix: Figure 19, Table 14). Strippings, and in particular the main milk, contain usually lower bacteria counts and are therefore advantageous regarding the detection of different strains of multiparous cows. In agreement with the findings made by Sears et al. (1991) and Godden et al. (2002) it was found that stripping samples are less likely to be contaminated because teats and teat canals were flushed during the milking process.

In August 2004 all cows of herd I were *S. aureus* negative in the 1st lactation. Phage type 30 was only detected in samples from older cows of herd C which had contact with cows with an IMI in summer 2004 (Table 21). High *S. aureus* counts in cheese were the reason for culling or treatment with penicillin in herds C and I. The detection of phage type 19 in herd C, and phage types 9 and 30 in herd I over a long period of time indicate that *S. aureus* can persist in the udder cistern in low concentrations without causing an IMI (Tables 19 and 21). The long persistence of the antibiotic resistant phage types 9 and 19, even after treatment, should be seen as the reason why the consequences of antibiotic treatment could remain unnoticed for a long time. Investigation of only 0.01 ml of foremilk is the main reason why *S. aureus* persisting in low numbers in the bovine udder are not detected. Furthermore, the high prevalence of antibiotic sensitive types causing bovine mastitis is a second explanation for the slow increase of antibiotic resistance in dairy herds.

The low fidelity of the bacteriological diagnosis on the quarter level is the reason why Østeras et al. (1991) recommended the antibiotic treatment of all quarters, instead of single quarters only during the dry off period (Appendix: Table 14). The strong variations in consecutive sampling intervals highlight the large problem of bacteriological examinations when samples are taken at only one point in time (Brolund, 1985; Zecconi et al., 1997). According to Brolund (1985) only 54 % of *S. aureus* shedding quarters are *S. aureus* positive in the second consecutive sampling interval (Appendix: Table 13). The explicit cause for the low accuracy of common bacteriological investigations investigating the antibiotic resistance in bovine mastitis is the consideration of only a single bacteria colony. However, Sommerhäuser et al. (2003) could culture from individual cows up to five different S. *aureus* genotypes, which all were only detectable at different consecutive samplings intervals.

The large effort involved in susceptibility testing and phage typing of many colonies including strains from different milk fractions is the reason why it is methodological not possible to determine exactly all different phage types of one cow in HCPS herds. Only the dominating phage type is usually detectable from cows shedding $\geq 10^3$ *S. aureus* cfu/ml. The detection of different strains at low concentrations is often impossible. In addition to the shedding rate the importance of the colony morphology in HCPS herds increases. Differences regarding the colony morphology are very often only visible after two days, which is not practicable for susceptibility testing. The reduction of the dominating strains in dairy herds through culling or treatment of cows shedding large numbers predominately leads in HCPS herds to an easier detection of *S. aureus* strains, which at low concentrations might be detectable at one time point of sampling only. The low number of udder associated bacteria is likely to be the reason why *S. aureus* classified as environmental mastitis pathogen culture easier on primiparous cows (Table 20).

Many strains with weak or no haemolysis were isolated from different cows with spontaneous infections, but usually in the same herd only in low numbers (Table 20). Many strains belong to *S. aureus* types which according to Sommerhäuser et al. (2003) are more similar to environmental udder pathogens. Munch-Peterson (1972) grouped the coagulase positive staphylococci with no or only weak haemolysis to CNS, which also showed a lower SCC than the haemolytic staphylococci classified as *S. aureus*. Klima (1972) found in agreement with the results obtained from the 10 herds (Table 21) a high prevalence of bovine strains with penicillin resistance of 40 %, and 39 % of isolates with β-haemolysis. Klima's publication (1972) was the single published investigation available which described the selective enrichment using 0.01 ml foremilk according to Heeschen et al. (1968). This indicates that the

commonly used approach frequently fails to isolate weak or non haemolytic bovine strains. Furthermore it shows that many strains without β-haemolysis were still present 30 years ago. According to Munch-Peterson (1970) it is not possible to recover *S. aureus* selectively even in mixtures with CNS, independent of the used medium (Blood agar, Baird parker agar, Neave's or Chapman's medium). 46 % of quarter samples from cows infected with subclinical mastitis are bacteriological negative, 25 % contain other bacteria species and additionally, in 23% are only staphylococci detectable (Kurzhals et al., 1985). The high prevalence of phage types with a weak or no haemolysis (Table 21), but with positive egg-yolk reaction, illustrate the problem involved when using BA instead of BP or PB-RPF, particularly in case of cows shedding a large number of CNS. Only by plating and determination of the clumping factor of each different colony-morphological-type on BA, or positive egg-yolk reaction as selection criterion on BP, it is possible to find *S. aureus* strains with no or only weak haemolysis. The high prevalence of CNS in herd J was the reason why no *S. aureus* was detected by the local veterinary laboratory during mastitis diagnosis (Table 21). Problems in the detection of *S. aureus* shedding cows and failure to reduce the *S. aureus* count in the bulk milk were the reason for pasteurization of the milk of herd J in October 2004.

Satisfying results for culturing bovine strains of the udder skin were observed by Fox et al. (1992) only using the Vogel-Johnson broth, whereas the BA had the lowest recovery rate (Appendix: Table 47). BA however have the highest recovery rate for bovine *S. aureus* of milking liners, respectively mastitis isolates (Fox et al., 1992). The typical haemolysis of most strains from milking liners, or IMIs in comparison to isolates of the udder skin, is obviously the reason, why BA has a better detection rate. The strains with typical haemolysis are probably the *S. aureus* types which according to Sommerhäuser et al. (2003) are initially detected in monitoring programs of dairy herds. The phage types 2, 13, 15, 17 as well as phage types 16 and 30 cause high *S. aureus* counts > 10^2 cfu/ml bulk milk, respectively > 10^4 cfu/g cheese, and lead to culling, respectively treatment of the *S. aureus* shedding cows. All of these dominating phage types produced α-and β-, with exception of phage type 30 which produces only α-. The successful elimination of these phage types, especially of phage types 15, 16 and 30 in case of the LCPS herds is shown in the results of the here presented study (Table 21).

The high prevalence of the clumping factor negative phage pattern I/119 (phage type 15 in Table 20) in dairy herds is according to Hummel et al. (1992) in agreement with the observations made by Larsen et al. (2000), who was able to detect continuously over a periode of 18 months phage types 52/52A/80+ as the dominating strains causing clinical

mastitis in many herds (Appendix: Table 22, Table 41). The great significance of phage types 15, 16, 30 and phage group IV (phage type 29) has been confirmed by the results of common monitoring programs (Table 7 and 11). These results show the low importance of *S. aureus* in common dairy herds when considering only SCC-level and total bacteria count as quality criteria for raw milk. Low shedding rates of cows in the LCPS herds were the reason for sampling the main milk, respectively the strippings. For instance, phage type 95 (phage profile 27 in Table 19) according to Larsen et al. (2000) is only occasionally detected in cows with IMI. This explains, in agreement with the here presented results (Table 20), the low concentration of this phage type, and of phage types with high sensitive for phage 85 in raw milk or raw milk products. During cheese making the *S. aureus* count usually in whey increases about 10^2 CFU/g (Zangerl, 1999a). An enrichment of foremilk samples from individual cows of > 0.1 ml milk and of > 1 ml bulk milk would be necessary to detect phage types 6, 9, 15, 22 and 27 in the cheese made of milk from LCPS herds. The strains in a herd, which are frequently detectable only in low concentrations and only from single cows, usually are not considered in the monitoring but can be very important with respect to the distribution of antibiotic resistant *S. aureus* strains in dairy herds.

According to Sabolic et al. (1980), no or only single cows in herds without *Str. agalactiae* shed penicillin resistant staphylococci. The large number of herds with only single or few cows shedding penicillin resistant staphylococci are a problem when attempting to define the effectiveness of antibiotics in monitoring programs (Sabolic et al., 1980; Appendix: Table 6). The crucial question regarding the distribution of antibiotic resistant strains, respectively the reduction in effectiveness of antibiotics, is the determination of the timepoint when instead of pathogens isolated from single cows, suddenly the majority of udder pathogens is resistant against the used antibiotic. Therefore one of the most important questions is how many antibiotic resistant strains are initially present in the background level of the herd. Only with this knowledge it is possible to obtain a comprehensive picture about changes in the distribution of antibiotic resistant staphylococci in response to frequent use of medication. Thus, not only the dominant strain but also the second and third most frequently detectable types in each herd have to be considered in resistance monitoring programs. The second and third most frequently detectable types frequently do not show typical β-haemolysis on BA, are penicillin resistant and egg-yolk positive or produce enterotoxin A. The probability for the detection of strains with *sea* of bovine origin increases in farms which produce raw milk cheese because only these farms treat or cull cows shedding the dominating strain in the bulk milk, which frequently do not produce enterotoxins.

4.2.2.4 Summary

The effective erradication of common contagious *S. aureus* strains leads to the strong reduction of phage type 119, 78, 116 and isolates with a high sensitivity for phages of phage group IV. These phages however were the cause for the creation of the BPS in the past. The lower sensitivity fo bovine phages let increase the relevance of not included human phages in the BPS as well additional phages and the perfomance of phage typing at 100×RTD, because of the high number of phage reactions without strong phage reactions. The shift in the *S. aureus* subpopulations is confirmed through the high distribution of egg-yolk positive isolates without β-haemolysis, low sensitivity for penicillin and occasionally detection of *sea*. The low fidelity of enterotoxin-profiles for typing agrees with the observation in chapter 4.1.4.

Only the investigation of many isolates together with sampling of different milk fractions and the consideration of such many phenotypic characteristics as possible allow the detection of a higher number of different strains at one timepoint of sampling. The high number of types shows the low importance of the number of investigated herds in agreement to appendix Table 34 indicating the great concern of applied methods. When using the common approach of sampling only one or two strains - usually the dominating *S. aureus* types causing IMI - were detected. When using the modified agar-plate and sampling procedure according to chapter 4.1.1 and 4.2.1 increased the number of *S. aureus* strains in single HCPS herds up to six or eight different types. These strains however were usually found on cows without IMI similar to the pathogensis of environmental mastitis pathogens. The detection of *S. aureus* in many LCPS-herds only in the product or in low concentration on single cows shows the necessity of investigating milk samples in lower volumes with at least 10 ml milk. This is very important for suceptibility testing or the detection of enterotoxin producing strains in the dairy chain.

4.2.3 SCC-values and their significance in bovine mastitis

The lack of success in reducing incidences of clinical mastitis during the past 40 years indicates that a low SCC-value alone is not a sufficient indicator for the good health of dairy herds (Appendix**:** Figures 1, 3). In most dairy farms high BMSCC are the only reason for mastitis monitoring programs concerning the entire herd. Usually, only the milk of cows with high SCC is investigated and cows with moderate SCC < 400,000 SCC/ml are not sampled. This procedure however is not a sustainable measure and provides no information about the real prevalence of udder pathogens in dairy herds. Many chronical shedders are not detected and subsequently spread the udder pathogens in the whole herd. Not the high SCC-values, but much more the critical SCC-values for chronical shedders are the distinct indicator for controlling bovine mastitis. This is the reason why SCC-values in bovine mastitis have to be investigated in greater detail. When discussing critical SCC-values, furthermore it has to be considered that SCC of cows is the average of the quarter total milk including the milk of different milk fractions (foremilk, main milk, strippings).

4.2.3.1 Problem and Goal

In 1967, somatic cell count was included as a criterion to define mastitis (Hillerton, 1999). Quarters with less than 500,000 SCC/ml and no pathogens were defined as having a normal secretion. Later, this limit for subclinical mastitis was set to 300,000 SCC/ml and finally to 100,000 SCC/ml (Kurzhals et al., 1985; Hillerton, 1999). Furthermore the somatic cell count (Smith and Schulze, 1967; Vries, 1976; Bruckmayer et al., 2004), the shedding rate of *S. aureus* (Murphy, 1943; Vries, 1976; Sears et al., 1991; Mavrogianni et al., 2006), and the somatic cell composition change during milking (Sarikaya et al., 2005).

The low correlation between clinical mastitis and SCC-values respectively subclinical mastitis and clinical mastitis is one crucial problem in the udder health. Even in herds with somatic cell counts of the bulkmilk between 86,000 and 300,000 SCC/ml *S. aureus* mastitis is one of the most important udder pathogen (Hoblet et al., 1988; Hutton et al., 1990; Busato et al., 2000). The detection of all *S. aureus* shedders, also the one with low SCC, is only the challenge in single farms producing raw milk free for consumer or raw milk products. The shedding cycle of cows with elevated SCC is otherwise only from veterinarian interest since these cows causing high SCC in the bulk milk (Sears et al., 1991; Godden et al., 2002). The low median however of 449,000 SCC/ml from *S. aureus* infected quarters as well the SCC of

frequently 10,000 SCC/ml from bacteriological not infected quarters according to Tolle et al. (1968) are the reason for the low SCC of *S. aureus* positive bulk milk samples. A "median SCC" of a cow with 120,000 SCC/ml would be not unusual, if the cow has one *S. aureus* infected and three bacteriological negative quarters.

Intramammary infections (IMI) are very important for the udder health and play a major role in the dynamic of mastitis. *S. aureus* IMI is according to Zadoks et al. (2001) defined with at least ≥ 100 cfu/ml in three consecutive foremilk samples, which is identically with the detection of chronically shedders causing elevated SCC in the bulk milk. The dry off period and age of cows are very important in this context (Sheldrake et al., 1983; Mylls, 1994; Enevoldsen et al, 1995; Zecconi et al., 2003). The high correlation between the SCC and number of lactation of cows is the best evidence for this problem (Zeidler et al., 1969; Milchprüfring Bavaria, 2001; Appendix: Figure 3).

The interdependence of number of lactations and correlation to IMI and SCC is one of the unsolved problems in bovine mastitis. The increasing dilution effect of the SCC and bacteria count in the order udder quarter, compositive quarter samples (cow level), bulk tank milk and number of lactations of cows is usually not taken into account. This study was conducted to reveal a realistic exposure of the SCC-values for *S. aureus* shedding cows under consideration of the number of lactations, IMI and prevalence of *S. aureus* in the herd.

4.2.3.2 Results

Eight cows in six farms shed more than 1,000,000 SCC/ml main milk. In all cases except of farm A they contributed significantly to exceeding the limit for somatic cell count in bulk milk (Table 22). These cows were causing the outliers with respect to the high SCC and therefore were excluded from the statistical calculations. A detailed analysis of the data from these eight cows revealed that three cows shed less than 1×10^2 CFU of *S. aureus*/ml milk. Five of the cows shed *Streptococci* in at least one fraction during milking. Two of these cows had severe mastitis one month after sampling for this study and one had been culled in the meantime. Sampling of cow 1 was performed one week earlier and revealed a mixed infection with *S. aureus* and *Streptococci* in two quarters.

However, cow number 5 - the only young cow in the first lactation period in this group - ranked second in shedding *S. aureus* in the main milk for this farm. Prior to this study, this cow had tested positive for *Streptococci* in one quarter, but negative for *S. aureus* at three consecutive samplings. Two cows shed no *Streptococci* and both were post patrum. One of

those cows had about 550,000 SCC/ml in a two-month period prior to the study and <300,000 SCC/ml in a two-month period after the study.The other cow had < 300,000 SCC/ ml in the same period before and after the study.

Table 22 Somatic cell count and numbers of *S. aureus* and Streptococci in the milk of *S. aureus* positive cows with more than 1,000,000 SCC/ml main milk

Cow	Number of lactations	SCC in thousand/ml					Milk fraction	*S.aureus* (CFU/ml)	*Streptococci* (CFU/ml)
		1st month	2nd month	**present study** 3rd month	4th month	5th month			
1[c]	5	291	681	**1,191**[a]	severe mastitis[b]	ill	foremilk main milk strippings	2.5×10^4 2.0×10^4 1.3×10^4	$< 1.0\times10^1$ $< 1.0\times10^1$ $< 1.0\times10^1$
2	5	585[a]	7,506[b]	**1,854**	culled	xxx	foremilk main milk strippings	1.5×10^2 4.0×10^2 4.0×10^2	1.0×10^3 2.0×10^3 7.0×10^2
3	5	1,980	2,982	**3,377**	1,496	3,190	foremilk main milk strippings	3.0×10^1 1.0×10^1 $< 1.0\times10^1$	$< 1.0\times10^1$ 4.0×10^2 4.0×10^3
4	2	559	525[a]	**2,173**[b]	250	263	foremilk main milk strippings	2.0×10^1 4.0×10^1 $< 1.0\times10^1$	$< 1.0\times10^1$ $< 1.0\times10^1$ $< 1.0\times10^1$
5	1	405	298	**2,485**	severe mastitis	culled	foremilk main milk strippings	2.0×10^1 1.5×10^3 2.5×10^2	8.0×10^3 4.0×10^3 4.2×10^3
6	10	499	1,517[a]	**2,399**[b]	1,247	6,128	foremilk main milk strippings	$< 1.0\times10^1$ 1.2×10^3 8.0×10^2	$< 1.0\times10^1$ 7.5×10^3 2.5×10^3
7	8	3,372[b]	190	**2,061**	51	148	foremilk main milk strippings	$< 1.0\times10^1$ $< 1.0\times10^1$ 2.0×10^1	5.0×10^3 1.5×10^4 1.3×10^4
8	4	43	70[a]	**2,802**[b]	166	219	foremilk main milk strippings	1.0×10^4 $< 1.0\times10^1$ $< 1.0\times10^1$	$< 1.0\times10^1$ $< 1.0\times10^1$ $< 1.0\times10^1$

[a] Before dry-off

[b] First measure after calving

[c] Number represents the detection limit of the microbiological method

The SCC increases linear together with the number of lactations in cows negative for *S. aureus*, and a negative correlation between the number of lactations and the number of cows with < 100,000 SCC/ml was observed (Figure 6).

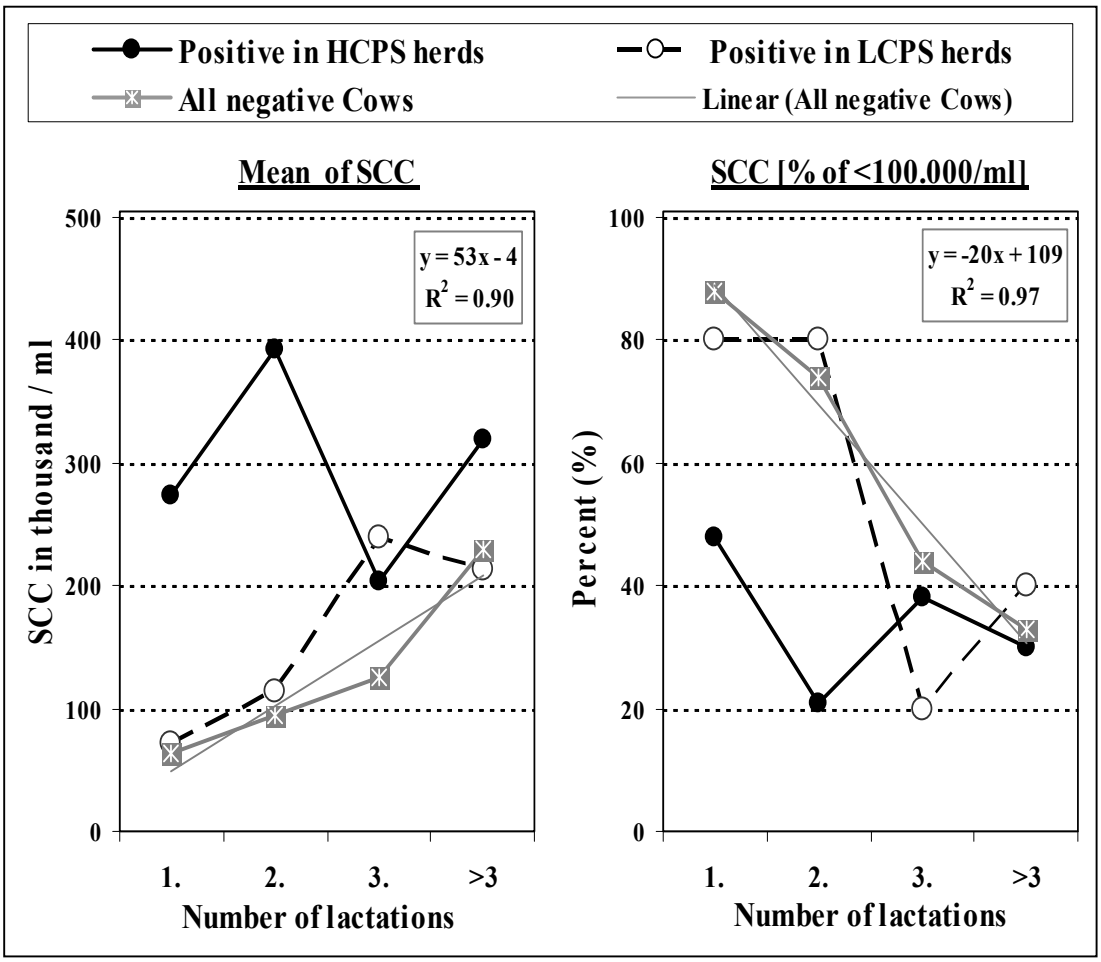


Figure 6 Relation of SCC (mean: in thousand/ml, SCC % < 100,000 SCC/ml) and number of lactations of *S. aureus* shedding and non-shedding cows in LCPS and HCPS farms

The difference is significant in the SCC between the LCPS and HCPS farms in the first and second lactation (P= 0.072; P=0.087), but insignificant for positive cows with more than three lactations (P= 0.581). No cow with IMI had less than 25,000 SCC/ml (Figure 7). A strong increase of cows with IMI was observed for SCC-values >100,000 SCC/ml. Many cows without an IMI but ≥200,000 SCC/ml shed streptococci.

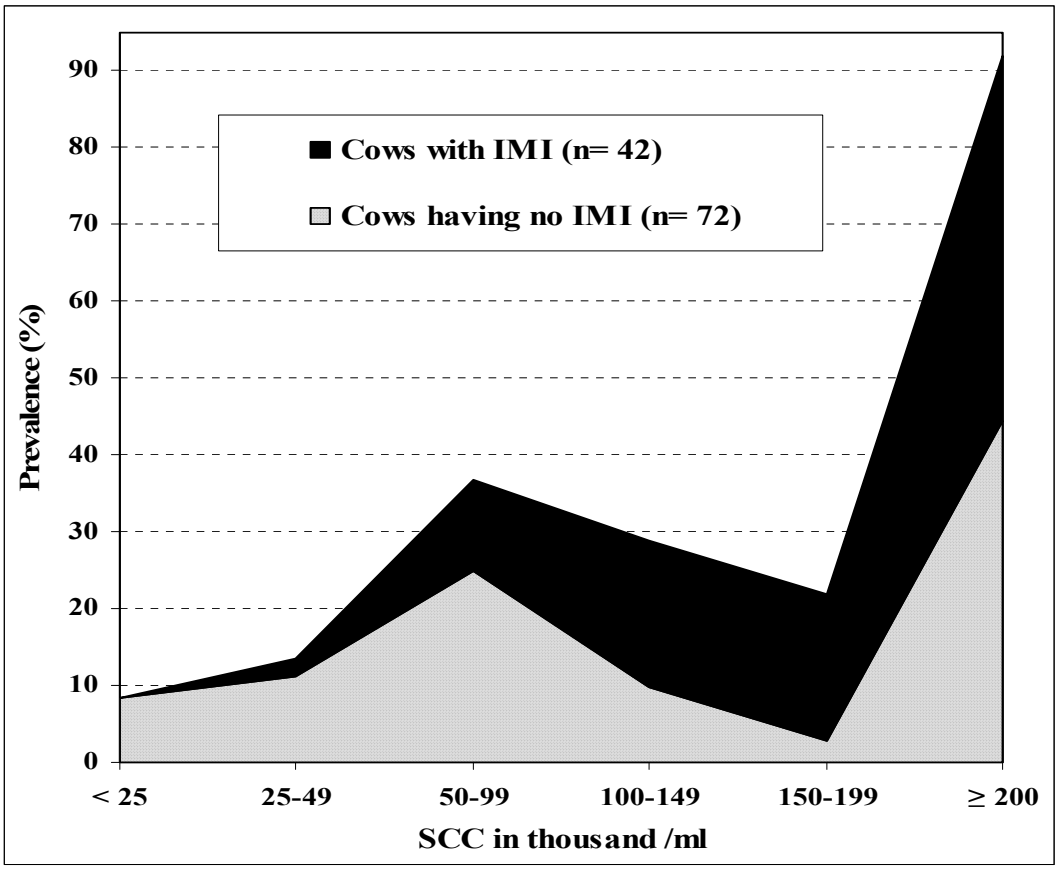
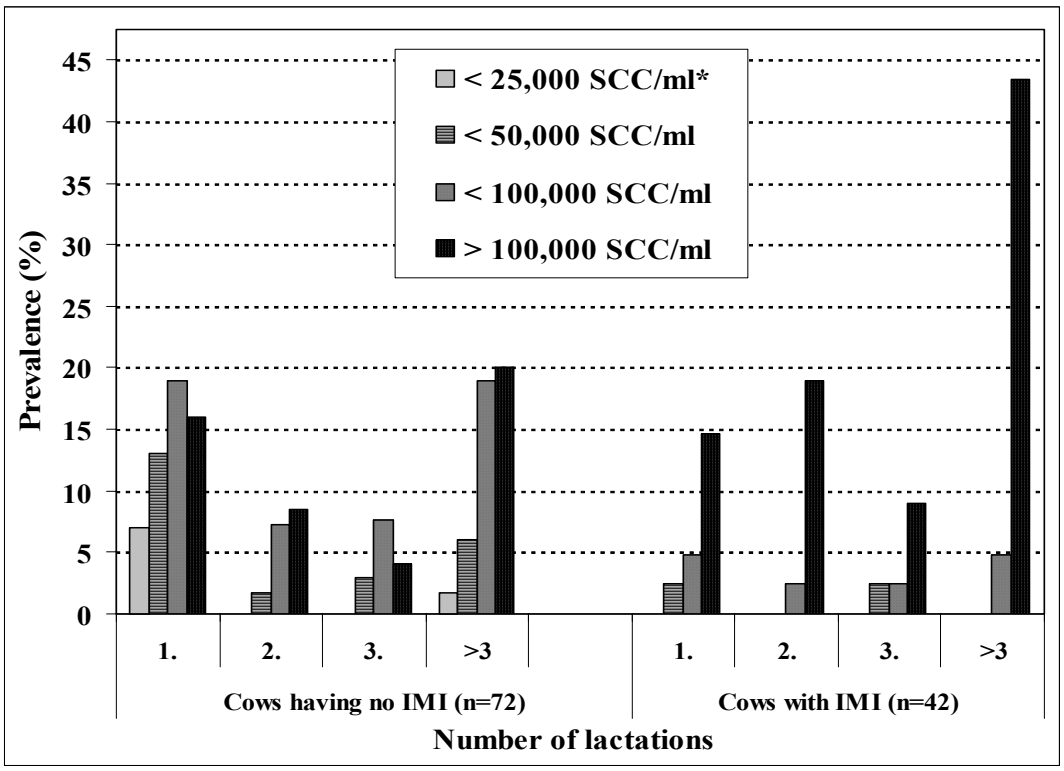


Figure 7 Classification in SCC-values between <25,000 and ≥200,000 SCC/ml for cows with IMI or spontaneus *S. aureus* infections (Cows having no IMI)

The classification of cows with IMI and *S. aureus* positive cows without IMI into different SCC-groups revealed that most cows with subclinical mastitis have less than 100,000 SCC/ml (Figure 8). Apart from one animal, all were *S. aureus* positive cows without IMI and <25,000 SCC/ml in the 1st lactation. *S. aureus* positive cows with less than 50,000 SCC/ml and no IMI were most frequently detected in the groups of cows with more than three lactations.



*All quarters have < 100,000 SCC/ml

Figure 8 Classification of cows with *S. aureus* positive samples but no IMI or *S. aureus* IMI in relation to the number of lactation and SCC-groups of < 25,000, < 50,000, <100,000, >100,000 SCC/ml

4.2.3.3 Discussion

Methodological problems of bacterial examinations are one explanation why the SCC is still the most dominantly used parameter for the definition of bovine mastitis. According to Miltenburg et al. (1996) one reason for the great importance of the SCC-value is the high prevalence of about 25 % of negative bacteriological samples obtained from cows with clinical mastitis. In 1967 infected quarters with less than 500,000 SCC/ml were according to Hillerton (1999) defined as having a latent infection. The high prevalence of udder pathogens in quarters with less than 500,000 SCC/ml, with exception of *Str. agalactiae* positive is the reason why the threshold for subclinical mastitis was defined first to 300,000 SCC/ml and finally to 100,000 SCC/ml (Hillerton, 1999). The median-value of only 174,000 SCC/ml of bacteriological negative quarter samples containing no udder-associated bacteria, or *S. aureus* with a median-value of only 187,000 SCC/ml or 449,000 according to Zeidler et al. (1968)

show as well that the SCC count is no suitable criteria for the classification of the infection status of single cows (Appendix: Table 5).

According to Hoblet et al. (1988), even herds with a BMSCC of approximately 130,000 cells/ml have a prevalence of about 20 % *S. aureus* positive composite foremilk samples, with many cows shedding only 21,000 up to 100,000 SCC/ml in the foremilk. These results illustrate the low relevance of SCC regarding the prevalence of udder pathogens in dairy herds. The SCC of a cow is according to Brolund (1985) considerably more informative about udder health than BMSCC, even through being less informative than the SCC obtained from single quarters. The herd size, respectively dilution effect of individual cows is the crucial factor of influence for extreme BMSCCs (Tolle, 1977). Single cows shedding more than 1,000,000 SCC/ml are the main problem in small herds regarding the BMSCC limits of 300,000 or 400,000 SCC/ml. Furthermore, in this context the average annual milk yield of a herd, its lactation average, and number of cows in late lactation are very important parameters (Appendix: Figures 2-6). The close correlation between clinical mastitis, subclinical mastitis, or high SCC and lactation is shown when comparing the frequency of clinical mastitis and IMI during this period. These observations are in agreement with the here presented results regarding cows shedding more than 1,000,000 SCC/ml in the bulk milk (Table 22). The first month in the lactation shows the highest numbers of new infections, clinical mastitis and high SCCs in relation to the subsequent month of the following lactation period (Appendix: Figures 3, 8, 17 and 18). The SCCs and new infections decrease during the second month and increase again to higher cell counts in the last month of the lactation period. The increasing SCC and a high frequency of clinical mastitis furthermore strongly correlate with multipareous cows (Barkema et al., 1998; Appendix: Figure 7).

The dilution effect of single quarters plays as well an important role for the SCC on cow level. Since more than 50 % of bacteriological negative quarters with less than 100,000 SCC/ml have a SCC of only 10,000 cells/ml did Hillerton (1999) underestimate the strong dilution effect of two or three quarters. According to Hillerton (1999) the grey zone of udder infections on the quarter level is between 100,000 and 400,000 SCC/ml. He states that in case of a cow with the SCC of one possibly infected quarter located in the grey area has to be consistently calculated for the entire udder with a SCC of only 33,000 SCC/ml in the main milk instead of a value between 100,000 and 200,000 SCC/ml.

Cows with bacteriological positive findings after different consecutive sampling intervals have according to Brolund (1985) usually a SCC value between 100,000 and 400,000 SCC/ml. According to Zeidler et al. (1968) the median value of bacteriological negative

quarters with only 174,000 SCC/ml shows the crucial problem when investigating 0.01 ml of foremilk, since according to Hillerton (1999) it is almost certain that quarter samples with a SCC lower than 100,000 cells/ml contains no microorganisms derived from the udder. The association between an earlier SCC and new IMI level is according to Zadoks et al. (2001) more or less linear for *S. aureus;* an previously high SCC is associated with a greater risk for a new IMI, in contrast to the original reference level of less than 50,000 SCC/ml. A SCC with less than 250,000 cells/ml was not significant for new IMI, in contrast to the SCC for cows with new IMI and a SCC of over 250,000 cells/ml, and in particular in cases with more than 500,000 cells/ml (Zadoks et al., 2001). The here presented results indicate that the grey area of the SCC calculated from the main milk of *S. aureus* positive cows is between 25,000 and 50,000 SCC/ml and for IMI between 50,000-100,000 SCC/ml (Figure 8). This grey area for *S. aureus* positive cows with less than 100,000 SCC/ml in the main milk is however only observed when analysing both the main milk and stripping samples.

Together with methodological difficulties for bacteriological examinations lead foremilk samples to an over- or underestimation of the SCC in the main milk, respectively of shedding rates of the corresponding cow in the bulk milk. The overall correlation coefficients between the SCC in the quarter foremilk and quarter total milk are in the order of 0.70 to 0.86 (Reichmuth, 1975; Mijnen et al., 1982). Other reports indicate that the correlations depend on the range of SCC and the sampling periods of diagnostic interests (Brolund, 1985). Vries (1976) found shedding cycles for somatic cells and bacteria counts only in quarters with more than 500,000 SCC/ml (Appendix: Figure 15). The shedding cycle differs not only between bacteria species, each quarter has even its individual shedding cycle (Huber, 1970; Vries, 1976). This fact recommends therefore the use of main milk, which is the total milk of all four quarters, as the best adequate representative average of all milk components; respectively SCC and bacteria count of a single cow. When based on lactation records the correlation of the SCC in the main milk includes according to Brolund (1985) several levels of information:

- an increasing SCC corresponds to an increase of positive samples for subclinical mastitis,
- an increasing SCC corresponds to an increase of the number of quarters with subclinical mastitis in the udder,
- an increasing SCC corresponds to a bacteriological panorama change towards an increased number of quarters infected with major pathogens.

The results shown in Figures 6, 7 and 8 indicate that probably a too low number of older cows contained in the analysis of Hillerton (1999) could be another reason why he defined the limit of an accepTable SCC on the cow level to 100,000 cells/ml. The good correlation in Figure 6 for an increasing SCC with rising number of lactations with 53,000 SCC/ml per lactation for non-*S. aureus* shedding cows fits very well with the average for Bavarian dairy herds of 46,000 SCC/ml per lactation (Appendix: Figure 3). These results confirm the observation of Brolund (1985) that not only the number of infected cows but also the number of infected quarters increases with each further lactation. However, only the low number of infected cows together with the low number of infected quarters causes the low distribution of older cows with less than 100,000 SCC/ml. This observation furthermore explains why almost exclusively individual *S. aureus* positive cows in the 1st lactation had less than 25,000 SCC/ml, or why young *S. aureus* positive cows without IMI had the highest prevalence of SCC-values lower than 50,000 SCC/ml (Figure 8).

The low number of *S. aureus* IMI in LCPS herds, in comparison to HCPS herds and especially in case of cows in the first two lactations, is the reason for an increased risk of elevated BMSCC in HCPS herds. A high BMSCC is according to Smith (IDF-Bulletin, 2001) further correlated to IMI with *Str. uberis*, *S. aureus*, *Str. agalactiae* and *Str. dysgalacticae*. The prevalence of only 4.5 % environmental streptococci in quarter foremilk samples from cows with subclinical mastitis with a SCC-value between 300,000-3,000,000 cells/ml according to Kurzhals (1985) indicates that many quarters with streptococci have high SCC (>3,000,000 cells/ml) and/or abnormal milk representing the transition to clinical mastitis. Only three out of eight cows with more than 1,000,000 SCC/ml shed no streptococci and the *S. aureus* shedding rates of five out of eight cows were under the detection limit of 0.01 ml foremilk of the quarter samples (Table 22). The results of Vries (1976) show that taking foremilk samples from cows with staphylococci IMI lead easily to an overestimation of the SCC, in comparison to samples from the quarter total milk of persistently streptococci shedding cows (Appendix: Figure 15 b, c). Furthermore, during the lactation cows with streptococci show subacute signs of mastitis and their milk is frequently not added into the bulk tank milk.

The CMT-test is still the most used method for screening cows with subclincial mastitis (Busato et al., 2000). The low median-value in relation to the average-value with respect to the SCCs of streptococci and *S. aureus* positive quarters according to Zeidler et al. (1968) are the main reason why the CMT-test is very effective for the selection of cows with a high SCC. However sampling of quarters with a CMT score of 2 or 3 selects quarter samples of

cows with 500,000 and up to 5,000,000 SCC/ml (Seelemann 1964, Appendix: Table 4; Godden et al., 2002). According to Zeidler et al. (1968) and Kurzhals et al. (1985), the high number of staphylococci positive quarter samples with less than 500,000 SCC/ml is realized only when using more sensitive methods or by sampling all cows in one herd (Appendix: Table 5). Therefore, one reason for the high prevalence of cows with streptococci mastitis could be the use of the CMT-test as a single test when screening dairy herds with high BMSCC (Appendix: Table 1) (Sobiraj et al., 1997; Schaellibaum (IDF-Bulletin, 2001). Furthermore, except from the selection of older cows the sampling of cows restricted to strong reactions in the CMT-test leads to an underestimation of *S. aureus* positive cows, respectively quarters, when the median value of 449.000 SCC/ml and the high prevalence for *staphylococci* positive quarters are considered (Zeidler et al., 1968).

Additionally the SCC-level as well *S. aureus* prevalence are not correlated with a high occurence of clinical mastitis. Zadoks et al. (2001) exclude all cows with clinical mastitis in their multivariable models because the cause-effect order of the associated variables was unknown. Dairy herds with less than 150,000 SCC/ml have according to Miltenburg et al. (1996) and Barkema et al. (1998) 20 % cases with clinical or subacute mastitis in one year in comparison to herds with more than 150,000 SCC/ml with 12 % cases annually. Only cows with the lowest mean SCC (<60,000 SCC/ml) in the 1st lactation showed a significantly lower risk for clinical mastitis in the 2nd lactation (Rupp et al., 2000). Furthermore the risk of serve clinical mastitis decreased significantly for slight increased individual SCC in the month before clinical mastitis was diagnosed (Peeler et al. 2002). The critical SCC is according to Peeler et al. (2002) 40,000 cells/ml on the cow level to suffer a serve rather than a mild case of mastitis. According to Schalm et al. (1964) quarter foremilk with 200,000-300,000 SCC/ml reflected a partial protection and 500,000 SCC/ml complete protections against experimental coliform infections. The BMSCC of about 300,000/ml in the 12 dairy farms of the presented resutls in herds with ≤ 36 cows per herd is the reason, why only single cows (usually statistical outliers) in each herds had more than 1×10^6 SCC/ml**.** The great concern of removing outliers from the data base in screening herds based on SCC was recently reported (Bartlett et al., 2001).

A high occurrence of clinical mastitis with environmental streptococci and coliforms is according to Miltenburg et al. (1996) and Barkema et al. (1998) especially observed in particular for herds with low BMSCC. E*scherichia coli, Str. uberis, Klebsiella spp.* and *Serratia spp.* are according to Smith (IDF-Bulletin, 2001) the most relevant pathogens for causing clinical mastitis in the United States of America. The coliforms respectively *E. Coli*

are according to Krabisch et al. (1999) also the most important udder pathogen in Bavarian dairy herds with a high occurrence of mastitis in contrast to randomized selected herds with a high prevalence of coagulase negative staphylococci (Appendix: Table 3). According to Zadoks et al. (2001) are clinical signs in the quarter significantly associated with new *S. aureus* infections (P<0.0001). Environmental streptococci have according to Hogan et al. (1989) the highest prevalence in cows with subacute mastitis, whereas coliforms respectively *E.coli* are the most important udder pathogen causing serve mastitis with systemic symptoms (Appendix: Table 2).

4.2.3.4 Summary

High SCC´s (> 1,000,000 SCC/ml) are usually detected short after calving or short before the dry off period. Streptococci are frequently involved when high SCC´s are detectable. Only cows with < 50,000 SCC / ml have no IMI. All *S. aureus* positive cows with < 50,000. SCC were only occasionally shedders and shed *S. aureus* only in lower numbers (< 100 / ml). They were usually primipareous or old cows with more than three lactations with a beginning colonization or a *S. aureus* infection in the past. Most cows with IMI belong to the SCC-value of > 100,000 SCC /ml, frequently with about 200,000 SCC/ml. This is especially observed for cows with more than two lactations. Considering the SCC of one *S. aureus* positive quarter with 449,000 SCC /ml according to Zeidler et al. (1968) have the other quarters about 120,000 SCC/ml. This observation explains the low suitability of the CMT for the selection of *S. aureus* shedding cows. Additionally *S. aureus* negative cows with more than two lactations had about 200,000 SCC/ml as well, which is the cause for the low suitability of the SCC when investigating *S. aureus* infections in dairy herds. Furthermore it demonstrates the low fidelity of SCC- values in dectecting IMI of all other udder pathogens as well. Finally, the high correlation between the SCC and age of cows is almost an evidence for the natural colonization of the udder with bacteria during the lactation period, which make progress with each lactation during the dry off periode.

4.2.4 Shedding patterns of *S. aureus* during milking in relation to the pathogenesis of bovine mastitis

The shedding patterns of *S. aureus* during milking are in the centre of the discussion about controlling this pathogen in the dairy chain. The interdependence between immune response and effects of the milking process determines the pathogenesis of mastitis. Shedding patterns, as well normal and exponential distributions of SCC and CFU data in groups with one, two and three positive milk fractions, demonstrate the importance of the milking process in relation to the immune response for the pathogenesis of mastitis. This further explains the commonly used approach of using 0.01 ml foremilk, and the significance of sampling different milk fractions, in particular when regarding the relevancy of high shedders in the strippings.

4.2.4.1 Problem and Goal

The conventional bacteriological mastitis diagnostic based on international standards resulted in 43.1 % ± 20.8 % false positives, which were mainly due to teat canal infections simulating mastitis (Giesecke and Viljoen, 1974). Daley et al. (1991) found a positive correlation between the somatic cell count (SCC) and phagocytosis resulting in an alternating, asynchronic shedding cycle of *S. aureus* counts (CFU/ml) in relation to SCC. At periods with lower SCC the cells are at least efficient enough to kill intracellular bacteria, and at this time, bacteria can often be non detectable (Daley et al., 1991). These findings might also explain quantitative and qualitative differences in DNA-based real-time PCR compared to traditional microbiological methods for the detection of *S. aureus* in raw milk (Hein et al., 2005; Studer et al., 2008). On the other hand, quarters with low somatic cell counts had no shedding cycle of somatic cells (Studer et al., 2008; Vries, 1976). For these quarters the type of milk fraction with the highest bacteria count was variable (Vries, 1976). These shedding cycles and the variability in the type of positive milk fraction pose a challenge to effective diagnosis of *S. aureus* infected udders and might result in major variances in predictive values in low prevalence situations (ISO 2006/408).

In the present chapter the shedding rates of *S. aureus* in foremilk, main milk and strippings of 269 cows in 12 farms were compared and number and type of positive milk fractions per animal were determined. Twelve herds were investigated and grouped into HCPS (high prevalence of coagulase-positive staphylococci) and LCPS (low prevalence of coagulase-

positive staphylococci) herds according to the classification of Roberson et al. (1994). The data obtained were analyzed with respect to different stages of the disease, number of lactations and correlated to the respective somatic cell counts. In addition, the diagnostic value of analyzing single milk fractions was assessed.

4.2.4.2 Results

Results were grouped with respect to the number of *S. aureus*-positive milk fractions per cow. The SCC- and CFU counts were tested before and after logarithmic conversion with respect to the distribution of data (Tables 23 and 24). In case of non-transformed SCC values of cows with two positive milk fractions an exponential curve fitted the distribution of the data well. In these cases, *P*-values of 0.91 (positive foremilk and main milk), 0.80 (positive foremilk and strippings), and 0.63 (positive main milk and strippings) were registered. For cows with one or three positive milk fractions, *P*-values for exponential distribution ranged from 0.07 to 0.013. For log-transformed SCC values the normal distribution fitted very well, with *P*-values ranging from 0.87 to 0.99.

In addition, for cows of farm A, which shed *S. aureus* in all three fractions, *P*-values of 0.86 and 0.99, respectively, indicated exponential distribution of non-transformed SCC data and normal distribution of log-transformed data. Non-transformed CFU data agreed better with normal distribution than with exponential distribution (Table 24).

Table 23 Fit of normal and exponential distribution to non-transformed and log-transformed SCC data of groups with one, two and three positive milk fractions.

Type of distribution	*P* - values						
	One positive fraction			Two positive fractions			Three positive fractions
	Foremilk	Main milk	Strippings	Foremilk + main milk	Foremilk + strippings	Main milk + strippings	
Non-transformed data							
Normal	0.02	**0.08**	0.04	**0.42**	**0.65**	**0.20**	<0.004
Exponential	**0.08**[1]	**0.11**	**0.13**	**0.91**	**0.80**	**0.63**	**0.07**
Log-transformed data							
Normal	**0.97**	0.96	**0.87**	**0.89**	**0.99**	**0.99**	**0.89**
Exponential	<0.004	<0.004	<0.004	**0.05**	0.01	<0.004	<0.004

[1] *P*-values above the level of significance (0.05) are indicated in bold.

Table 24 Fit of normal and exponential distribution to non-transformed and log-transformed CFU data of groups with one, two and three positive milk fractions.

Positive milk fraction	Distribution	*P*-values				
		One positive fraction	Foremilk + main milk	Foremilk + strippings	Main milk + strippings	Three positive fractions
Non-transformed data						
Foremilk	Normal	0.01	**0.54**[1]	**0.09**		<0.004
	Exponential	<0.004	**0.62**	0.01		<0.004
Main milk	Normal	0.01	**0.70**		0.03	0.01
	Exponential	<0.004	**0.23**		0.02	<0.004
Strippings	Normal	**0.09**		0.04	**0.09**	<0.004
	Exponential	**0.26**		**0.07**	**0.051**	<0.004
Log transformed data						
Foremilk	Normal	**0.93**	**0.98**	**0.93**		**0.83**
	Exponential	0.02	**0.23**	**0.25**		<0.004
Main milk	Normal	**0.61**	**0.98**		**0.90**	**0.99**
	Exponential	<0.004	**0.19**		0.01	<0.004
Strippings	Normal	**0.95**		**0.80**	**0.81**	**0.09**
	Exponential	<0.004		**0.06**	0.01	<0.004

[1] *P*-values above the level of significance (0.05) are indicated in bold.

However, *P*-values were generally low and only the strippings of cows with one positive milk fraction, the foremilk of cows with positive foremilk and strippings, and the strippings of cows with positive main milk and strippings, showed significant *P*-values. The data of stripping samples of cows with one positive milk fraction, foremilk of cows with positive foremilk and main milk, and strippings of cows with positive main milk and strippings, were more likely to be exponentially distributed than normally distributed. All log-transformed CFU data were normally distributed, with *P*-values ranging from 0.8 to 0.99. Main milk from cows with one positive milk fraction ($P = 0.61$) and strippings from cows with three positive milk fractions ($P = 0.09$) had lower *S. aureus* counts. The latter may have been due to the fact that this group included six cows which shed higher numbers of *S. aureus* (>1000 - 58,000 CFU/ml) than the remainder of the group.

When these cows were excluded, the *P*-value rose to 0.23. The separately analyzed *P*-value of the six cows was 0.98. Apart from the data for foremilk, the log-transformed CFU data of farm A were normally distributed, with *P*-values of 0.73 and 0.75 in main milk and strippings, respectively. The low *P*-value ($P < 0.48$) in foremilk may have been due to difficulties in determining the exact number of *S. aureus* on agar plates. 50% of cows in farm A with three positive milk fractions had *S. aureus* counts of about 30,000 CFU/ml. Thus, more than 1,000

colonies were present on agar plates had contained 0.01 ml in one sample. The correlation between SCC and CFU values was rather poor. In most cases there was a negative correlation between these parameters, with regression coefficients (R^2) ranging from -0.03 to -0.24. A positive correlation was observed only for the foremilk of cows with one positive milk fraction, foremilk and strippings of cows with both fractions positive, and the main milk of cows with three positive milk fractions, with corresponding R^2 ranging from 0.13 to 0.25. When analyzing the data of cows with three positive milk fractions of farm A separately, the correlation in strippings was 0.51, and in both foremilk and main milk it was 0.18.

The median of SCC was for all *S. aureus* positive cows 146,000 /ml. The median count and mean count for *S. aureus* CFU in relation to SCC-values of positive samples in foremilk, main milk or strippings are shown in more detail in Figure 9. The biggest differences between the mean count and median count of the foremilk were observed for SCC-values with less than 50,000 SCC/ml and more than 400,000 SCC/ml. The mean count in the strippings increased in a high extent for cows with more than 100,000 SCC/ml. The increasing median count in the strippings was linear for cows with more than 100,000 SCC/ml in contrast to the foremilk. The median *S. aureus* count in the strippings of cows with less than 200,000 SCC/ml increased from 50 CFU/ml up to 210 CFU/ml for cows with more than 200,000 SCC/ml.

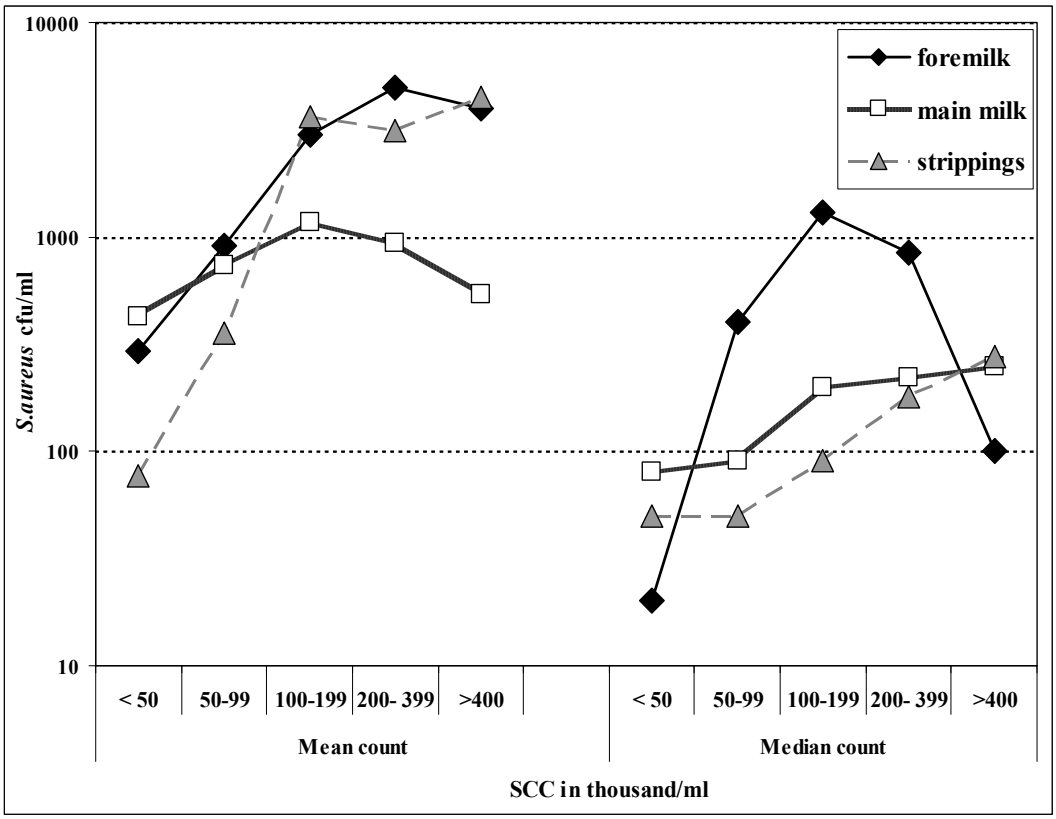


Figure 9 Mean count and median count of *S. aureus* CFU/ml in positive foremilk, main milk and stripping samples in relation to SCC-values of up to ≥ 400,000 SCC/ml

All cows with only one positive milk fraction had median SCC values of below 100,000/ml, with no statistically significant difference between the SCC values of cows with positive foremilk, main milk or strippings (foremilk vs. main milk: $P = 0.3$; foremilk vs. strippings: $P = 0.36$; main milk vs. strippings: $P = 0.86$). Median numbers of *S. aureus* were 60, 150 and 50 CFU/ml in foremilk, main milk and strippings, respectively. No statistically significant difference was registered in the counts for foremilk and strippings ($P = 0.77$). Cows with positive main milk and strippings had similar values as cows with positive foremilk and main milk ($P = 0.43$), and cows with positive foremilk and strippings ($P = 0.12$). Median *S. aureus* counts in the first positive milk fraction ranged from 210 to 380 CFU/ml and were always higher than those in the second one, which ranged from 50 to 60 CFU/ml. Cows with two positive milk fractions showed median SCC values ranging from 69,000 to 483,000/ml. SCC and CFU counts in relation to the number of positive milk fractions per cow are shown in Figure 10. For detailed information see Table 25. As the majority of cows from farm A shed *S. aureus* at very high levels in all milk fractions, this farm is shown separately.

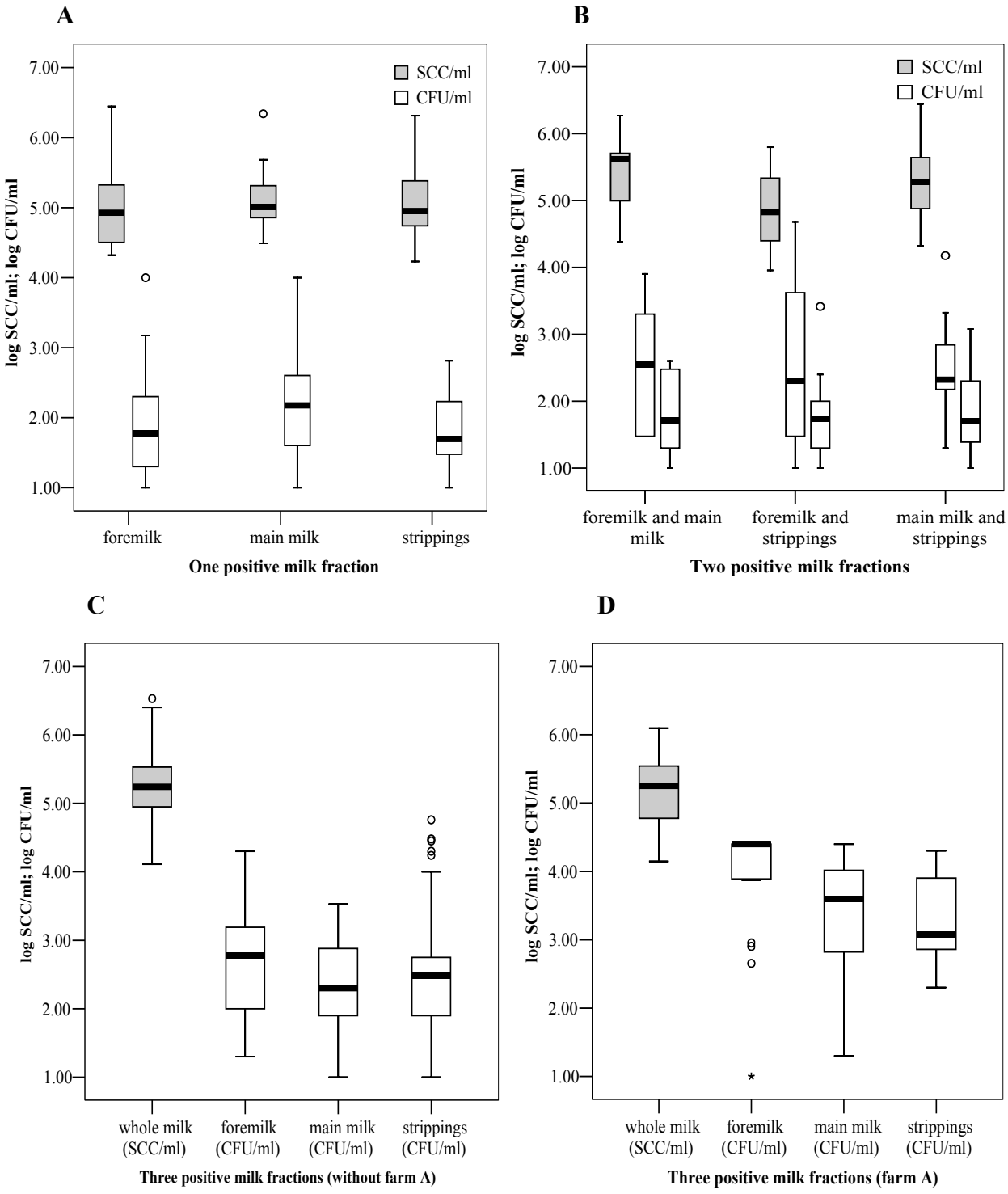


Figure 10 Somatic cell counts and CFU numbers of *S. aureus* per ml milk in relation to the number of positive milk fractions per cow. Asterisks indicate extreme values.

Table 25 Somatic cell count and CFU counts in *S. aureus* positive milk samples of different fractions.

		P - values								
		One positive milk fraction				Two positive milk fractions				Three positive milk fractions
Milk fraction		Foremilk	Main milk	Strippings	All	Foremilk + main milk	Foremilk + strippings	Main milk + strippings	All	
SCC`000/ml	Median	85	99	89	95	483	69	190	134	161
	Mean	318	147	273	239	659	149	328	327	273
		(228)†	(84)	(181)	(204)	(105)	(129)	(146)	(145)	(149)
Foremilk	Median	6.0×10^1				3.8×10^2	2.5×10^2		3.3×10^2	6.0×10^2
(cfu/ml)	Mean	2.0×10^2				1.8×10^3	8.3×10^3		5.9×10^3	1.7×10^3
		(207)				(173)	(201)		(229)	(215)
Main milk	Median		1.5×10^2			6.0×10^1		2.1×10^2	2.0×10^2	2.0×10^2
(cfu/ml)	Mean		1.0×10^2			2.0×10^2		5.2×10^2	4.1×10^2	5.9×10^2
			(230)			(118)		(129)	(144)	(145)
Strippings	Median			5.0×10^1			6.0×10^1	5.0×10^1	5.0×10^1	3.0×10^2
(cfu/ml)	Mean			1.5×10^2			7.0×10^1	1.8×10^2	2.3×10^2	4.7×10^3
				(134)			(106)	(171)	(235)	(251)

† Relative standard deviation in percentage is mentioned in brackets.

SCC values of cows with positive foremilk and strippings were similar to those of cows with positive foremilk and main milk, but slightly above the level of significance ($P = 0.08$). For the comparison of foremilk and main milk the *P*-value was rather low (0.15), but above the level of significance. Pairwise comparison of both positive fractions revealed good correlation between *S. aureus* counts in foremilk and main milk ($R^2 = 0.73$). CFU counts in foremilk and strippings, and main milk and strippings, were not well correlated ($R^2 = 0.07$; $R^2 = 0.25$). *P*-values for this comparison suggested a statistically significant difference between the counts in foremilk and strippings ($P = 0.02$), as well as in main milk and strippings ($P = 0.01$). Cows with three positive milk fractions had a median SCC value of 161,000/ml. Median *S. aureus* counts ranged from 200 to 600 CFU/ml in the different milk fractions. *S. aureus* counts in foremilk and strippings showed no statistically significant difference ($P = 0.80$), whereas the counts in foremilk and main milk as well as in main milk and strippings were rather different ($P = 0.04$, $P = 0.13$). Correlation between *S. aureus* counts in different milk fractions was poor (foremilk and main milk: $R^2 = 0.03$; foremilk and strippings: $R^2 = 0.36$; main milk and

strippings: $R^2 = 0.12$). As the majority of cows from farm A shed *S. aureus* at very high levels in all milk fractions, this farm is shown separately. In farm A the median SCC count for cows with three positive fractions was 134,000 SCC/ml.

When comparing SCC values of the different groups, cows with one positive milk fraction differed slightly from cows with two positive milk fractions ($P = 0.26$), and differed to an even greater extent from cows with three positive fractions ($P = 0.08$), whereas cows with two positive milk fractions and those with three positive milk fractions had rather similar SCC values ($P = 0.51$). Farm A and the cows of other farms with three positive milk yields showed quite similar SCC values ($P = 0.51$). When comparing *S. aureus* counts in the milk fractions of the different groups, the counts in the main milk were most similar (Table 26). The correlation between SCC and CFU values was rather poor. In most cases there was a negative correlation between these parameters, with coefficients of regression (R^2) ranging from -0.24 to 0.18.

Table 26 T-test comparison of CFU counts in *S. aureus* positive milk fractions.

Comparison	Positive milk fraction		
	Foremilk (P - values)	Main milk (P - values)	Strippings (P - values)
One positive fraction vs. Two positive fractions	0.03	0.86	0.62
One positive fraction vs. Three positive fractions	<0.004	0.94	0.01
Two positive fractions vs. Three positive fractions	0.75	0.89	<0.004

4.2.4.2 Discussion

Zeidler et al. (1968) explained the normal distribution of SCC log transformed data by the exponential growth of udder bacteria. They however did not investigate the role of bacteria count and SCC in other milk fractions in consideration to the pathogenesis of bovine mastitis. Godden et al. (2002) observed differences between shedding rates in the foremilk and strippings of single quarters but did not consider the correlation between foremilk and postmilking of the same quarter sample as well the pathogenesis of mastitis. The weak but continuously increasing median value of *S. aureus* only in strippings with a rising SCC is not only important in diagnosis of subclinical mastitis. It represents furthermore the shedding

cycle of *S. aureus* in bovine mastitis and confirms the crucial role of residual milk represented of stripping samples of chronic shedders (Figure 9).

The overall lack of correlation between somatic cell count and *S. aureus* count concurs with the data reported in the published literature, and may be explained by the reduction of bacterial counts in milk due to high somatic cell counts (Steck, 1920). This should be the reason why according to Zeidler et al. (1968) not more than 50 % of foremilk samples with more than 10^7 SCC/ml are bacteriological positive. Only cows with very low somatic cell counts (<50,000/ml) were found to have no shedding cycle in somatic cells (Smith and Schulze, 1967; Vries, 1976). A significant increase in SCC count in strippings correlated with an increase in the number of pathogens and vice versa, illustrating the upward and downward regulation of SCC counts in connection with the CFU count (Beech, 1967).

Daley et al. (1991) found a positive correlation between the somatic cell count (SCC) and phagocytosis resulting in an alternating, asynchronic shedding cycle of *S. aureus* counts (CFU/ml) in relation to SCC. At periods with lower SCC the cells are at least efficient enough to kill intracellular bacteria, and at this time, bacteria are often non detectable (Daley et al., 1991). These findings might also explain quantitative and qualitative differences in DNA-based real-time PCR compared to traditional microbiological methods for the detection of *S. aureus* in raw milk (Hein et al., 2005; Studer et al., 2008). On the other hand, quarters with low somatic cell counts had no shedding cycle with respect to *S. aureus* counts (Studer et al., 2008; Vries, 1976). For these quarters, the type of milk fraction with the highest bacteria count was variable (Vries, 1976). These shedding cycles and the variability in the type of positive milk fraction pose a challenge to effective diagnosis of *S. aureus* infected udders and might result in major variances in predictive values in low prevalence situations (Anonymus, 2006).

According to Nickerson (1989), the high unspecific phagocytosis activity of the deeper area in the distal end of the teat cistern might be responsible for the large number of negative foremilk samples (73.9%). The unspecific phagocytosis at the initial stage of mastitis should further be the cause for the varying level of 1-100 cfu/ml between the first foremilk and foremilk after cleaning of quarters with 100,000 up to 300,000 SCC/ml (Vries, 1976; Appendix: Figure 15, A). The residual milk in the udder cistern (250 to 300 ml) is followed by secretion of sterile alveolar milk induced by oxytocine (Bruckmayer et al., 2004). The *S. aureus* count in each udder cistern and the dilution effect of sterile alveolar milk determine the *S. aureus* count in the main milk. Five thousand CFU of *S. aureus*/ml in one udder cistern would be adequate to obtain about 100 *S. aureus*/ml in whole main milk if the alveolar milk

volume per milking would be about 10 liters. The effective unspecific immune response in the distal teat cistern together with the dilution effect of alveolar milk might be responsible for the frequent detection of *S. aureus* only in the main milk of cows with less than two lactations in the HCPS herds.

According to Knight et al. (1994) at peak lactation the cisternal milk increases from about 600 g to 2,60 kg at late lactation inspite of the decreasing daily milk yield. The reduction of alveolar milk in relation to the cisternal milk is correlated with the increasing SCC and new IMI during the lactation periode and confirms the great importance of the dilution effect through the alveolar milk for the pathogenesis of mastitis (Appendix: Figure 3 and 17). In comparison to data gathered 30 years ago, in 2001 high SCC levels were observed during the first two month of the lactation, which can be explained with increasing metabolic disorders due to increased milk yields (Appendix: Figure 3, Table 16). On the other hand lower milk yields in the past should induce higher SCC levels at the late lactation stage because of the increasing significance of cisternal milk.

The data gathered from cows with one, two or three positive milk fractions could be related to different stages of *S. aureus* infection. The low SCC of young cows is only explainable with the initial stage of teat canal infections or the beginning colonisation of the udder cistern in one of the four quarters. Cows with one positive milk fraction corresponded to the first stage of mastitis respectively colonization of the teat canal or deeper area in the teat cistern. These cows have usually low SCC [<100,000/ml (median number)] and low *S. aureus* counts [<100 CFU/ml (median number)]**.** Only some cows with one positive milk fraction had high shedding rates of somatic cells or *S. aureus*. These cows however were usually shedders of streptococci or other udder bacteria. The increase of intramammary pressure due to oxytocin release during milking may remove *S. aureus* cells attached to the surface of the udder epithelium. Especially older cows have had contact with *S. aureus* in the past. This together with the high antibody production of cows with more than two lactations according to Brown et al. (1974) could explain the high prevalence in HCPS herds of cows shedding *S. aureus* in low numbers only in the strippings**.**

The link in the pathogenesis of IMI is provided by cows with two positive milk fractions, which had an IMI. The invasion through the teat canal and colonization of the udder, represented by SCC-values of $<10^5$ SCC/ml, follow the multiplication to high *S. aureus* counts in the udder cistern, which induce higher shedding rates of phagocytes. The exponential distribution of somatic cell counts observed in the present study for cows with two *S. aureus*-positive milk fractions -especially positive foremilk and main milk- may be

explained by the exponential growth of bacteria. The low probability of sampling exactly at this stage of the disease might have been the reason for the small number of animals in this group (2.5%). The increase in somatic cell counts after the growth of bacterial pathogens might limit the disease to the subclinical stage by eliminating vast numbers of bacteria. Cows with two positive milk fractions and an IMI shed *S. aureus* always in the strippings. High phagocytosis activity may reduce the *S. aureus* count in cisternal milk, resulting in low *S. aureus* counts in the foremilk, below the detection limit of the method used (Studer et al., 2008). Cows with a high phagocytosis activity would have only main milk and strippings as positive milk fractions. For cows with positive foremilk and main milk, the dilution effect of sterile alveolar milk might have been the reason for significantly higher *S. aureus* counts observed in foremilk than in milk fractions collected later during milking from the same cows.

With the exception of one cow, all cows with three positive milk fractions had >50,000 SCC/ml and the highest shedding rates in the strippings [3×10^2 CFU/ml (median number)]. Higher shedding rates in the foremilk and strippings of cows with IMI in relation to the main milk agree with the observation of Vries (1976). For cows of farm A the shedding level in the foremilk was so high that the dilutive effect was not observed until shortly before the end of the mechanical milking process. Thus these cows showed no decrease of *S. aureus* counts in the main milk. The critical CFU count for effective phagocytosis in order to avoid the colonization of the udder cistern is about 100 *S. aureus*/ml (Newbould and Neave, 1965a). Higher numbers might survive the first immune response in the udder cistern and single *S. aureus* cells multiply to ≥100 CFU *S. aureus*/ml in the foremilk. This assumption provides the basis for the conventional use of 0.01 ml milk for analysis and explains why the recovery of a single *S. aureus* colony in 0.01 ml quarter foremilk according to Zecconi et al. (2003) and 0.05 ml of composite milk samples according to Roberson et al. (1994a) are considered a positive result indicating an IMI.

Especially the presence of *S. aureus* in biofilms of the milk cistern respectively in the udder epithelium according to Almeida et al. (1996) would favour the multiplication of bacteria and stimulate an increase in somatic cell count with a long duration. The percentage of epithelium cells in the SCC increases according to Kurzhals et al. (1985) up to 0.52 % when the SCC increased by 1000 SCC/ml. All cows in LCPS herds with IMI had experienced more than one period of lactation. Thus, *S. aureus* in the teat canal or teat cistern could colonize the udder cistern during the dry period and IMI occurred in the following lactation.

The high production of antibodies such as IGG, IGM and IGA according to Leitner et al. (2000) is the crucial characteristic of cows with IMI and their high phagocytic capability in

the udder cistern. The distribution of frozen or fresh milk samples are however only statistical significant for strippings and not for foremilk samples (Godden et al., 2002, Appendix: Figure 14). The high number of intracellular *S. aureus* in mastitis milk according to Godden et al. (2002) lead to lower CFU especially in fresh stripping samples. Very important in this context is the high fat content of stripping samples according to Sarikaya et al. (2005) in relation to other milk fractions**.** The high fat content leads not only to a lower phagocytic ability according to Paape et al. (1978) and is according to Ali-Vehmas (1997) associated to a lower sensibility of *S. aureus* to penicillin. The content of IGA additionally determines according to Honkanen-Buzalski and Sandolm (1981) how many *S. aureus* cells are bound to fat globules. This observation in agreement to Godden et al. (2002) would also explain the missing normal distribution of log and non log transformed data in the strippings (Table 24). On the other hand it would be an adequate explanation why single cows show a high shedding cycle especially in the strippings.

4.2.4.3 Summary

The presence and median count of *S. aureus* in foremilk, main milk, strippings and somatic cell count (SCC) were determined for 269 cows in 12 dairy farms. Grouping the herds in HCPS (high prevalence of coagulase-positive staphylococci) and LCPS (low prevalence of coagulase-positive staphylococci) herds separated the farms exceeding the limit of 100 *S. aureus*/ml raw milk and 1,000 *S. aur*eus/g raw milk products from those not exceeding this limit. In addition to that, the major difference between HCPS and LCPS herds was the frequency of cows with three positive milk fractions (39.5% vs. 1.9%). None of 21.8% cows with one *S. aureus* positive milk fraction had an intramammary (IMI) infection. They had low SCC with <100,000 SCC/ml and *S. aureus* counts between 50 and 60 colony forming units (cfu) of *S. aureus*/ml. 15.5% of the cows shed *S. aureus* in all three milk fractions and had all an IMI. These cows had the highest somatic cell count (161,000 SCC/ml) and between 600 and 300 *S. aureus* cfu/ml in the different milk fractions. Cows with two positive milk fractions (12.6%) occupied an intermediate position in respect of somatic cell count (134,000 SCC/ml) and single cows within this group had an IMI. Since in all cases of IMI the strippings were positive, investigating this milk fraction together with the foremilk might be very useful for the detection of cows with IMI.

4.2.5 Dynamic of subclinical mastitis in dairy herds

Finally, the relationship between the dynamic processes of subclinical mastitis and prevalence of positive milk fractions will be illustrated in more detail. Distinct differences between the *S. aureus* count in the bulk milk, and shedding patterns of foremilk, main milk and strippings in LCPS- and HCPS herds, have to be revealed in order to control this pathogen in a sustainable manner. The reduction of numbers of young infected cows has the highest priority in this context.

4.2.5.1 Problem and Goal

Even for farms that met the criteria of somatic cell count and total bacterial count, it was nearly impossible to produce raw milk cheese consistently containing < 10,000 colony forming units (cfu) of *S. aureus*/g (Anonymus, 1992). Since the detected limit of *S. aureus* has recently been elevated to a more practicable level of < 100,000 /g, the production of raw milk cheese is now being considerably facilitated (Anonymus, 2005).

In spite of culling or treating of infected cows the dynamic of *S. aureus* mastitis is so fast that new intramammary infections (IMI) are still detected (Zadoks et al, 2001; Sommerhäuser et al., 2003). The risk of an *S.aureus* IMI increases with the number of lactation (Enevoldsen et al., 1995). The high correlation of the prevalence of positive cows and the concentration of *S. aureus* specific antibodies to the number of lactations is an indicator for the problem of controlling this pathogen in this context (Brown et al., 1974). The initial immune response to *S. aureus* leads frequently only in an increase of SCC and the succeeding IMI spread these pathogens on the rest of the herds (Zadoks et al, 2001). The significant difference of new IMI between herds with a high *S. aureus* prevalence and herds with a low *S. aureus* prevalence of primiparous cows is only observed during lactation and not at the time of parturition (Roberson et al., 1994b). In contrast to *Str. agalactiae***,** only a low correlation (r=0.46) between *S. aureus* counts in the bulk tank milk and the prevalence of positive foremilk samples of lactating cows in the herds was found (Gonzalez et al, 1986). Especially in farms with a low *S. aureus* prevalence this pathogen was detected in raw milk and raw milk cheeses, whereas each lactating cow was tested negative for *S. aureus* by using the common approach of investigating 0.01 ml of milk sampled from each quarter (Gutser, unpublished results). Methodical difficulties in detecting *S. aureus* in dairy herds and the influence of the

pathogenesis of mastitis are shown and discussed in chapter 4.2.1 and 4.2.2 and 4.2.4. These chapters before show all the fast dynamic of mastitis but give no solution for this problem.

This chapter was conducted to determine all important parameters, which leads to the wide distribution of *S. aureus* in dairy herds and the bulk tank milk, one of the central problems for this pathogen. This information is necessary to discuss about possible solutions for this phenomenon. For that purpose all available results were considered and assumed to a final evaluation. The twelve herds were ranked according to the *S. aureus* prevalence in the foremilk, main milk respectively IMI and grouped into HCPS (high prevalence of coagulase-positive *S. aureus*) and LCPS (low prevalence of coagulase-positive *S. aureus*) farms according to the classification of Roberson et al. (1994a). Additionally the average of shedding rates in relation to the number of lactations were separately calculated for each single milk fraction (foremilk, main milk and strippings) for HCPS as well LCPS herds .

4.2.5.2 Results

During an analysis of bulk milk over a 14-month period within the survey, constantly >100 CFU of *S. aureus*/ml were detected on farm A, with 2.800/ml at maximum. No HCPS farm had *S. aureus* counts <10 CFU/ml in bulk milk (Figure 11). On the HCPS farms 68.6 % of all samples exceeded 100 CFU/ml, whereas on LCPS farms only 16.7% exceeded this limit. The differences of *S. aureus* counts between the HCPS and LCPS herds were not significant (P = 0.127), whereas the distribution to the corresponding classification according to the former *S. aureus* limit for milk free for consumption (Anonymus, 1992) was significant (P = 0.012).

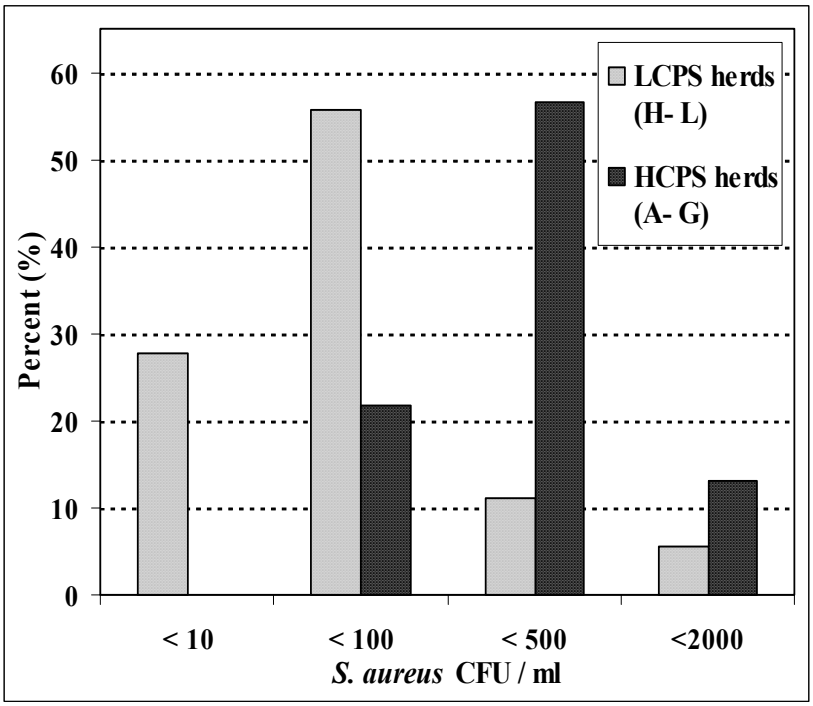


Figure 11 *S. aureus* count after consecutive sampling of bulk milk (four bulk milk samples from each herd) in LCPS and HCPS herds classified in the dectection threshold (<10) and the limits for milk free for consume or raw milk products according to Anonymus (1992)

LCPS farms showed the lowest and HCPS farms the highest *S. aureus* prevalence during the first lactation, while in the third lactation the prevalence pattern was inverted (Figure 12). Culling or treatment of cows with IMI infections in LCPS herds were the reason why chronic shedders were not detectable in case of cows with >3 lactations.

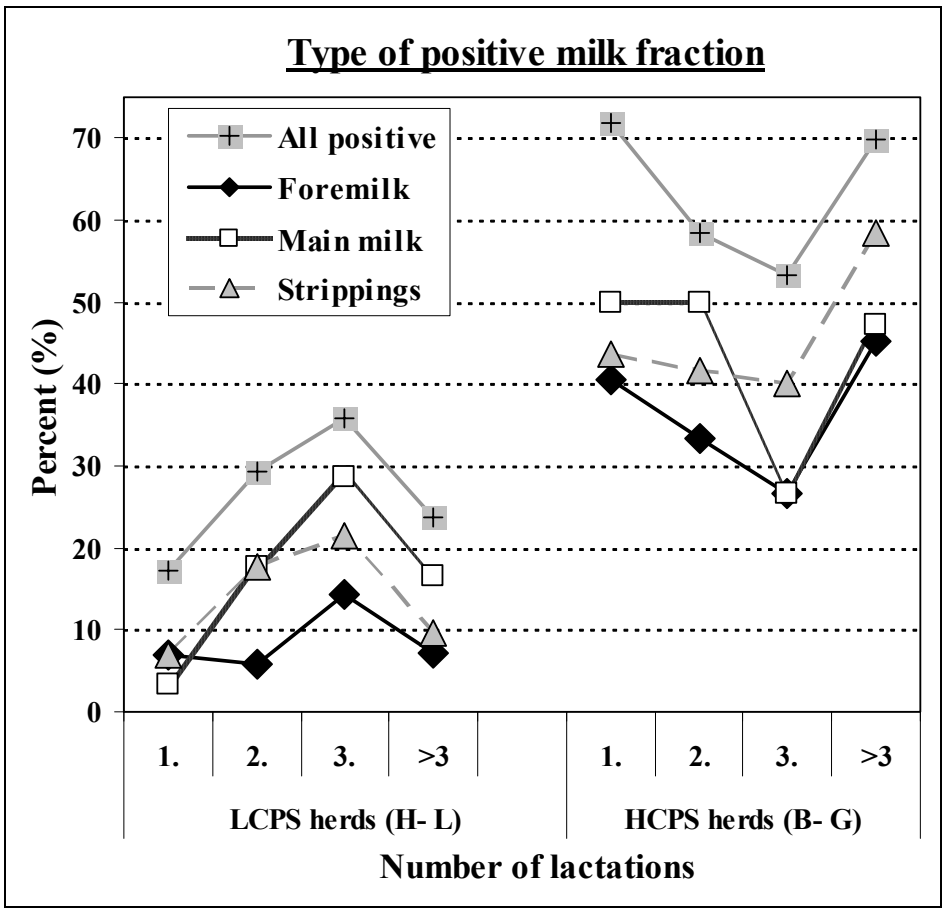


Figure 12 Prevalence of *S. aureus* in HCPS and LCPS herds in relation to type of milk fraction sampled and number of lactations

The *S. aureus* counts in foremilk, main milk and strippings on HCPS farms and LCPS farms were compared and put in relation to the number of lactations (Table 27). When analyzing the relatively small amount of 0.01 ml aliquots, the percentage of *S. aureus* positive cows ranged between 34.0–49.1 % (HCPS farms: herds B-G) and 0.0– 41.7 % (LCPS herds), depending on the milk fraction sampled. When comparing HCPS and LCPS herds, only the *S. aureus* counts of strippings showed significant differences (P = 0.026) (Table 27). Regarding this milk fraction, 86.2 % of the cows in HCPS farms and 69.2 % of the cows in LCPS farms shed < 500 CFU/ml, but 6 % in HCPS and only 0.8 % of the cows in LCPS farms shed >1000 CFU/ml. The percentage of positive strippings with < 100 CFU/ml was > 40 % in both the HCPS and LCPS herds, in contrast to the percentage of positive foremilk (HCPS: 34. 0 %, LCPS: 12.5%) and main milk (HCPS: 36.2 %, LCPS: 0.0 %). This difference is significant with a P-value of 0.045.

Table 27 Shedding rate of *S. aureus* positive cows in foremilk, main milk and strippings in relation to the prevalence of *S. aureus* in the herd and the number of lactations

		Foremilk	Main milk	Strippings
HCPS- farms (herd B- G)	**number** (n)	49	57	59
	Mean (cfu/ml)	2.6×10^3	7.0×10^2	3.2×10^3
	Median (cfu/ml)	4.0×10^2	4.4×10^2	1.0×10^2
	< 100 cfu/ml (%)	34.0 %	36.2 %	47.0 %
	RSD %	260	161	310
LCPS- farms (herd H- L)	**number** (n)	8	15	12
	Mean (cfu/ml)	5.4×10^3	1.7×10^3	2.5×10^2
	Median (cfu/ml)	6.0×10^2	6.0×10^2	2.0×10^2
	< 100 cfu/ml (%)	12.5 %	0.0 %	41.7 %
	RSD %	196	157	128
1. Lactation	**number** (n)	15	15	14
	Mean (CFU / ml)	6.6×10^2	2.5×10^2	6.0×10^2
	Median (CFU / ml)	1.0×10^2	1.0×10^2	1.2×10^2
	RSD %	176	164	91
2. Lactation	**number** (n)	9	15	13
	Mean (CFU / ml)	9.2×10^3	6.7×10^3	8.0×10^3
	Median (CFU / ml)	3.5×10^3	3.0×10^2	1.6×10^2
	RSD %	145	126	152
3. Lactation	**number** (n)	6	8	9
	Mean (CFU / ml)	5.3×10^2	1.9×10^2	2.2×10^2
	Median (CFU / ml)	1.5×10^2	1.7×10^2	0.9×10^2
	RSD %	127	52	143
> 3 Lactations	**number** (n)	27	30	23
	Mean (CFU / ml)	2.1×10^3	8.1×10^2	3.4×10^2
	Median (CFU / ml)	4.0×10^2	2.3×10^2	1.0×10^2
	RSD %	278	145	188
1. / 2. Lactation	**P- value**	0.090	0.096	0.048
2. / 3. Lactation	**P- value**	0.087	0.048	0.049
3. / > 3 Lactations	**P- value**	0.520	0.008	0.612

The difference of the shedding rates in relation to the number of lactations is significant in all milk fractions for the first and second lactation ($p< 0.10$). Cows in the second lactation had the highest *S. aureus* counts in foremilk and main milk. No difference in shedding rates was observed between foremilk and strippings of the third and subsequent lactations. For the main milk samples the lowest P-value was calculated to be $P = 0.008$. The foremilk (RSD = 278 %) together with the strippings (RSD = 188 %) showed the highest variation coefficients for

cows with more than three lactations. The large differences between shedding *S. aureus* in one, two or three positive milk fractions in HCPS (164 cows) herds and LCPS (105 cows) herds are shown in detail in Figure 13.

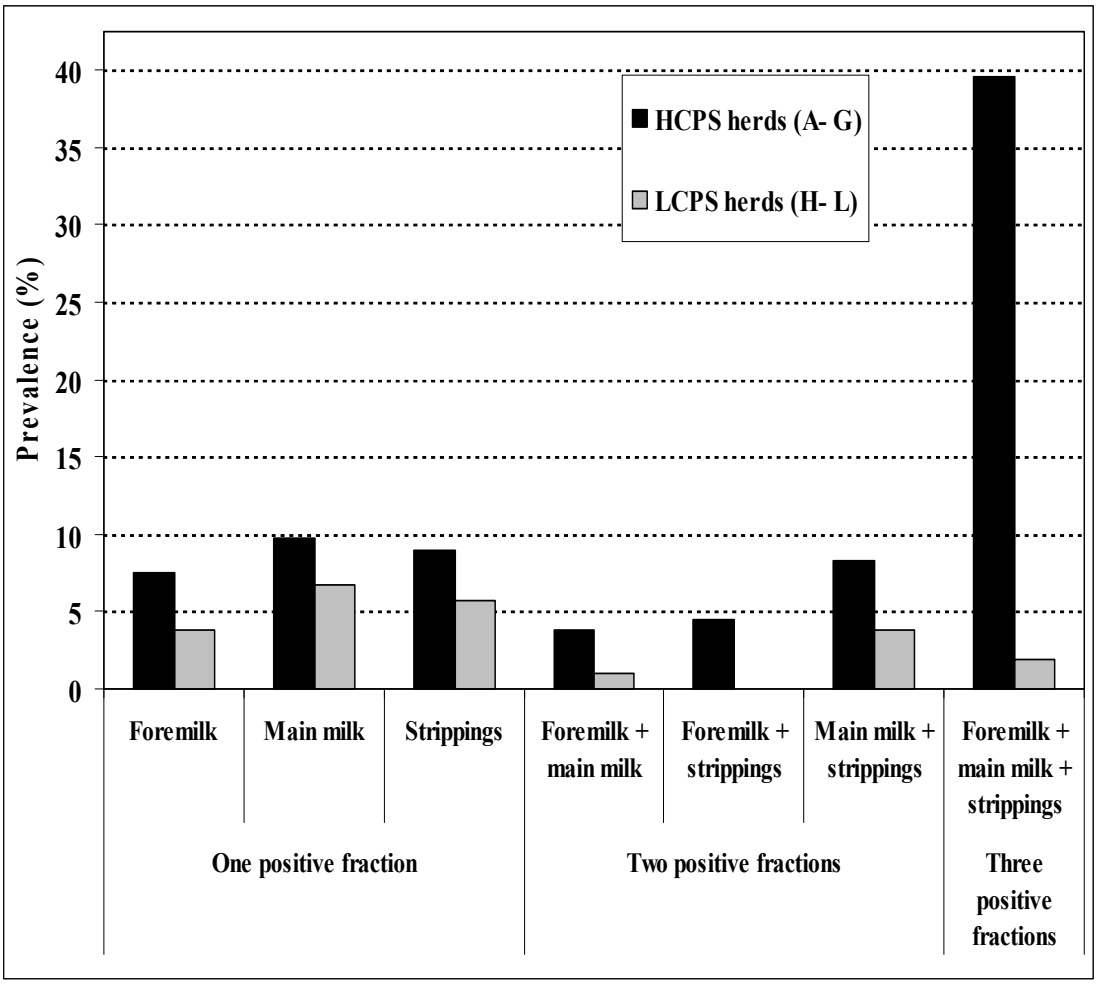


Figure 13 Prevalence (%) of *S. aureus* positive cows in relation to the number and type of milk fractions sampled in the HCPS herds (A-G) and LCPS herds (H-L)

Farm A was considered in Figures 13 and 14 for comparison of the results with Roberson et al. (1994a). Excluding farm A 27.6 % of the 133 cows in HCPS herds B-G were positive in three milk fractions. The twelve farms (A-L) investigated in this study were ranked according to shedding rate of *S. aureus* in foremilk (highest farm A with 93.5 %; lowest farm L with 4.5 %) to highlight the large correlation between all positive tested cows and cows with IMI (Figure 14). According to Figure 8 the regression equations and regression coefficient (R^2) for the foremilk and strippings were very similar to the correlation coefficients (R^2) >0.78 for cows with IMI and three positive milk fractions (foremilk: Y= -7.6+ 81.6, R^2= 0.80; strippings: Y= -7.5+ 86.3, R^2= 0.84; Three positive fractions: Y= -6.1+ 62.2; R^2= 0.79).

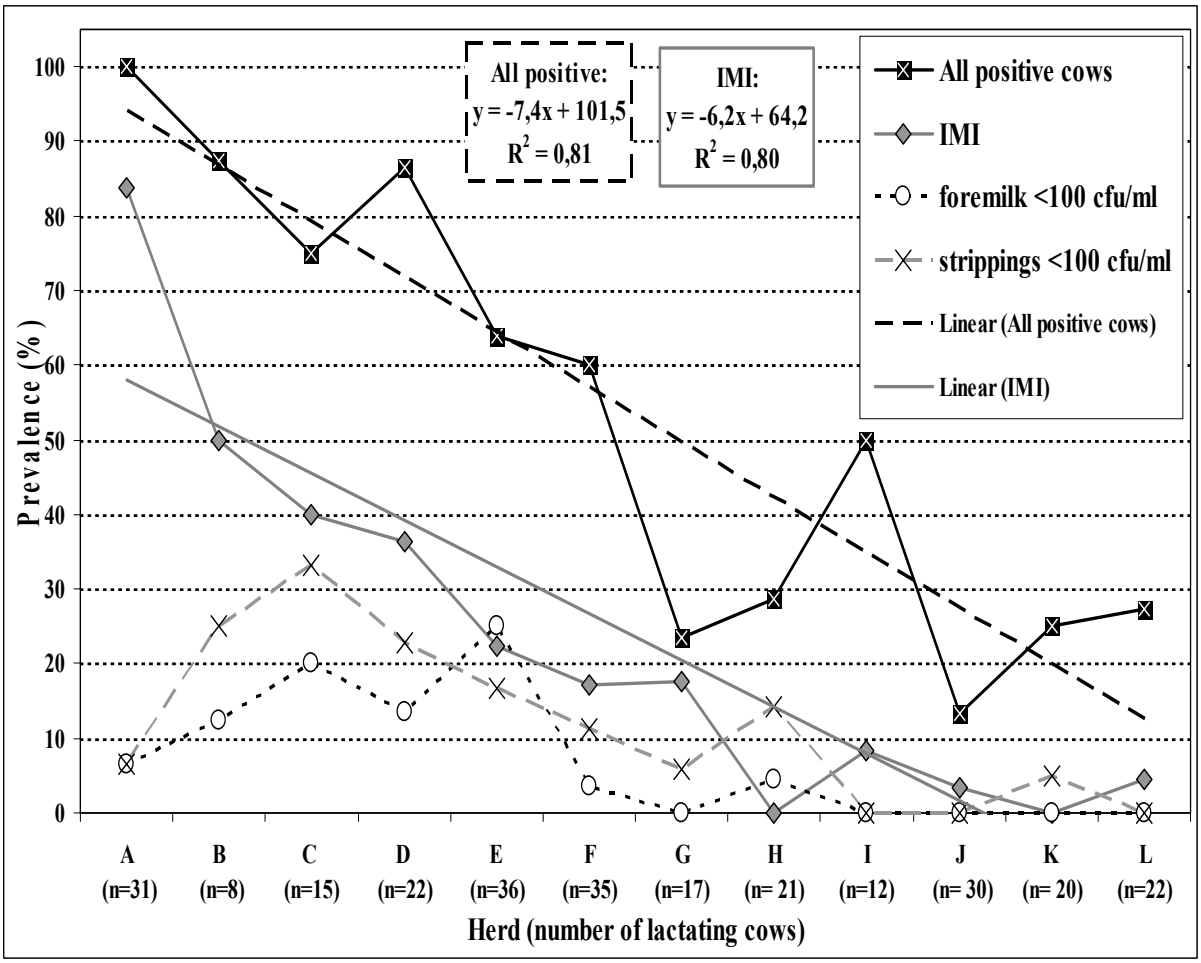


Figure 14 Prevalence (%) of *S. aureus* in herds A-L of cows with intramammary infections (IMI) and all positive cows with respect to positive foremilk and stripping samples < 100 CFU/ml

The same was not observed for the main milk and especially for cows with two and one positive milk fractions (Figure 15). Concerning the main milk, the regression coefficient of *S. aureus* prevalence is 0.73, instead of 0.60 when excluding data from herd I. Only the cows in the first lactation of herd I tested negative in all milk fractions.

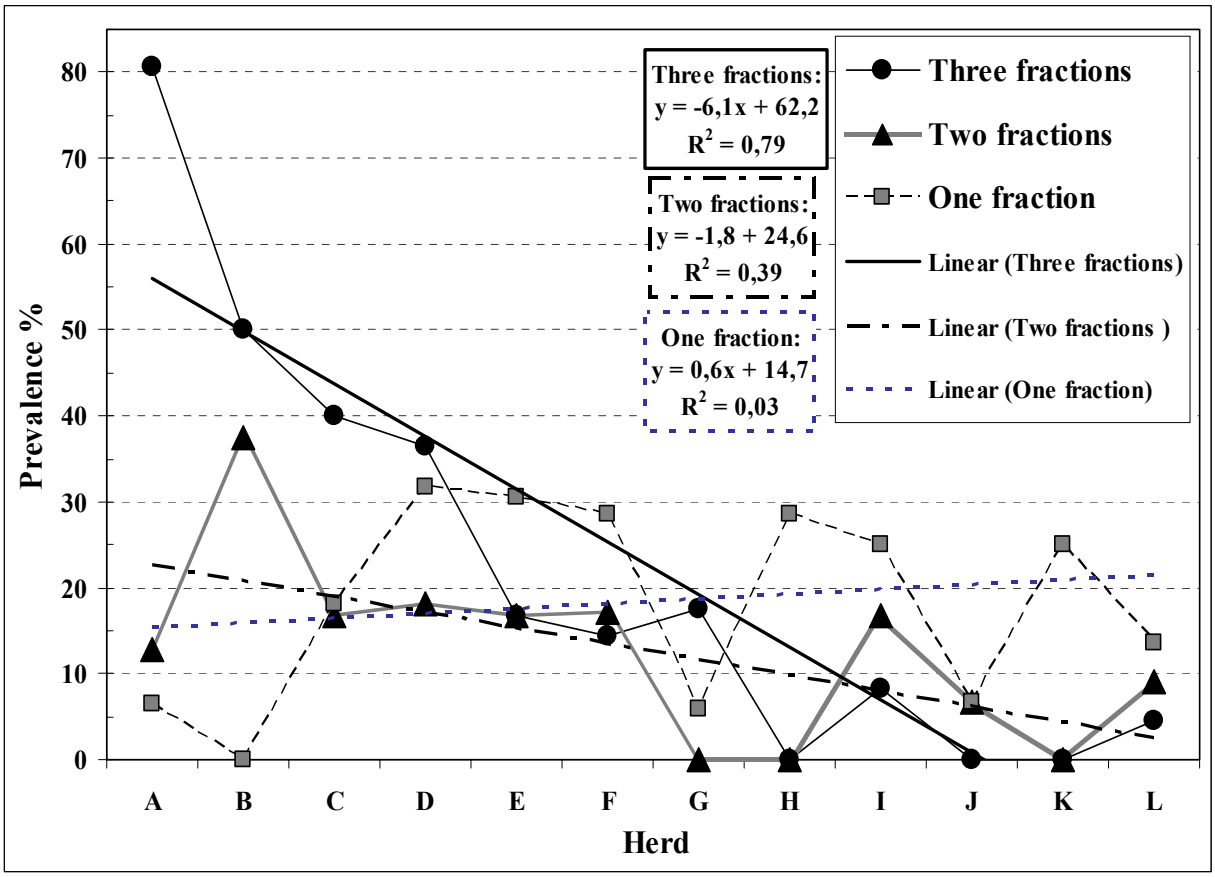


Figure 15 Distribution (%) of cows with three, two and one S. aureus positive milk fractions for herds A-L (ranked according to prevalence of positive foremilk samples)

All older cows, including one cow before drying off in the second lactation, shed between 1,000 and 5,200 *S. aureus* CFU/ml into the bulkmilk, in which 1,100 *S. aureus* CFU/ml could be detected on the same day**.** All other cows except one tested *S. aureus* negative in their foremilk. Three cows shed *S. aureus* only in the main milk with < 100,000 SCC/ml. This was due to an extensive heat period August 2003 together with a reduced amount of drinking water, inducing too low food absorption and thus resulting in low dairy milk production. With reduced temperatures in October the bulkmilk samples of the same untreated cows showed 30 *S. aureus* CFU/ml, as usual. During the statistical calculations it turned out that all outliers in the corresponding cell count groups shown with high *S. aureus* counts in the main milk (Figure 16) had a negative energy balance with close fat-to-protein rations, respectively high urea content according to Steinwidder and Wurm (2005). The mean *S .aureus* counts of the main milk (main milk Corr) excluding these outliers showed good correlation to the SCC-values (Figure 16).

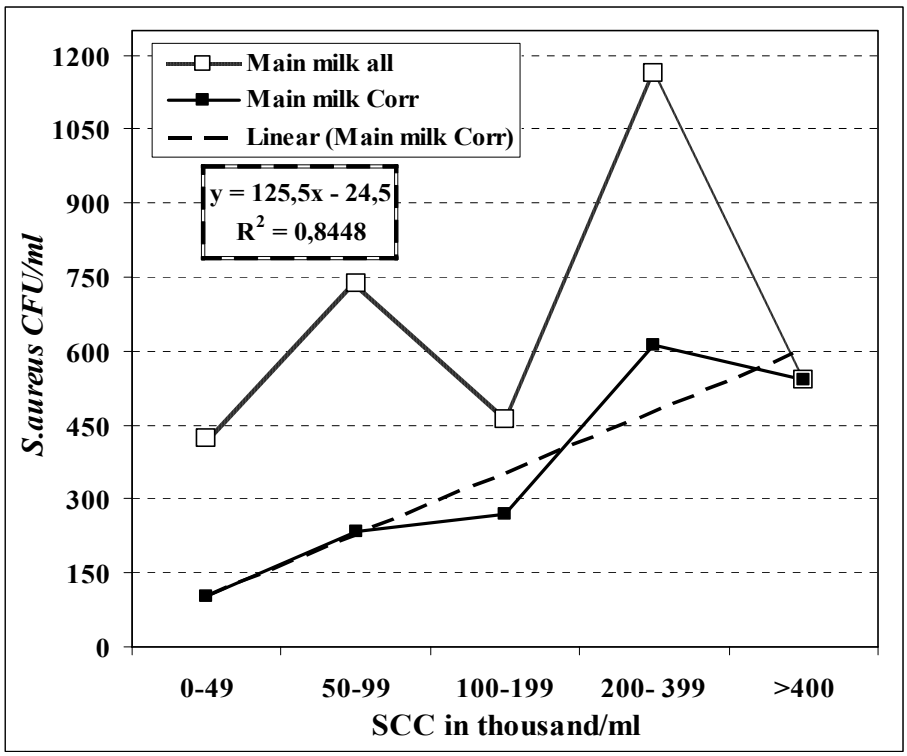


Figure 16 Mean values of all *S. aureus* shedding cows in the main milk (n=89) and mean values in the main milk (main milk Corr) excluding the statistical outliers (n= 78), respectively cows with a negative energy balance in relation to the SCC-values

4.2.5.3 Discussion

The dynamic of sublinical mastitis is according to Brolund (1985) an unsolved problem in dairy herds. Roberson et al. (1994a) observed no significant difference of *S. aureus* IMI for primiparous cow after calving between LCPS and HCPS herds in opposite to all lactating cows with one and more lactations (Appendix: Table 12). The fast dynamic of the udder colonization remains unnoticed in control programs when using only 0.01ml foremilk.

The prevalence of positive quarters increases according to Zecconi et al. (1997) from 8.8 % in the first five days after calving in the second weak up to 13.9 % with 0.01 foremilk, but when using 10 ml centrifuged quarter foremilk samples, Zecconi et al. (1997) observed an increase in the same period from 13.8 up to 30.6 %. The most quarters (48 -57 %) are just one time during the lactation *S. aureus* positive, whereas only 11-15 % of quarters were tested six times *S. aureus* positive during the lactation period (Brolund, 1985; Appendix: Figure 21). The dynamic of subclinical mastitis is not only responsible for the problems defining the *S. aureus* prevalence in dairy herds. The dynamic of subclinical mastitis is furthermore the

reason, why the HCPS herds have without a big effort no success in reducing the *S. aureus* count in raw milk and usually prefer the pasteurization of milk for the production of raw milk products.

Consecutive sampling of the bulk milk is a suitable parameter to asses the benefit of the management system in dairy herds and is a means to check for cows with IMI. Of great concern is, that sampling the bulk milk gives no information about the number of infected cows, because of the high percentage of cows in HCPS farms shed low numbers of *S. aureus* in the main milk at one time point of sampling. Consecutive sampling is necessary in farms with more than 10 *S. aureus* cfu/ml in the bulkmilk, reasoned from the fact that bulk milk samples containing less than 10 *S. aureus* cfu/ml are solely counted on the LCPS farms. After the treatment or culling of high shedders in the LCPS herds the limit < 100 *S. aureus* cfu/ml bulk milk is reconstituted very soon there after in contrast to HCPS herds. The high number of cows in HCPS herds with low shedding rates (Table 27) in comparison to only single cows shedding *S. aureus* in high numbers in the main milk is the reason, why the *S. aureus* count of the bulk milk gives no information about how many cows are infected. This is furthermore the explanation for the low correlation between *S. aureus* infected cows and *S. aureus* count in the bulk milk according to Gonzalez et al. (1986).

The *S. aureus* infections are continuously observed over the whole lactation period without seasonal influence (Hughes, 1960; Hillerton et al., 1993; Zadoks et al., 2001). The increasing risk of infection with the number of lactations is according to Brolund (1985) the own independent variable of bovine mastitis. The presence of two multiparous *S. aureus* shedding cows in herd G (Figure 14) were exemplarily the single difference between LCPS herds during the last sampling round in July 2004. The most new IMI are according to Zecconi et al. (2003) observed during the 1^{th} and 2^{nd} lactation period. Culling or treating of cows with less than four lactations in the LCPS herds is sufficient enough that *S. aureus* remains on an endemically low level and explains the low number of *S. aureus* shedding cows in the second and third lactation in the LCPS herds (Figure 12). The high number of cows with IMI and more than three lactations in the HCPS herds however is exemplary for the problems in control of transmission in these herds with only culling or treating of single infected cows.

The lack in defining the interval of consecutive sampling for IMI is a further crucial problem when discussing about the dynamic of subclinical mastitis. It is to expect that weekly sampling according to Griffin et al. (1977) or shorter intervals have a higher percentage of quarters, which are positive in two of three consecutive sampling intervals, as sampling intervals of three weaks or two month according to Zadoks et al. (2001) and Zeconi et al.

(2003). The variation in the protein content and energy content of rations fed to cows or climate rises with longer sampling intervals without considering new infections during the sampling period. The results of Zadoks et al. (2001) show that the real problem when investigating the dynamic of subclinical mastitis is the lack of confidential control herds with only single cows having an IMI over a longer period. The role of independent variables from the environment and management can be only revealed in control herds respectively LCPS herds with a constant low rate of *S. aureus* infections over a lactation period as it was observed in herd I. The relevance and frequency of spontaneous infections in dairy herds are realized only when investigating different milk fractions of each cow and the *S. aureus* count in the bulk milk.

The main problem according to Dodd et al. (1977) and Zadoks et al. (2001) is the definition of the herd level of infection. Dodd and Neave (1970) discriminate between the actual infection rate of one quarter during the lactation period, the incidence of infection during the lactation period of one cow and persistently mastitis with respect to positive findings at consecutive sampling in one quarter. The dynamic of subclinical mastitis without IMI is according to Brolund (1985) only observed when samples are taken every month during the whole lactation periode. The SCC of single cows reproduces better the dynamic of subclinical mastitis in comparison to bacteriological examinations of individual cows (Appendix: Figure 4, Figure 20). According to Brolund (1985), the increasing percentage of bacteriological positive results in cows with >50,000 SCC/ml from ≤10 to about 40 % with the number of lactations shows the low suitability of using only 0.01 ml foremilk samples. Mainly the actual infection rate and the incidence of *S. aureus* increase slightly with each lactation (Appendix: Figure 20). The investigations of samples during the whole milking process as we did or the examinations of higher volumes according Zecconi et al. (1997) is neccessary for a comprehensive picture regarding the dynamic of subclinical mastitis. According to Dodd et al. (1977), the infection rate when considering only findings at one time point of sampling and the incidence when considering all findings during the lactation period are not suitable to define the herd level of infection.

Only 34.4 % of the IMI from primiparous cows after calving had according to Roberson et al. (1994a) still an IMI on the end of the study. They concluded that the most of these primiparous cows had only teat canal infections with a spontaneous cure or were eliminated with the onset of milking. This agrees with our observations for cows shedding *S. aureus* only in one or two milk fractions. The fast colonization of the teats is obviously the cause for the high number of *S. aureus* infections in the HCPS herds, which are spontaneously recovered or

eliminated before persistently mastitis occurs. The spontaneously elimination through phagocytosis and high dilution effect of sterile alveolar milk during milking should be the cause for the high number of *S. aureus* positive quarters with a short duration of infection and low shedding rates in spite of the high prevalence of teat canal infections.

Dodd et al. (1977) found only a satisfying correlation (r= 0.77) between the herd level of infection at the end of the year and the average length of infection. This is in accordance to our results for cows shedding *S. aureus* in all three milk fractions (Figure 13, 14). The difference in linear regression of all positive cows in comparison to cows with IMI is about 40 % (Figure 3). The good correlation of the proportional same decrease between cows with IMI and all positive tested cows in Figure 14 is not only the best evidence for the strong correlation between persistently mastitis and spontaneous infections**.** These data explain furthermore the prevalence of about 43.1 % ± 20.8 % false positives according to Giesecke and Viljoen (1974) and confirm their observation of high prevalence of teat canal infections simulating mastitis. The percentage of about ± 20.8 % false positive teat canal infections simulating are also in agreement with Dodd et al. (1977) and Rainard and Poutrel (1982). About 20 % of staphylococcal infections were spontaneously eliminated (Dodd et al., 1977) or the animals recovered (Rainard and Poutrel, 1982). The spontaneous elimination or detection of *S. aureus* can be explained by the unspecific immune response or growth of this pathogen in the udder cistern of cows, respectively.

The strong increase of *S. aureus* positive cows especially during the 1th lactation in the HCPS herds in comparison to the LCPS herds according to Roberson et al. (1994b) and our results has to be linked with the colonization of the teats, which leads to the fast dynamic of subclinical mastitis in these herds (Figure 12, 13; Appendix: Table 12). According to Roberson et al. (1994b) heifers with teat skin colonized with *S. aureus* have 3.34 times more IMI risk than non-colonized heifers. High shedding rates in the strippings are the crucial difference between HCPS and LCPS herds (Table 26, Table 28). The S. *aureus* counts in the strippings should be almost the same as the *S. aureus* count of milk, which remains in the teat cup of shedding cows and is therefore equal to the infection level for the teats of the next cow. As little as about 200 *S. aureus* CFU/ml were sufficient to colonize the teat apex (Bramley et al., 1979). The high S. *aureus* counts in the strippings cause obviously through transmission via the teat cups of the milking machine the high prevalence of teat canal infections and mastitis with a short duration in HCPS herds.

The influence of the breed of cows is according to Brolund (1985) one more important factor when the dynamic of mastitis during the lactation period is investigated as well the prevalence

of IMI in relation to the number of lactations (Appendix: Figure 4 , 20). The risk of infection increases with large milk flows. The term for the milk flow (milk flow in kg milk per minute) is milkability. The milkabilty is correlated with breeds of cows. The Holstein-Frisian breed is known to have the highest milkability with the highest SCC-values, the Simmental have the lowest milkability of the most common breeds with the lowest SCC-values in Germany (Appendix: Figure 5). The increased milkability is according to Grindal and Hillerton et al. (1991) one crucial problem why the cows have an increasing risk of mastitis and are more susceptible since than 40 years ago. The consequence is a higher SCC on a stabile level of cows respectively breeds with a higher milkability, as it is observed for Holstein Frisian cows (Appendix: Figure 5). Short teats and wide teat canals are high correlated to an increased milkability. The shorter the teats and the wider the teat canals the sooner bacteria can colonize the udder. This induces probably furthermore the risk of the infection of the udder cistern respectively only positive main milk samples. During the milking process with the milking machine is it possible that 10 *S. aureus* cells are pressed form the teat cistern in the udder cistern, which is enough to release mastitis in quarters with less than 100,000 SCC/ml (Tolle, 1977). On the other side it is possible that the pathogens are flushed out of the udder cistern again (Tolle, 1977).

The prevalence of *S. aureus* in dairy herds according to Zadoks et al. (2001) however cannot only be explained by IMI alone, even through chronic shedders are responsible for the transmission of these pathogens from cow to cow. The dynamic of infection is presumably different in outbreaks or epidemic situations when virtually all cows of one herd are susceptible and have not been exposed to the pathogen yet, compared to steady states or endemic situations in which most individuals of the population have had contact to the pathogen (Zadoks et al., 2001). The “real” infection status of cows, which had contact to the pathogens such as the older cows in herd I, is obviously only observed in situations, where the cows are stressed or have a negative energy balance (Figure 16).

The efficiency of phagocytes in the udder cistern is obviously correlated to the energy balance of single cows, especially for cows with low SCC-values. Stress or a negative energy balance cause increased shedding rates in the main milk and lead to a low correlation between the *S. aureus* count in the bulk milk and number of infected cows (Figure 16). These infected cows can have low SCC, no IMI and shed high *S. aureus* counts possibly only in the main milk and cause high *S. aureus* counts in the bulk milk. An increased urea- and decreased fat content respectively a negative energy balance was generally found for cows in the pasture during late summer month, which is in accordance to the observation of Wendt et al. (1998). This fact

was especially from great concern for single older cows or cows in the early lactation shedding high numbers of *S. aureus* in the bulk milk in spite of moderate SCC.

In summary, the dynamics of mastitis in herds is too fast to control the transmission during the lactation via the milking machine of bacteria through treating, separating or culling of cows with IMI. Consecutive sampling is according to Zadoks et al. (2001) the sole method to diagnose IMI. Consecutive sampling however is part of the dynamic process itself. Only in the farm, disinfecting the milking machine with hot water new IMI could be almost eliminated eight month after implementation of this measure (Zadoks et al., 2001; Appendix: Figure 23). The energy balance of single cows during the lactation period is additionally an important influencing factor with respect to spontaneously *S. aureus* infections and high shedding rates. The great importance of this paramter and increasing importance for mastitis together with the milkability are confirmed when comparing the SCC-levels during the lactation period observed now with those observed fourty years ago (Appendix: Figure 3). In spite of the high number of younger cows, the SCC-level increased during the past 40 years in the first month after calving to levels above 250,000 SCC/ml and the level during the second month remained above 200, 0000 SCC/ml.

4.2.5.4 Summary

12 dairy farms (farm A – L) in Germany were grouped according to the prevalence of *S. aureus* in foremilk in HCPS (high prevalence of coagulase-positive staphylococci: >10 % positive foremilk samples) and LCPS (low prevalence of coagulase-positive staphylococci: <10 % positive foremilk samples) farms. A stabile low *S. aureus* level in the bulk tank milk could not be guaranteed, because of the dynamic of new *S.aureus* intramammary infections (IMI) and the problem in dispatching cows shedding *S. aureus* in the main milk. Even in the LCPS herds was the difference between cows with IMI and all positive tested cows on a stable level of about 36 % representing the ubiquitious distribution of *S. aureus* on the teats and teat canals of the mammary gland in dairy herds.

75.5 % of cows in HCPS herds shed *S. aureus* in a wide range in each herds for foremilk, main milk or strippings (17.6- 97.1 %). The level was with 25.7 % positive cows and the highest variability in the main milk of 0-50 % significant lower in LCPS herds. The high prevalence of negative foremilk samples in 0.01ml was irrespective of the high level of shedding cows the distinct problem in HCPS herds (34.0 %) in contrast to LCPS herds (12.5 %). The higher number of *S. aureus* counts in the strippings was the crucial difference

between HCPS and LCPS herds ($P = 0.026$). No cow in the first lactation of LCPS herds had an IMI or shed *S.aureus* in two or three milk fractions in contrast to HCPS herds. The real challenge in the control of subclinical mastitis is to implement a stable level of < 100,000 SCC/ml in the bulk milk of those small herds, mainly consisting of cows with more than three lactations. It is concluded that the prerequisite for controlling infection and reinfection during lactation, disinfection of the milking machine is the single practicable measure to controll mastitis. The negative energy balance of cows - especially in the late summer month for cows on pasture- plays an important role for high *S. aureus* counts in the bulk tank milk or raw milk products. It agrees with elevated BMSCC during this periode (Appendix: Figure 6).

5 General discussion

Even though intensive preventive measures have been applied for decades, the prevalence of bovine mastitis has not decreased and pathogenic bacteria are becoming progressively less susceptible to available therapies (Mylls et al., 1994). The ubiquitous distribution of *S. aureus* in dairy herds is according to Davidson (1963) and Roberson et al. (1994a, b) an explanation why *S. aureus* can be found in every dairy herd when conducting long term monitoring programs. Environmental influencing factors and phylogenetic relationships, respectively the adaption of the *S. aureus* species to ecotypes according to Hajek and Marsalek (1971) and Devriese (1976), are the two crucial parameters determining the biodiversity of this pathogen in the dairy chain when methodological problems related to bacteriological examinations are not being considered. The changing environmental conditions in dairy herds over the last 50 years have been the most important influencing factor on the population of udder pathogens. Mylls et al. (1994) found a strong correlation between the population of bovine udder pathogens and herd size, the use of milking machines, respectively the increase in milk production per cow. The results of Mylls et al. (1994) furthermore verify the strong association between the role of *Str. agalacticae* and *S. aureus* in bovine mastitis (Appendix: Table 12 A). The continuously development of phage typing and genotyping together with methodological difficulties during sampling programs in dairy herds complicates comparisons between population analysis especially over longer periods.

5.1 Important environmental influencing factors for the prevalence and biodiversity of *S. aureus* in the dairy chain

Not only the variable distribution of certain bacteria types but even the presence or absence of certain udder pathogens, as well as extreme differences between some HCPS and LCPS herds are in many cases not explainable with the dynamics of bovine mastitis. Over the past years, attempts to eradicate mastitis have resulted in a shift in the prevalence of the causative bacteria (Bramley and Dodd, 1984). Strong differences for the distribution of dominant *S. aureus* clones between the geographical locations have been observed despite of the world wide distribution of cows belonging to single European breeds. According to Smith et al. (2005) the high variability of environmental conditions may lead to localized strong selection pressures favouring certain biotypes.

The cumulative risk for mastitis has increased over the past 30 years (Østeras; IDF-Bulletin, 2001; Appendix: Figure 1). Hess und Meyer (1978) observed an increasing risk for acute mastitis of cows with > 6.500 kg milk per lactation, which according to Hogan et al. (1989; Appendix: Table 2) and Mylls et al. (1994; Appendix: Table 12 B) should be seen linked to the large number of cows with clinical mastitis through CNS and environmental udder pathogens during the past 20 years. According to Sheldrake et al. (1983) the low SCC of infected quarters during the first 100 days post partum corresponds with the negative energy and protein balance of cows described by Beerda et al. (2007). The incidence of IMI is according to Grindal and Hillerton (1991) further correlated with the milk flow rate of single cows. According to Brolund (1985) the flow rate may explain differences of SCC-values, respectively the dynamics of subclinical mastitis, between different breeds. However an increase in milkability through shorter teats as well as wider teat canals was the aim in breeding of most different breeds, so that the differences in milkability are likely to be reduced in comparison to 20 years ago.

According to Suriyathapora et al. (2000) the high prevalence of acute mastitis, caused especially by staphylococci and environmental udder pathogens after calving (Hogan et al., 1989) can be explained with a negative energy balance, leading in ketotic cows in particular during the 1st month of the lactation period to lower numbers of leukocytes. Regarding the coherence of negative energy balance and mastitis the results of Gröhn et al. (1990) agree as well with the observations made by Wendt et al. (1998) (Appendix: Tables 16, 17). According to Miltenburg et al. (1996) the prevalence for acute mastitis continuously decreases with each lactation month, whereas the high prevalence of *S. aureus* IMI after calving increases again, particularly during late lactation (Appendix: Figures 8, 17). According to Breeda et al. (2007; Appendix: Figure 24) the low intake of feed of young cows during the 1st month post partum is therefore an additional relevant risk factor for heifers with clinical mastitis. This is also an explanation why chronic mastitis in comparison to acute mastitis is more often diagnosed in the period with positive energy balances, highlighting the great importance of adequate feeding of cows (Appendix: Figure 24, Table 16).

The correlation between season and BMSCC render the seasonal influence on the prevalence of mastitis (Appendix: Figure 6). In Switzerland subclinical mastitis increases in alpine dairies according to Busato et al. (2000) especially during summer months. According to van Schaik et al. (2002) the seasonal influence is strongly expressed in herds with high SCC. The results of Larsen et al. (2000) indicate that a larger number of different *S. aureus* types are cultured in spring and summer months compared to the autumn and winter period. According

to Morse et al. (1988) climatic effects in Florida lead during the months with high temperatures and humidity to an increase of over 50% above the annual incidence. This is very important in countries with different climatic zones, as for example the United States of America. The high correlation between intake of feed and temperature is observed during the summer months for cows on pasture, as well as elevated SCC in the consequence of protein surplus (Wendt, 1998; Steinwidder and Wurm, 2005). Therefore, according to Reneau (1986), are climatic effects together with the feeding of cows one of the best explanations for the day-to-day variations in bovine mastitis and have to be considered when discussing the regional differences within one country or between different countries. In southern countries heat stress in connection with antibiotic treatment can explain the high prevalence of penicillin resistant *S. aureus,* as reported for isolates from South Africa (Swartz et al., 1984), Iraq (Abbar et al., 1986) or Zimbabwe (De Oliveira et al., 1999).

Furthermore Barlett and Miller (1993) revealed a lower risk for *S. aureus* infections in case of smaller herds. Larger herds have according to van Schaik et al. (2002) a significantly lower SCC but contain "subherds" of substantial size with elevated SCC and more antibiotic violations. The herd with second highest *S. aureus* prevalence in the study described by Roberson et al. (1994a) was for example the smallest herd with additionally the lowest SCC of all HCPS herds (Appendix: herd J in Table 11). Therefore it should be investigated why the effects of herd size and the housing of cattle, respectively different milking systems in the dairy chain are frequently not addressed in the population analysis of *S. aureus* based on monitoring programs at an international level. According to Zecconi et al. (2003) the control of *S. aureus* IMI's can only be achieved when a control program with hygienic and therapeutic protocols based on segregation is used. The use of hygiene protocols, respectively the milking of cows in the same order, is easier to implement in smaller herds and with bucket or pipeline milking installations, compared to loose housing systems with parlour milking installations which are generally only used for herds with more than 30 cows or on organic farms. Finland, Norway and Switzerland have in average the smallest herd sizes and the lowest BMSCC (Appendix: Table 37).

Penicillin resistant and SED or SEA producing *S. aureus* strains show usually a high sensitivity for phages of phage group III, and are frequently associated with cows with chronic mastitis and elevated SCC (Olson et al., 1970; Lombai et al., 1976; Appendix: Table 37). The larger number of free stall barns in Germany and Italy with milking parlour systems (IDF-Bulletin, 2001) would explain the high prevalence of *S. aureus* strains with *sed* (Zschöck et al., 2003; Morandi et al., 2007) in comparison to Norway and Switzerland

(Stephan et al., 2002; Jørgensen, 2005) having many small herds. The low BMSCC observed in Norway and Switzerland shows that chronic shedding cows are possibly more effectively eradicated or controlled in smaller herds. On the other hand, the high prevalence of penicillin sensitive *S. aureus* in phage group I shows the general shortcomings in the control of *S. aureus* strains causing acute mastitis (Appendix: Table 37). The strongly increase in prevalence of phage pattern I/119 over the past 40 years is obviously not only a German phenomenon. It is rather that the breeding of cows with high milk yields and high milkability, rising metabolic disorders and the lower age of cows has increased the risk for acute mastitis. The best evidence in this context is the rising prevalence of phage type 78 in Germany, which according to Gudding (1978) is highly correlated to acute mastitis of young cows.

Sabolic et al. (1980) found that the prevalence of *Str. agalactiae* is a very important parameter when discussing frequent use of penicillin and the shift of *S. aureus* populations in dairy herds (Appendix: Table 6). According to Seelemann (1941) *Str. agalactiae* is the only serious udder pathogen causing frequently the complete drying out of the milk production from single quarters. The strong association of *Str. agalaticae* to the bovine udder is probably the best explanation for the high prevalence of this pathogen in the past. *Str. agalactiae* is up to three days detectable on the hand of milking farmers (Wendt et al., 1998). In agreement with our results (not shown) Larsen et al. (2000) could detect only one bovine *S. aureus* strain from a milking hand. This could explain the high distribution of *Str. agalactiae* until the 1950´s, when all cows were still milked by hand and when antibiotic treatment was not uncommon (Appendix: Figure 12).

Several studies conducted around the world have reported susceptibility of *S. aureus* from bovine mastitis to antimicrobial agents. In the majority of these studies a frequent occurrence of penicillin resistance has been found, while only limited resistance to other antimicrobial agents has been reported (Vintov et al., 2003b; Werckenthin et al., 2001). The antibiotic treatment with penicillin in dairy herds is closely associated with *Str. agalactiae* (Dodd, 1980). The effective eradication of *Str. agalactiae* with penicillin in comparison to the time consuming disinfection of milking machines in herds with bucket milking installations were the reasons why Wilson (1961) justified the treatment of acute mastitis with antibiotics. The frequent use of penicillin specific sanitation programs almost caused the elimination of *Str. agalagtiae* in dairy herds. This development together with machine milking, a large therapy resistance and the fact that the teat skin of cows is the main reservoir of staphylococci lead to an increased prevalence of *S. aureus* in dairy herds.

The introduction of penicillin for the treatment of cows with mastitis induced a high variability of penicillin resistant *S. aureus* strains in different geographical areas (Wilson, 1961). Staphylococci have in comparison to streptococci a penicillinase producing subpopulation which gained more and more significance during the past 40 years. According to Gedek (1978) the high prevalence of penicillin resistant *S. aureus* strains in the Bavarian district of Swabia is exemplarily explainable with the traditional intensive milk production combined with the treatment of many older cows having streptococci mastitis. The frequent use of drugs and the overestimation of susceptibility testing are of great concern in this context. While an increase in penicillin resistance has been observed during the mid-1980´s in most countries, major differences can be observed between countries (Aerestrup and Jensen, 1998). Geographical differences and fluctuations of penicillin resistant strains may reflect the occurrence of different clones of *S. aureus* in different countries and may be related to national policies for the use of antimicrobial agents (Vintov et al., 2003b).

Penicillinase producing strains tend according to Nyhan and Archer (1967) and Pearson (1952) (quoted by Williams and Smith, 1959) and Frost (1962), when present, to be predominant. This indicates however that the high prevalence of penicillin resistant isolates does not always need to be associated with the frequent use of penicillin. In Ireland the natural high prevalence of penicillin resistant strains belonging to phage pattern I/III/(IV) agree according to Nyhan (1967) with observations made by Mackie et al. (1987) and Vintov et al. (2003b). The high BMSCC in Irish dairy herds demonstrates that this *S. aureus* type still contributes to the since decades unsolved problem of controlling chronically shedding older cows. A similar situation is observed in Norway for the penicillin sensitive phage type 78, which according to Gudding (1978) is responsible for the most cases of acute mastitis in Norwegian dairy herds. According to Vintov et al. (2003b) the high prevalence of penicillin sensitive strains from Norway which are not typable with the HPS are due to many strains belonging to phage type 78 or phage group IV.

The high degree of similarity between the antibiogram and the prevalence of antibiotic resistant strains regarding conventional and organic farms seems according to Pol and Ruegg (2006) to be surprising when considering the selective pressure of antibiotics. Strong similarity in the distribution of antibiotic resistant strains between conventional and organic farms however can only be understood when the history of organic farming and a possible lack of random samples from conventional farms are taken into account. Organic farming is characterized by low input systems with no use of antibiotics. The distinctive increase of penicillin resistant *S. aureus* strains was according to the results of Gedek (1978) observed in

Germany in the period 20 years ago between the 1970`s und 1980`s. However 20 years ago most organic farms were producers of conventional milk. The application of antibiotic drugs show as secondary effect that up to date there are no detectable differences regarding the antibiotic resistance between organic and conventional production systems. Furthermore, only conventional farms with mastitis problems and a frequent use of drugs showed a significantly elevated prevalence of penicillin resistant *S. aureus* strains (Krabisch et al., 1999). Pol and Ruegg (2006) did not distinguish between mastitis problems on conventional farms with high respectively low use of drugs.

5.2 Control of major udder pathogens in dairy herds

Save detection mechanisms, the bovine udder as single reservoir, and the lack of a natural resistance for common antibiotics are the most crucial parameters for the almost complete eradication of *Str. agalactiae* in contrast to the unsuccessful eradication of *S. aureus* (Appendix: Figure 12B). *Str. agalactiae* is the own udder pathogen with a good correlation between the occurrence of positive foremilk samples and levels in bulk milk. The correlation coefficent (r^2) between the proportion of infected cows and the level in the bulk milk for non *Str. agalacticae*–streptococci is only about 0.02, in comparison to 0.42 for *S. aureus* and 0.78 for *Str. agalactiae* (Gonzalez et al., 1986; Zadoks et al., 2004). This demonstrates in agreement with the here presented results that without the investigation of the main milk it is not possible to obtain a sufficient correlation between number of shedding cows and bacteria count of udder pathogens in the bulk milk. The only exception in most cases is obviously *Str. agalactiae*. In this context the most important criterion for *Str. agalactiae* free herds are according to Wendt et al. (1998) negative results for this pathogen in three consecutive samples over a period of three months.

Similar to the here presented findings for *S. aureus* observed Zadoks et al. (2004) methodological difficulties in the detection of the same ribotype of *Str. uberis*, or all mastitis causing streptococci in the herd as well as in the bulk milk of the same farm. Zadoks et al. (2004) explained this mismatch with an infected cow going undetected, a mixed infection in a cow going undetected, or the isolate originating from teat skin or environmental contamination rather than from mastitis milk. According to Zadoks et al. (2004) the considerable lower median from staphylococci with only 1,550 CFU/ml in comparison to a streptococcal count of 11,750 CFU/ml indicates general methodological difficulties when using only foremilk samples for the estimation of the level of mastitis pathogens in the bulk milk. Much more the results of Zadoks et al. (2004) show that herds with the same or similar

bulk milk streptococcal count can have < 10% or > 50% of streptococci positive cows, demonstrating the great risk for underestimation of the effects of shedding animals when investigating only foremilk samples (Appendix: Figure 22).

S. aureus is obviously more associated with machine milking. The long duration of *Str. agalactiae* on milker´s hand according to Wendt et al. (1998) in comparison to the low prevalence of *S. aureus* according to Larsen et al. (2000) and own results (results not shown) explain the high prevalence of *Str. agalactiae* until 50 years ago. The most important observation regarding the increased distribution of *S. aureus* in dairy herds between the 1950`s and 1970`s is the high correlation between a growing number of milking machines and the number of *S. aureus* positive samples (Appendix: Figure 12). Seelemann (1941) still reported that those cows which were machine milked after the milking of cows having mastitis had a higher risk to be infected due to the transmission of udder pathogens via the teat cups of the milking machine. Zadoks et al. (2002) agree with these findings when analysing their epidemiological data. Davidson and Slavin (1958), Wilson and Davidson (1961) and Davidson (1963) were able to significantly reduce the prevalence of the contagious mastitis pathogens *S. aureus* and *Str. agalactiae* by disinfecting the milking machine prior to milking of the next cow. In this context Davidson (1961b) has shown that the infection with staphylococci does not necessarily start with an immediate entrance into the milk secreting tissues; staphylococci are frequently found on the intact skin of the udder and teats where they may remain and multiply for some considerable period of time before actually penetrating the udder tissues.

According to Roberson et al. (1994a, b) the fast colonisation of *S. aureus* on teats and the lack of differences between *S. aureus* IMI of heifers in LCPS and HCPS herds demonstrates the difficulties when discussing the prevalence of mastitis pathogens in dairy herds. Additional measures, such as routine culling of chronically infected cows as well as teat dipping, did not significantly reduce IMI in dairy herds (Zadoks et al., 2001). The day-to-day variations in the detection of new infections were large (Appendix: Figure 23, Table 15). Zadoks et al. (2001) detected for example no new *Str. uberis* IMI during the first eight sampling intervals in the herd with most *Str. uberis* infections (herd B), but 24 of 46 new infections were found in herd B in the 13^{th}, 14^{th} and 15^{th} sampling intervals. A significant reduction of new IMI was only detectable in herd C when disinfecting of the milking machine after the milking of *S. aureus* infected cows (Appendix: Figure 23, Table 15).

Raw milk producing farms which changed from tee stalls to loose housing systems experienced suddenly high *S. aureus* counts in raw milk and problems reducing this pathogen

because of the dynamics of subclinical mastitis (results not shown). Single infected cows in smaller herds with free stall barns and milking parlour systems spread the udder pathogens very fast in the whole herd. This is a problem especially for organic farms because of the high prevalence of free stall barns and small herds. Zadoks et al. (2001) could as well detect only in the smallest herd (herd C) new *S. aureus* IMI's until the 12th sampling interval (Appendix: Figure 23). The number of new *S. aureus* infections decreased in herd C from 39 in the first until the 13th sampling interval to only three between the 14th and 26th sampling interval. With an average of 41 cows in herd C this corresponds to a reduction from 95 % to 7 % (Appendix: Figure 23). A similar development was observed for new *Str. uberis* infections during these periods (decrease from 17 to 7 cows, respectively from 41% to 17%).

According to Zadoks et al. (2001) the strong reduction of IMI, respectively almost the elimination of *S. aureus* infections after a period of 12 sampling intervals in herd C is very important to the understanding of the dynamics of mastitis in dairy herds. Continuous sampling and disinfection of the milking machine in case of all *S. aureus* infected cows during a period of 36 weeks obviously prevented the transmission of this pathogen to all cows or heifers which were in the dry-off period or have had no contact with udder bacteria earlier. Furthermore, all *S. aureus* infected cows went during this time into the dry-off period in which the antibiotic treatment or a strong immune response effectively suppresses *S. aureus* from the udder. In the here presented study only the older cows of a single herd with more than one lactation shed for example during one sampling interval the same phage type in the main milk, whereas all younger cows in the 1st lactation period were *S. aureus* negative (phage type 78 in herd I; Table 20). The phage type 78 was 14 month before the dominating strains in raw milk products. The fact, that only the heifers were *S. aureus* negative, whereas all cows with more than one lactation, which one year earlier had been in contact with older cows which subsequently had been culled, were infected, shows that the transmission of contagious udder pathogens during the lactation period is of great concern.

The recorded "outbreak" of *Str. uberis* in one herd (herd B, Appendix: Figure 23) indicates that the milking machine can as well act as a vector for most other udder bacteria in a herd. In this context the same ribotype for *Str. uberis* strains from individual cows from two herds, and the high prevalence of one ribotype in different herds is according to Zadoks et al. (2004) an important link. According to Roberson et al. (1994a; Appendix: Table 12) decrease the prevalence of other udder pathogens with the increasing prevalence of *S. aureus*. This would explain the high number of IMI with CNS or environmental udder pathogens in LCPS herds for cows with two and more lactations. The transmission of *S. aureus* from older to younger

cows via the milking machine should be also seen as the reason for the increasing prevalence of penicillin resistant *S. aureus* strains during the 1st lactation period (Tenhangen et al., 2006). In the past, milking by hand, which promote the distribution of one *S. aureus* type on a lower level in comparison to machine milking, could explain the lack of predominating strains in many herds according to Cole and Eisenstark (1959b) and other investigators (quoted by Cole and Eisenstark, 1959b) 50 years ago.

5.3 Control of *S. aureus* in the dairy chain of raw milk

Milk may also be contaminated after pasteurization by staphylococci present in aerosols, in the equipment or on food handlers (Gutser, 2001; Niskanen and Koiranen, 1977). Spontaneous infections with lower bacteria counts simulating mastitis through teat canal infections are only from interest when bulk milk samples or raw milk products are investigated. The high risk of staphylococcal IMI during the first month after calving is according to Roberson et al. (1994b) and Zecconi et al. (2003) one crucial problem controlling *S. aureus* infections in dairy herds. This dynamic in bovine mastitis is the main problem, why the risk of *S. aureus* contaminating raw milk is always present. The low level of *S. aureus* contaminating raw milk however is the reason for the low risk for food poisoning outbreaks in raw milk cheeses. Even 10^7 cfu/ml of enterotoxin A producing *S. aureus* in milk is not always sufficient enough for detecting enterotoxins in dairy products (Tatini, 1971b). The low prevalence of cheeses with $\geq 10^6$ *S. aureus* cfu/g is the best indicator in this context (Appendix: Table 9). The median *S. aureus* count of 490 raw milk samples from these 90 farms was in accordance to the literature and Zangerl (1999a) about 8×10^1 cfu/ml. 10^5 *S. aureus* cfu/ml in the bulk tank milk is only observed for 0.01 %.

The bacteria content is one of the most relevant influencing factors with respect to the growth rate of *S. aureus* in milk. Takahashi and Jones (1959), Baumgarter et al. (1968) and Tatini et al. (1971b) could show that multiplication to high *S. aureus* counts is correlated with milk samples with low bacteria contents. Tatini et al. (1971a) found only enterotoxins in artificial contaminated low heated skimed milk samples with $\leq 10^4$ cfu/ml but not in milk samples with a total bacteria count of $\geq 10^6$ cfu/ml (Appendix: Figure 13). The high bacteria count was in the past frequently caused through an insufficient cleaning of the milking machine (Abo-Elnaga and Kandler, 1965c). The total bacteria count of $>10^9$ cfu/ml in the slime of milking machine according to Abo-Elnaga and Kandler (1965c) demonstrates that the strong reduction of the bacteria count respectively distinctive progress in the dairy hygiene was the increasing standardization of cleaning the milking equipment. Abo-Elnaga and Kandler (1965c) isolated

in the slime of some milking machine *S. aureus* more than > 10^6 cfu/ml as well a high prevalence of streptococci indicating the great concern of the milking machine as vector for udder pathogens but especially of lactic acid bacteria 40 years ago.

The strong reduction of the total bacteria content from about 1.5×10^6 cfu/ml according to Zeidler et al**.** (1969) to now usually ≤ 10^4 cfu/ml is therefore one crucial problem in food poisoning outbreaks with *S. aureus* in dairy products (Appendix: Table 7). Primary infections of mastitis pathogens and udder bacteria predominate in milk samples with low bacteria contents (Tatini et al., 1971a; Vries, 1976). Bulk milk samples with a total bacteria count of ≤ 10,000 CFU/ml have usually > 50 % staphylococci and micrococci but only about 10 % lactic acid bacteria (Kurzweil und Busse, 1973). The predomination of udder pathogens in the bulk milk is at least the consequence of the bacteria limit of 100,000 CFU/ml. Forster et al. (1987) exemplarily found in the Switzerland a geometric mean of the bacteria count in bulk milk of <10^3 cfu/ml, which is about 10 % of the *S. aureus* count according to Stephan et al. (2001) in raw milk samples. The average of the bacteria count with good hygienic standard during the 1960´s was according to Seeleman et al. (1963) and Kurzweil und Busse (1973) about ≤ 4×10^3 cfu/ml without any crucial difference in the *S. aureus* level of raw milk.

The reduction of the total bacteria count is therefore in comparison to the reduction of the *S. aureus* count from great concern, especially when single outliers with 10^5 *S. aureus* cfu/ml according to Stephan et al. (2001) are taken into account. Furthermore the dilution effect of composite bulk milk samples in relation to bulk milk tanks of single farms is frequently not considered when investigating the *S. aureus* count and total bacteria count of raw milk. Outliers in single farms induce not only high *S. aureus* or bacteria count in composite bulk milk samples, they are further the reason why cheeses from lowheat skim milk of one farm can have elevated *S. aureus* counts. The reduction time of *S. aureus* strains through heating differ according to the literature in high extends (Zangerl, 1999a). This is in accordance to our experiments (results not shown). Short- time pasteurization was according to our results only sufficient enough for negative samples in one ml milk when a normal distribution with <10,000 *S. aureus* /ml as usual for raw milk samples were calculated. The heat resistance of *S. aureus* with respect to short- time pasteurization is confirmed through microbiological investigations in the past. Lukasova and Vavrova (1974) investigated *S. aureus* from milk products with a collection *S. aureus* strains from pasteurized milk. Many strains showed no lipolytic activity. Lukasova and Vavrova (1974) concluded that these *S. aureus* isolates represent the bovine biotype. Cheese, butter, butter-milk, skim milk, cream, ice cream, kefir, dried and condensed milk yielded staphylococci lysed according to Munch-Pertersen (1963)

mainly by phages of Groups III and IV, especially by phage 42D using the IPS. The low bacteria content in raw milk is especially of great concern for cheese types where only lactic acid according to Tatini (1971a) and no lactic acid bacteria are given to the fat milk. The increasing number of *S. aureus* in the rim of cheese according to Nooitgedagt and Hartog (1988) is in accordance to our observations (results not shown). This is very important when discussing about enterotoxin production in connection with decreased temperature, lower growth rate and oxidation-reduction potential. The distinct influencing factor regarding production of enterotoxins is therefore the acidification during cheese making (Zangerl, 1999a). A growth rate up to 10^5 during cheese making is only detected when the acidification is completely inhibited through phages (Tatini et al., 1971b; Ibrahim et al., 1981b). This is the reason, why the acidification has to be controlled during each cheese making. A disturbance in starter activity can be furthermore expected when the temperature optimum of used acid bacteria is not adapted to the production process of corresponding cheeses.

S. aureus usually grows between 15°C und 45°C (Bergey´s manual of bacteriology, 1984). *Lactobacillus bulgaricus* for example can not grow during the first hours in scalded hard cheeses (≥50°C) or is galactose negative, whereas *Lactobacillus helveticus* can grow until 54°C and is galactose positive (Bergey´s manual of bacteriology, 1984). This is according to Zangerl (1999a) the reason, why Tatini (1971c) could detect enterotoxins in their experiments with scalded hard cheese. Inhibited acidification respectively the use of no suitable acid bacteria induce furthermore in farms producing raw milk cheese high enumeration of *E. Coli* counts (1,000,000 CFU/ml) in cheese samples with ≥ 10^5 *S. aureus* CFU/g (Appendix: Table 10). Hüfner (1988a, b) could demonstrate the great concern of fast acid production with respect to low *E. coli* counts in dairy products as well. A high number of cheese samples with >10^3 *S. aureus* cfu/g have additionally >10^4 *E. coli* CFU/g (Appendix: Table 10). This is the best evidence that the most relevant problem of raw milk producing farms is the disturbance in starter activity. Only a *S. aureus* count of cheeses with <10^3 cfu/g ensures a regular acidification when a growth rate between 1.5×10^1-10^3 cfu/g according to Zangerl (1998) is accepted as a normal multiplication of *S. aureus* during cheese making. This explains the low prevalence of cheeses with *E. coli* > 10^2 cfu/g and <10^3 *S. aureus* cfu/g (Appendix: Table 10).

The presence of antibiotics in milk is additionally to the contamination of the starter by bacteriophages an important risk factor for the production of enterotoxins in food (Zehren and Zehren, 1968). The presence of antibiotics in milk may according to Zehren and Zehren (1968) arrest the effect of competition and allows the growth of staphylococci. The high association of penicillin resistant *S. aureus* in phage group III to food poisoning outbreaks

according to Parker and Lapage (1957) and Munch-Petersen (1963) indicates the risk regarding residues of antibiotics in food. In Great Britain the yearly incidence of food-borne intoxication from 1950 to 1962, due to strains of phage group III ranged from 64.5 to 94.7% (quoted by Munch-Petersen, 1963). This is possibly associated with the frequent use of penicillin since 1950 in dairy herds (Appendix: Figure 12 B).The high prevalence of antibiotic resistant strains is according to Wenworth (1963) and Gedek (1972) common for phage group III and not correlated to the host or origin of *S. aureus* isolates. Williams and Jevons (1961) found not only an increasing prevalence of *S. aureus* in phage group III from inflammatory infections of hospital patients (41.4 %) in comparisons to healthy carriers (14.9 %). They could confirm the results of Parker and Lapage (1957) with respect to *S. aureus* strains from food poisioning outbreaks with 78 % of 210 isolates belonging to phage group III.

5.4 Interdependence between the shift of *S. aureus* populations and introduction of new phages to international phage sets

The *S. aureus* strains contained in phage group II, phage group IV and phage type 119 of the international human and bovine phage set are according to Davidson (1972) and Hummel et al. (1992) to the most predominating *S. aureus* types in dairy herds (Appendix: Table 34). The dominating phages are five bovine phages (102, 107, 116, 117 and 119), which were cultured in three different countries (England, Japan and the USA). These phages were cultured from bovine milk because human phages of the IPS, which frequently were obtained from human material, were less sensitive for the typing of strains obtained from bovine milk. The coevolution of host, phages and virulence factors is not only the base for the epidemiological distribution and efficient discrimination of different *S. aureus* types, it is as well the cause for the high number of typable strains with a limited number of phages in one phage set, independent of the origin of the *S. aureus* strains.

The high distribution of single European cattle breeds all over the world as well as the fact that the bovine population in most countries descended from a limited number of individuals increase the probability of a wide distribution of individual clonal *S. aureus* types. According to Kappur et al. (1995) only eight clones from global sources represented 90 % of bovine isolates, and these clones rarely overlapped with those responsible for human disease (Heron et al., 2002). Considering all available results about phage typing, eight dominating *S. aureus* subpopulations in bovine mastitis are detectable in accordance with Kappur et al. (1995), excluding the high diversity of subtypes with weak phage reactions. Phage types 78, 119 and 96 (AC1) represent the common penicillin sensitive *S. aureus* subpopulations. Isolates in

phage group IV, phage group II and phage type 95 represent the *S. aureus* subpopulations with a high variability regarding penicillin resistance. The isolates which only show a high sensitivity to phages of phage group I or III, inclusive phage 81, belong to the *S. aureus* subpopulations which include many penicillin resistant strains.

The high number of non-typable bovine isolates in many collaborating laboratories according to Davidson (1972) might be explained by the only use of the lowest phage dilution at RTD and a too low number of specific phages. The collaborating laboratory from Bulgaria (Bajljosov and Grigorova according to Davidson (1972)) had the highest prevalence of non-typable strains with about 60 %. Bajljosov and Grigorova (1968) demonstrated that the prevalence of non- typable strains from Bulgaria was decreased from 69 % to 38 % for phages of the BPS and from about 65 % to 28% for the phages of the BPS and IPS when applying 1000×RTD and not only at RTD for phage typing. This shows that additionally to the standardized application at 100×RTD a higher number of phages is necessary for reliable results of bovine staphylococci typing. The large correspondence with results of PFGE and FTIR indicates the suitability of classifying strains to certain typing profiles and confirms the discrimination between strong and weak phage reactions by using phages at 100×RTD (Chapter 4.1.4). The classification of phage profiles avoids the separation of strains belonging possibly to one clonal complex. The specificity of weak phage reactions is frequently confirmed through other typing methods and/or phenotypic characteristics and confirms the need of additional specific phages in one phage set. Ruys and Borst (1959) suggested that the "tail" of weaker reactions is extremely important for true differentiation of strains and demanded more informative methods for reporting all visible reactions. However, the international Committee has decided that this method is too complicated for routine use (Subcommittee on Phage typing, 1959, quoted by Wenworth, 1963).

The composition of a phage set and the phage types included always depend on the investigated *S. aureus* populations at the point in time when the study is conducted. The initial underestimation of the diversity of *S. aureus* populations lead to an underestimation of the importance of individual phages and is the reason for both a too small a number of phages in the common basic phage sets and large number of weak phage reactions. The low prevalence or completely lack of the typical contagious *S. aureus* types, first of all in the LCPS but also in the HCPS herds of raw milk cheese producing farms, was the consequence of sanitation programs after the detection of $>10^4$ *S. aureus* CFU/g in individual dairy products (Table 21). Effective contagious mastitis control may prevent the spread of typical *S. aureus* strains from cow to cow, essentially leading to the generation of environmental style pathogens and

reducing the likelihood of eradication (Griffin et al., 1977; Larsen et al., 2003; Sommerhäuser et al., 2003; Zecconi et al., 2003). Sanitation programs obviously promote *S. aureus* types which initially represent only a minority in each herd, thereby circumventing the control programs (Sommerhäuser et al., 2003). After a few years the strains from these farms could only be satisfactorily discriminated when using a set of 30 phages, including additional human phages which are not part of the BPS. The prevalence of isolates with a high sensitivity for only bovine phages, which are highly correlated to *S. aureus* causing persistent mastitis in monitoring programs, was low (Figure 5). The bovine phage set was only developed for the typing of bovine strains from contagious mastitis and nor for bovine *S. aureus* with a high affinity to environmental style pathogens**.** The high prevalence of strains with weak or no haemolysis, positive egg-yolk reaction, penicillin resistance and high sensitivity to human phages show according to Hajek and Marsalek (1971) that the human biotype A could be detected in almost every of the ten herds from farms producing raw milk cheese (Table 20). Many strains showed many phage reactions without semi-confluent or confluent lysis (< 1000 plaques). This demonstrated the relevance of the instantaneous performance of phage typing at 100×RTD using additional phages (Table 19, Figure 5).

An interdependence between the composition of a phage set and the specimen source of *S. aureus* populations was observed when a collection of nasal human strains and a collection of strains from human milk was phage typed with our mixed set consisting of 30 phages (Appendix: Tables 38-40). The higher sensitivity of strains from human milk in comparison to nasal strains for bovine phages, especially for phage 116 and phages of phage group IV (Appendix: Table 40) can be explained with the adaptation of staphylococci to the mammary gland. Furthermore, a large number of weak phage reactions from the same phage profile would not be considered without the immediately performance of phage typing at 100 ×RTD (Appendix: Table 39). In this context, phage reactions between 20-100 plaques of phage 79 for strains with strong reactions with phage 95 from human milk are of special relevance , because exactly these isolates would be grouped with the old IPS to phage type 79 (phage group I). These results indicate furthermore that since 1954 the main problem associated with the first basic set was according to Blair and Williams (1961) the low number of specific phages from different specimen sources, as well as phage typing with different phage dilutions. Most human strains classified to one human phage group were not sensitive to bovine phages, as it was observed for bovine mastitis isolates (Table 13, Appendix: Table 38). This is very important in case of human milk isolates in phage complex- 80, and is the best evidence that these strains are most specific only for the human host.

The multi-resistance of one phage type from human milk belonging to the 80-complex additionally indicates that according to Gröschel (1968) multi-resistant strains in hospitals have their origin in a minority of the multi-resistant *S. aureus* population belonging to human milk. According to the the Subcommittee on Phage typing (1971), the successive appearance of new strains of antibiotic resistant staphylococci in hospitals caused the introduction of several new phages into the phage set which lead finally to the HPS. The sensitivity of many antibiotic resistant strains from bovine mastitis (Table 13; collection C) for several phages (human miscellaneous phage, phages of phage group III, bovine phage 108 and 111 of phage group IV) show the lack of specific phages for antibiotic resistant strains. In a study of 276 human *S. aureus* isolates conducted before the usage of penicillin became widespread, the distribution of phage types and groups was according to Blair and Carr (1960) the following: group I 9,9%, 80-complex 22.1 %, group II 21.6%, group III 8.8% group IV (phage type 42D) 4.6%, type 187 2.6 %, mixed group 7.7 % and non-typable 29.7%. During the late 1950´s still 41.4 % of *S. aureus* from hospital patients belonged to phage group III (Williams and Jevons, 1961). Phages 80 and 81 were introduced to characterize members of the "52, 52A, 80, 81-complex" of phage group I strains which globally distributed between 1954 and 1960. The phages of the 83A- complex (83A, 84 and 85) were introduced to characterize members of non-typable strains of phage group III strains that obtained a worldwide significance between 1958 and 1966. The phages 94, 96 and 95 were introduced to characterize members of non-typable strains distributed worldwide between 1966 and 1973 (Subcommittee on Phage typing, 1975).

An increasing number of non-typable MRSA´s in hospitals led again 20 years later to the introduction of new experimental phages. Richardson et al. (1999) observed an increase in the percentage of typability from 75% to 93% when using for the typing of 744 isolates of MRSA from hospital outbreaks 44 experimental phages in comparison to the international set. The number of identifiable lytic pattern increased from 192 with 23 phages of the HPS to 424 by using 44 experimental phages. The number of identifiable lytic patterns increased from 192 when using 23 phages of the HPS, to 424 when using 44 experimental phages. This is a very good further example for the strong correlation between the number of used phages and the discriminatory efficiency.

The high sensitivity to phages 81, 95 or 96 when typing a collection of human nasal strains or strains from human milk shows not only the natural wide distribution of strains belonging to phage type 81 (80-complex), 29/81 95 or 96 (Appendix: Table 38, Table 39). Furthermore these results obtained with human strains agree according Blouse et al. (1979) and Blair and

Carr (1960) very well with predominating phage types of newborn infants. The high prevalence of phage types 95 and 96 from healthy human carriers shows according to Larsen et al. (2000) and the here presented results (Appendix: Table 41) that these types do belong to the common human *S. aureus* population. These phages however were not added to the phage set for a better determination of the human *S. aureus* population. Phage types 95 and 96 were only added to the IPS because of non-typable S. *aureus* in epidemiological related clusters of new hospital outbreaks. The continuously adaption of the IPS since 1954 had therefore first of all only practical reasons for the typing of new outbreak strains and did not aim at a comprehensive determination of the entire human *S. aureus* population. Similar to the human set, the bovine phage set had only been developed for the typing of strains from bovine mastitis, not for the typing of bovine isolates derived from the udder skin.

The high discriminatory efficiency of our set with 30 phages at 100×RTD in combination with the important phenotypic characteristics (clumping factor, antibiogram, haemoylsis and egg-yolk reaction) can be commended with slightly variations as a "basic set" for phage typing of bovine strains. One phage however representing one phage group is according to Meyer (1967) only suitable for grouping but not typing of strains. Furthermore the criterion for the selection of phages for a phage set, which allow the classification of phage types only in one phage group according to Williams and Rippon (1952), induced methodological problems. Five of nine added phages (81, 817, 95, 94, 96) to the first basic set from Williams and Rippon (1952) do not belong to the common phage group and the phages of the 83-complex should be only used ad RTD (Subcommittee on Phage typing, 1975). The heterogeneity with respect to the sensitivity of penicillin and phenotypic characteristics belonging to phage type 78, 95 and 96 according to Vintov et al. (2003a, b), Niskanen and Korianen (1977) and our results show the necessity of additional phages for typing bovine isolates.

Results from the literature commend to replace phage 80, 52 and 55 through the phage AC1 (Coles and Eisenstark, 1959b), 101 and 110 (Davidson, 1961). This would allow a better discrimination of bovine strains, which are sensitive for phages of phage group I, III and IV. Furthermore bovine isolates with a high sensitivity for phage 96 can be better distinguished with the additional use of phage AC1. The bovine phages 101 and 110 solved according to Davidson (1961) many isolates, which had additionally a high sensitivity for phages of phage group I, III and IV. When Gedek (1966) used these phages of Davidson, he could classify only 45 % in phage group IV. Without the consideration of phage 101 the prevalence of strains in phage group IV increased up to 60 %.

5.5 Interdependence between enterotoxigeneity, host specificity and traditional election criteria in epidemiological investigations

Enterotoxin A (SEA) is the most important enterotoxin in food poisoning outbreaks (Parker and Lapage, 1957; Niskanen and Koiranen, 1977; Cha et al., 2006; Kérouanton et al., 2007). The high association of SEA producing isolates to phages of both phage group III and mixed phage group I/III was observed by almost all studies investigating the enterotoxin production of *S. aureus* isolates related to food poisoning outbreaks and other sources (Parker and Lapage, 1957; Williams and Jevons, 1960; Hajek and Marsalek, 1973; Mochmann et al., 1976; Niskanen and Koiranen, 1977; Adesiyun, 1998, Kérouanton et al., 2007). Hajek and Marsalek (1973) grouped all strains with SEA to the human biotype A (Appendix: Biotype A in Table 20 and 21). The *S. aureus* isolates of phage group IV, one of the predominating bovine strains in the past (Biotype C, variety bovis), have the lowest prevalence of strains (SEA-SED) (Figure 2) and have not such importance for outbreaks of food poisoning (Appendix: Table 21). Almost all strains from food poisoning outbreaks belong according to Kérouanton et al. (2007) to the human biotype (Appendix: Biotype A in Table 20; Figure 25). Enterotoxin C (SEC) is usually correlated to food poisoning outbreaks in dairy products from ovine or caprine milk with a high sensitivity to phage 78 and 740 (De Buyser et al., 1987; Witte, 2004; Jørgensen, 2005).

According to Kérouanton et al. (2007), single food poisoning outbreaks in cheese of sheep milk are caused by SEA from strains with typical ovine characteristics (Appendix: Table 49). Morandi et al. (2007) detected single bovine and caprine strains producing SEA as well, confirming the results of Kérouanton et al. (2007). Furthermore Mayer (1975), Mochmann et al. (1976) and Niskanen and Koiranen (1977) detected SEA producing *S. aureus* types with a high sensitivity for phages of phage group IV. This finding, being in line with the results of Appendix Table 24, explains the strong and fast coagulation of bovine plasma after 3h of all SEA producing isolates despite of the lack of β-haemolysis. The two mastitis samples of SEA from national monitoring programmes belonged to phage type 117 (phage group IV) and were αβ-haemolysis as well egg- yolk positive.

The sensitivity for phages of phage group IV, particularly for the bovine phages 117 and 111 is an indicator for their bovine origin in the context of mastitis. According to Stephan et al. (2001) and Morandi et al. (2007) SEA and SED producing isolates belong to endemic strains, which cause bovine mastitis and which are frequently found in bovine raw milk samples

(Appendix: Table 26). Penicillin sensitive isolates with SEA and SED are the single outbreaks strains, which were found in France and characterized by the same PFGE pattern (Stephan et al., 2001; Kérouanton et al., 2007; Appendix: p45 in Table 42). The close relationship of the penicillin resistant subtypes causing food poisoning outbreaks with SEA and SED (Kérouanton et al., 2007; Appendix p6, p7 and p 27 in Figure 25) is confirmed by the isolate 2, 34, 326 and 327 in Appendix: Table 24. Cha et al. (2006) could show that their majority of strains from food poisoning outbreaks belong to only three different PT`s or even close related types.

The high similarity regarding phenotypic characteristics of SEA producing strains to human isolates is the explanation why the lack of personal hygienic standards is frequently the cause for outbreaks of food poisoning. In this context it is important to consider that SEC is the most frequent enterotoxin of human strains (Hajek and Marsalek, 1973; Gonano et al., 2009). The probability of food poisoning outbreaks through contamination of human strains during the production of cheese is however very low. Still, the dilution effect of only l00 kg milk for making cheese (10^5 /ml) is very high even when a contamination of two ml human blood with 10^8 enterotoxigenic *S. aureus* /ml is calculated. The contamination level of enterotoxigenic *S. aureus* from the personal is likely insufficient for food poisoning, since during regular conditions $\geq 10^4$ *S. aureus* CFU/ml raw milk are at least necessary for the production of enterotoxins during cheese making. Even the contamination of *S. aureus* from festering wounds of single workers before reneting is unlikely to lead to a production of enterotoxins during the manufacture of milk during regular conditions with normal acidification. Rather, the primary infection of individual high shedding cows without clinical signs in combination with low hygienic standards of the milking equipments might be responsible for high *S. aureus* counts in the bulk tank milk. The probability that individual cows in a herd of ten or more animals are infected occasionally with SEA producing *S. aureus* strains is much more realistic than an infection caused by an individual person.

Because of the high diversity of the *S. aureus* populations must further be scrutinized to confirm the scheme for biotyping of *S. aureus* strains according to Hajek and Marsalek (1971, 1973) (Appendix: Tables 20 and 21). A serious problem to realize this project is the large distribution of *S. aureus* in different concentrations in food, environment, in animals or human beings. In this context the definition of isolates, strains, endemic strains and outbreaks strains is critical (Appendix: page 52). Biotyping, respectively the basic research for biotyping fourty years ago, is based on endemic strains. This is the crucial cause for the high correlation between biotypes and site or host specificity. Outbreak strains, however, belong to a minority

of biotypes in comparison to endemic strains because they have very specific characteristics such as the resistant to methicillin or production of enterotoxins in food. This is one reason for the low suitability of biotyping with respect to the source of infection in food poisoning outbreaks. A second problem is the poor declaration of the origin of products. According to Munch-Petersen (1963) is the main source of enterotoxin producing *S. aureus* meat and dairy products. The results of Kérouanton et al. (2007) however suggest that the most if not all strains from dairy products are associated with mastitis milk. Kérouanton et al. (2007) mentioned the kind of food in comparison to many investigators in the past. They did not distinguish between raw milk and pasteurized milk or bovine, ovine and caprine milk. This information, however, is critical when considering food poisoning outbreaks in their correct epidemiological context.

Different agar plates and methodical difficulties are one other important source of error (see Chapter 4.1.1). ´The strength of reactions, especially weak reactions of the egg-yolk reaction, haemolysis and coagulase reaction are critical for the correct identification of isolates, particularly when considering small differences between agar plates from different productions. Grün (1970) observed a stronger egg-yolk reaction or haemolysis when streaking the bacterial material in one lane on the agar plate (which was confirmed by the here presented results). Hajek and Marsalek (1971, 1973) unfortunately did not mention used methods for plate procedures. Devriese (1976) detected *S. aureus* isolates from different hosts with different methods particularly concerning agar plates almost for each different species.

The close relationship between biotyping, enterotoxigeneity and the isolation of different strains are reflected by the results of Tables 15, 19 and 20. Both, the great concern of election criteria and the number of investigated colonies per samples are in line with results of Loncarevic et al. (2005; Appendix: Table 45). Loncarevic et al. (2005) detected in 14 samples of 28 bovine milk and cheese samples different *S. aureus* strains with various Enterotoxin genes when the samples were investigated with BP or BP-RPF. In most cases they were able to confirm the differences of isolates from different agar plates with PFGE. Two strains in one outbreak according to Kérouanton et al. (2007) belong as well to two different biotypes (outbreak strain 23 and 29 Appendix: Table 42). The low number of investigated isolates might explain, why enterotoxin D producing strains were not detected in the outbreak 29 of Kérouanton et al. (2007) (Appendix: Table 42). Therefore, both the method of strain isolation and the number of isolates from a single sample have to be considered, particularly when investigating *S. aureus* populations from different sources or food with different ingredients.

The traditional use of BA in mastitis diagnosis with haemolysis as selection criterion for bovine *S. aureus* types might explain, why SEA producing strains with only weak haemolysis are not detectable. The negative egg-yolk reaction might explain why many bovine *S. aureus* types are not detected on BP in contrast to human biotypes. Schmidt (1962) was able to identify two different *S. aureus* types in a sample from clinical material using the egg-yolk reaction. According to Meyer (1966b) the egg-yolk reaction is an important tool for analyzing both food poisoning outbreaks and *S. aureus* isolates obtained from animals or food. Almost all isolates producing SEA, SEB or SED are egg-yolk positive (Tables 15 and 20, Appendix; Tables 24 and 26). The high prevalence of egg yolk positive strains is observed for SEA and SED producing isolates (Mayer, 1975 and Stephan et al., 2001). A further explanation for the low number of SEA enterotoxigenic isolates in δ- haemolysis based on BA (Appendix: Table 27) is the fact that δ- haemolysis is a weak, respectively narrow form of α- haemolysis. In the presence of CNS, weak haemolysis, such as δ- haemolysis, is not an appropriate selection criterion for SEA isolates, since only β- haemolysis is a reliable characteristic for the presence of *S. aureus* isolates.

Furthermore, environmental factors might influence procedures underlying selection criteria. Stress of frozen strains or isolates might explain variations regarding phenotypic characteristics. Our results confirm these suggestions in line with Batish et al. (1989) and Ali-Vehimas et al. (2001). Any type of stress, which is linked with phage-conversion, influences the expression of β-haemolysis and causes additional problems for the correct biotyping of *S. aureus* isolates. Bacteriophages mediate the simultaneous single or double-lysogenic conversion of enterotoxin A (sea) and β-hemolysin (*hlb* gene) (Colman et al., 1989). Milk affected by mastitis reduces the lytic activity of β- and δ- on BA (Ali-Vehimas et al., 2001). The reduction of lytic activity results from the production of specific antibodies similar to SEC (Kuroishi et al., 2003). Batish et al. (1989) observed the loss of coagulase activity or enterotoxigeneity for many *S. aureus* strains in relation to heat stress.

Finally, the extension of phage sets during the 1970´s together with the results of Hajek and Howak (1978) demonstrates the limitations of the biotyping scheme based on the results of Hajek and Marsalek (1971) (Appendix: Table 19). The most relevant characteristic is host specificity of phages (Appendix: Table 20). The main mistake of the scheme for biotyping since the 1970´s is the strong underestimation of the host specificity of phages according to Hajek and Marsalek (1971) and Devriese and Oeding (1976). The high sensitivity of poultry strains to the phage set with 22 poultry phages of Shimizu (1976) demonstrated the low suitability of phenotypic characteristic in comparison to other phages (Hajek and Howak,

1978). Hajek and Marsalek (1971) neither distinguished between phage typing results at RTD and 100×RTD nor considered that the Davidson phage set includes human as well bovine phages. Furthermore, their isolates came from only a limited number of locations and collections were too small to represent the biodiversity of the corresponding population of a single host or side.

S. aureus strains of phage group V (phage-complex 94/96) are strongly related with food poisoning outbreaks by SEB (Kérouanton et al., 2007). Soon after the addition of phage 94 and 96 to the new phage set (HPS), Asheshov et al. (1977) reported the relationship between phage type 94/96 and production of SEB. Results of Kérouanton et al. (2007) confirm the homogeneity and independency of isolates in phage group V. The high association of phage 29 in phage group I to *tst* and *sea* is well known (Meyer and Rische, 1984). Hajek and Marsalek (1971, 1973) grouped ovine *S. aureus* isolates, which frequently produce enterotoxin C, together with bovine strains into the biotype C (Appendix: Table 20). According to Buyser et al. (1987) produce the ovine *S. aureus* biotype usually SEC together with *tst* and has a high sensitivity for phage 78 and phage 740. Furthermore almost all strains with a high sensitivity for the phage complex 94/96 were also positive for *sei* and *seg* (Table 5 and 20, Appendix: Table 24). The strong relationship between enterotoxin genes and PFGE-clusters observed by Jørgensen (2005), Cha et al. (2006), Kérouanton et al. (2007), Gonano et al. (2009) suggests that the results of Figure 2 represent a general phenomenon. The enterotoxin-profile is obviously independent form the source of food, location and time, which together constitute the crucial characteristics of outbreak strains.

The results of Kérouanton et al. (2007) suggest further a low discriminatory efficiency of phage typing. Kérouanton et al. (2007), however, considered only phage groups and not single phage types; also they did not use bovine phages despite the high prevalence of bovine types in raw milk cheese and dairy products, respectively (17 of 31 outbreaks are directly associated with milk or ingredients such as cream, Appendix: Table 42). The results shown in Table 15 and 20 and Appendix Table 24 demonstrate the significance of phage types, phage profiles, phage reactions as well as the use of bovine phages. Isolates with *sed* show a high sensitivity for phage 6, 47, 53 but also for the bovine phage 111, 117 or 108. Isolates with *sea* have a high sensitivity for phage 29, 79, 75, 85, 95 but also for phage 117 and/or phage 111 and 42F. The phages, which carry *sea*, belong obviously to miscellaneous phages and attack strains with a sensitivity for phages of one of these characteristic phages. The possibility of strains being sensitive for phage 117 and phages of phage group I and III was shown by Bonin and Blobel (1967). On the one hand this might explain the various numbers of different

S. aureus types producing SEA with a high sensitivity for phage 117. On the other hand, the sensitivity of *S. aureus* types with *sea* for phages of different hosts explains why ovine biotypes cause food poisonings with SEA (Kérouanton et al., 2007). In summary, the high degree of host specificity of phages contains the distinctive information about the origin of *S. aureus* strains, which are involved in food poisioning outbreaks. The use of phages from different animal species together with the consideration of phage reaction strength might be faster to apply but also probably more specific than genotyping methods. Furthermore, the employment of about 30 phages from all useful animals for all critical *S. aureus* types in addition to the HPS might allow the determination of the source of infection during the manufacture of food. This is accordance to Munch-Petersen (1963), who observed the importance of a set with various phages and criticized the low suitability of using only human phages.

5.6 Interdependence between sampling, typing methods and the biodiversity of *S. aureus* in the dairy chain

Still in the 1950s Cole and Eisenstark (1959a) demonstrated that in most herds of cattle more than one strain is present, and a single cow may harbour more than one type of *S. aureus* in her udder. Over a longer period of measuring, Gedek (1972) found different *S. aureus* types in one quarter of single cows. The most SEA producing biotypes or strains with no or only weak haemolysis were only detected by the use of samples with different phenotypic characteristic (see Table 20). Apart from the sampling procedure and sampling plan, the contaminating flora is the critically limiting factor when focusing on single strains in low concentrations. *S. aureus* from the teat skin is only detectable by assessing many samples with different methods (Fox et al., 1991, 1992; Appendix: Tables 46 and 47). Zadoks et al. (2002), who typed these strains with PFGE, concluded that the teat skin is not an important reservoir for bovine IMI; this result is in significant contrast to findings of Fox et al. (1991), who used only phage typing without PFGE.

The isolation of *S. aureus* based on BP-RPF is superior to that based on BP, when the growth of the contaminants from raw milk samples is effectively supressed through the composition of supplements (Zangerl, 1999a). The high number of different bacteria especially in milk samples of older cows (Munch-Peterson, 1972; Brolund, 1985 and Østeras et al., 1981) causes problems for the preparation of antibiograms (this might explain the recommendation of Barkema et al. (2006), not to treat older cows (Appendix: Tables 13 and 14, Figure 19). The high number of bacteriological negative samples or the lower level of contaminants during the

1th lactation (Munch-Peterson, 1972 and Brolund, 1985) is the main reason for the high diversity of *S. aureus* biotypes, which are detected on primipareous cows (Table 20). The beginning colonisation of the udder during the 1th lactation may explain why Tenhagen et al. (2006) found an increasing number of penicillin resistant strains only in this period.

Zadoks et al. (2002) were not able to distinguish between single strains from human skin and isolates from teat skin. The authors explained this observation by the existence of side specificity. The significance of site specificity was already indicated by epidemiological investigations. Hajek and Marsalek (1973) observed that 12.2 % of *S. aureus* from cows carriers (n= 79) belong to biotype A in comparison to *S. aureus* from cows mastitis (n= 74) with only 2.5 % strains (Appendix: Table 21). However the small number of samples (40 or less) of the studies of Hajek and Marsalek (1973), Mochmann et al. (1976) and Devriese (1976) might explain why the human biotype is frequently not detected in populations from bovine mastitis strains or other animals. The high prevalence of *S. aureus* in low concentrations is the main reason, why only the dominating strains are detected by the common bacteriological investigation of 0.01 ml foremilk (see chapter 4.1.2).

The high number of phage types in herd L (Table 21) with less than ten cows in one sampling round show that the herd size is not a critical factor, how many *S. aureus* types are detectable in one herd. Davidson (1972) found a high biodiversity of *S. aureus* in one herd only during a period of three years (Appendix: Table 34, collaborating laboratory 15). Apart from methodical difficulties in detecting all various strains at one timepoint of sampling, these results confirm not only the sampling procedure but also the sampling plan as crucial steps determining results of *S. aureus* population analysis. The sampling plan is probably the most important factor when interpreting epidemiological results or determining the discriminatory efficiency of different typing methods. The large importance of the selection of specific *S. aureus* strains demonstrated by antibiotic resistant isolates in collection C (Table 13). The data of Jørgensen (2005) came to similar conclusions when considering only penicillin resistant isolates for SE positive isolates. 17 from 258 *S. aureus* strains (6.6%) according to Jørgensen (2005) were penicillin resistant. However six of eight strains with *sea*, *seb* or *sed* belonged to the 17 penicillin resistant isolates. This would mean, that the prevalence of strains with *sea*, *seb* and *sed* for the antibiotic sensitive *S. aureus* polulation (n=241) is only 0.008 % in comparison to 35.2 % for the penicillin resistant *S. aureus* strains.

In one dairy herd usually one strain predominates (Davidson, 1961; Nyhan, 1967; Hummel et al., 1992; Peles et al., 2008). The consideration of only a single strain per herd is the crucial problem of monitoring programs in *S. aureus* population analysis based on randomized

sampling. The consequence is that the second or third most distributed *S. aureus* phage types are not considered in population analysis of *S. aureus* in dairy herds. These phage types however can be the antibiotic resistant and/or enterotoxigenic strains. It further promotes the observation regarding the importance of geographical locations in population analysis in the dairy chain. The results of Hummel et al. (1992) are the best example for problems with randomized sampling in monitoring programs in this context. According to Hummel et al. (1992), the phage pattern I/119 is the dominant strain in the most herds (Appendix: Table 22)**.** Strains belonging to phage pattern I or III were only occasionally detected in single herds, which might explain the low percentage of <5 % in national monitoring programs in collection B. However strains belonging to phage group II are according to Hummel et al. (1992) isolated in almost each herd, but these phage types never had distribution of ≥ 50 % in one herd. This finding might explain why the strains of phage group II in spite of a wide distribution in dairy herds play only a subordinate role in many national monitoring programs (Table 13, Appendix**:** Table 34, 36 and 37).

Doubts with respect to the correct classification of epidemiological relevant *S. aureus* types from different hospitals were present since the evaluation of phage typing (Wenworth, 1963). The doubts did not decrease with the development of modern typing methods. The more new typing methods were developed the more increased the number of subtypes and varieties of *S. aureus* types. The results of Tenover et al. (1994, Appendix: Table 52) demonstrate that big difficulties in classifying single strains are mainly observed when investigating strains from different sets (collections from different hospital outbreaks) with various typing methods. A careful analysis of the data of Tenover et al. (1994) demonstrates that epidemiological data from different typing methods can be only compared when using results of one hospital outbreak. Four of eleven PT`s from different hospital outbreaks (Tenover et al., 1994) are not distinguished when considering only all PFGE data. The strains of PT`s B, C, D and E were confidently distinguished by Phage typing and almost all other methods used (Appendix: Table 52).

First of all typing methods have to guarantee a confidential discrimination between outbreak strains and other isolates because of the assessment of hygienic conditions in one hospital or just in one location. The large relevance of the sampling plan in this context is demonstrated by the comparison of data with a different relation between numbers of samples per herd. Larsen et al. (2000) detected for example only 11 phage types and 16 ribotypes in comparison to Aarestrup et al. (1997), who detected 25 phage types and 87 ribotypes. Both however investigated the same number of samples (n=404) and used identical methods. The strains of

the study of Larsen et al. (2000) however were detected in 10 herds, whereas the isolates from Aarestrup et al. (1997) came from monitoring programs from 5 Nordic countries.

The challenge of an objective interpretation from epidemiological data and an adequate sampling plan in the dairy chain show the population analysis of Zadoks et al. (2002); Zadok and colleagues explored the role of skin, milking machine unit liners and bovine milk as reservoirs for *S. aureus* in the dairy chain with phage typing, PFGE and binary typing. They concluded that PFGE is the most suitable method for distinguishing between strains from bovine milk when investigating 225 strains from 40 herds from the study of Fox et al. (1991). Only the Binary types 5715 and 1107, 1619 had a similar distribution of strains from all three sources (Appendix: Table 44, Figure 26 and Table 45). These are single types confirming the hypothesis, that the transmission from the milking machines lead to the colonisation of the teat skin and finally to an IMI. However the deficiencies of the study set up of Fox et al. (1991) do not allow such conclusions. Zadoks et al. (2002) did not give detailed information about the number of identified *S. aureus* types except of the remark that not more than 20 strains per herd were used for epidemiological investigations. Even the presentation of the same data using only phage typing (see Fox et al., 1991) give no further information in this context (Appendix: Table 45). Each source of samples (liner, teat skin and bovine milk) was only taken in 10 herds. Swabs of liners and teat skin but no bovine milk were sampled in ten 12 herds. Bovine milk and swabs of liners but no swabs of teat skin were sampled in 18 herds. Additionally milking unit liners were sampled in each but only after milking all cows and not between each milking of each single cow. Only Fox et al. (1991) gave these relevant but still unsatisfying informations about the sampling procedure.

The most problematic and most relevant phage type 060000 was only sensitive for one phage (Fox et al., 1991 and Zadoks et al., 2002). The low number of phages and the use of an unknown phage set in this study was still criticized (chapter 4.1.3.2). However the differences of the discriminatory efficiency between PFGE and phage typing were only small. Phage typing revealed 21 types and PFGE 24 PT`s (Zadoks et al., 2002). The discrimination of PT A in six b

inary types including five or more isolates indicates deficiencies of the discriminatory efficiency of PFGE (Appendix: Table 49). The PT A is separated in many different binary types (Zadoks et al., 2002)). Particularly the binary types 5175 and 9811 with many mastitis isolates confirm that some isolates of PT`s G and H are subtypes from PT A. The binary type 9811 was found in one herd only (Appendix Table 49: the single isolates, which was classified by PFGE not by PT Q, belonged to PT A). This result indicates that the strains from

bovine milk samples belonging to the most frequent phage type 060000 are not misclassified and the used phage set with only 18 phages just fail to distinguish in many cases between different subtypes. Jørgensen (2005) observed similar results for their dominating strain from bovine and caprine milk samples (Appendix: Table 44, Figure 25).

A detailed analysis of Zadoks et al. (2002) and Fox et al. (1991) further indicate that the sampling procedure and sampling plan of the study set up only simulate the better suitability of PFGE (Appendix: Tables 48 and 49, Figure 26). The lack of an equal distribution of samples from different sources considered in epidemiological questions simulates a high prevalence of single strains and explains why single *S. aureus* types do not have one isolate from the teat skin, bovine milk or liner (Appendix; Table 45, 46). Usually only the isolates from the predominant phage and PFGE types, which came from one or two herds only, were confirmed by binary typing (Appendix: boldface type in Table 49, Table 46). The observation (Zadoks et al., 2002) that the binary type 9811 was present in only one herd is the best example in this context. The binary types 5715 and 1107, 1619 are probably the strains, which were isolated in the ten herds by sampling the teat skin, bovine milk and milking unit liners. The high number of strains from teat skin and single isolates from milking unit liners belonging to binary type 17491 or 22099 might be explained by the fact that in 10 herds only teat skin and milking unit liners were sampled.

Furthermore, Fox et al. (1991, 1992) considered each strain without detailed descriptions which methods and how many methods from the same sample have been tested positive. The number of strains from the skin and liners is exactly the same (Appendix: Tables 46 and 47). Fox et al. (1991) additionally investigated bovine milk samples only by using BA favouring haemolytic staphylococci. We suggest that platting on different agar plates might lead to a selection of certain types or, much more problematic, at least the enrichment in broth only allow the isolation of single *S. aureus* types (Fox et al., 1991, 1992). For each method the agar plates and the enrichments the same volume (0.1ml) was used (Fox et al., 1992)**.** Only *S. aureus* count in the Vogel Johnson-broth of at least 10^3 CFU/ml guarantee according to Fox et al. (1992) a sufficient recovery rate for isolates from teat skin due to the high presence of CNS or non- haemolytic *S. aureus* strains (Appendix: Table 46). The high prevalence of strains from liquid Baird- Parker or Liquid Vogel- Johnson in contrast to only 12.5 % positive samples on BA from *S. aureus* of teat skin indicates that the most *S. aureus* showed no or only weak haemolysis (Appendix: Table 46). Unfortunately neither Fox et al. (1991) nor Zadoks et al. (2002) took consideration of traditional selection criteria, in spite of the important role especially of haemolysis when investigating *S. aureus* from the skin and IMI.

Munch-Petersen (1971, 1972) grouped non-haemolytic *S. aureus* to micrococci because only haemolytic strains were correlated to high SCC and IMI. The high prevalence of 44.4 % positive samples from milking liners on BA in contrast to only 12.5 % positive samples from teat skin only indicates that most *S. aureus* from liners were αβ- haemolytic strains causing IMI in contrast to isolates from teat skin (Appendix: Table 47).

In summary, the results of Zadoks et al. (2002) confirm our study design and the large importance of comparing first of all only strains form a single herd or chain. A quantitative determination of udder pathogens is indispensable when investigating the interdependence between IMI, teat skin and transmission from cow to cow. Very important in this context is the prevalence of dominating strains. The investigation of milking liners is not necessary, because of the high correlation between *S. aureus* types from IMI and the bulk tank milk. Only the examination of the teat cups and teat skin after milking each cow together with the bulk milk and different milk fractions would give a comprehensive picture about the role of *S. aureus* from the teat skin. The high biodiversity of *S. aureus* in dairy herds is the underlying cause for this high effort.

5.7 Discriminatory efficiency versus comparability of different typing methods

Tenover et al. (1994) observed slight variations of duplicates almost for each typing method apart from the low standardization independent of the typing method used. Various mutations, in which the primers are located, may cause non-typable results in *spa* typing or MLST (Baum et al., 2009). However, neither the reproducibility nor the low standardisation of typing is the critical problem of *S. aureus* population analysis (Hayes and Borodovsky, 1988). The continuously chancing environmental conditions and methods during a period of 50 years play a major role in this context. The development of typing methods with a different discriminatory efficiency is the real challenge for comparing epidemiological results especially over longitudinal periods. Not only a too low but also a high discriminatory efficiency such as it is observed for PFGE, lead to a higher number of misclassified strains (see Tenover et al., 1994 and Appendix: Table 53). Further PFGE analyzes genetic markers that undergo rapid evolutionary changes: Strains exhibiting differences of 1 to 6 fragments in their banding patterns may still be clonally related; all of this has to be considered in evaluating population analysis over a longitudinal period (Blanc et al., 2001).

The variation of intra- and inter-laboratory results may decrease in the order of biotyping, phage typing and genotyping. However the high agreement between biotyping, phage typing

and genotyping from different investigators confirm the great concern and discriminatory efficiency of phenotypic characteristics for typing *S. aureus* isolates. The high reproducibility of phenotypic characteristics, phage typing and especially the antibiogram with many chemicals was demonstrated by Kerouanton et al. (2007) (Appendix: Table 42 and Figure 25). Almost all outbreak strains belong to an individual PT with a characteristic antibiogram (Kerouanton et al., 2007). The homology from the dendrogram of 60% (Kerouanton et al., 2007), the chosen clusters for all outbreak strains and the homology of the phage profile and biotypes indicate that the strains belong to one single clonal complex (Ikawaty et al., 2009).

A lack of discriminatory power of typing methods is frequently observed when both a higher number of samples and a larger number of phenotypic characteristics and strains from different locations are investigated. Therefore the underestimation of the high diversity of *S. aureus* populations from different locations is complementary to the overestimation of the discriminatory efficiency of single typing methods. The results of Zadoks et al. (2002) and Tenover et al. (1994) are the best examples for difficulties in interpreting epidemiological data by using methods with a different discriminatory efficiency, especially when samples from the same reservoir came from different locations or herds (Appendix: Tables 44 and 49). PFGE distinguishes *S. aureus* isolates, which belong to one binary types or phage type and vice versa (Appendix: Table 44, Table 45). Similar results were observed by Jørgensen et al. (2005) and Ikawaty et al. (2009). Jørgensen et al. (2005) separated the MLST type 133 in 20 different PT`s. Ikawaty et al. (2009) separated the same *spa* type in different MLST or PT`s (Appendix: Table 42, Figures 25 and 28). The low reliability of both methods characterized by a high discriminatory efficiency and methods of classification of strains from different hosts for epidemiological studies are demonstrated by the results of Aires de Soursa et al. (2009). Neither *spa* typing and MLST nor PFGE could distinguish between single isolates from the ostrich and bovine strains (Appendix: Table 50). A detailed description about the origin and cultivation of isolates is therefore indispensable.

The choice of methods depends further on the local strain types and circumstances of investigation (Blanc et al., 2001). The high diversity of the *S. aureus* population with respect to phenotypic and biochemical characteristics, the antibiotic resistance and virulence factors cannot be considered all together during one microbiological study. Many investigators use genotyping methods with an unknown discriminatory efficiency, unsuitable phage sets, or antibiograms, phenotypic and biochemical characteristics with low relevancy for their source of strains. Population analyses agree therefore frequently more with “randomized sampling and typing”. The missing consideration of these relevant characteristics is one reason for the

wrong interpretation of epidemiological results. This default induces an ineffective infection control in the dairy chain or wrong conclusions about hygienic measurements during the manufacture of food. The classification of strains in phage group II as abattoir biotype (cluster F and K in Appendix: Table 42; Isigidi et al., 1990) is one example for difficulties regarding true conclusions in this context. The most human *S. aureus* from the nose belong to Phage group II and are not a special characteristic of the butcher's nare (Appendix: Tables 38 and 41).

Phage typing is based on the negative or positive reaction of single phages, which can gain strong importance because of the limited number of phages in one phage set. That is the reason, why the discriminatory efficiency of phage typing is highly related with the number of specific phages in one set of isolates in comparison to genotyping and why genotyping methods are more universal in use. Studies comparing new genotyping methods and phage typing in the 1990`s were achieved by using only phages of the HPS. They did not use more specific phages for typing MRSA or bovine strains in comparison to Richardson et al. (1997) or Davidson (1961). Furthermore the change of *S. aureus* populations in phage typing is frequently only indicated by the loss of sensitivity from single phages. This would be a further crucial advantage of genotyping methods, especially for *S. aureus* population analyses over longer periods. On the other side big scale screenings based on genotyping are not practicable and expensive. However the low prevalence of antibiotic resistant *S. aureus* strains in the dairy chain, in comparison to clinical veterinary strains during the last decade, might be the main reason for the lack of epidemiological data in more detail.

The complex regulation of different virulence factors within an innate immune evasion cluster by bacteriophages (Norvick, 2004; van Wamel et al., 2006; Kumagai et al., 2007; Marquies et al., 2007) explains why many strains cannot be allocated to host specific biotypes or the prevalence of one biotype in single PFGE-cluster´s with different phenotypic characteristics (Appendix: Figure 25, Tables 41 and 42). Not solid banding patterns of PFGE in inter-laboratory studies seem to be specific for *S. aureus* and may account for the variable numbers of lysogenic phages in genomes (Hayes and Borodovsky, 1988). Unfortunately phage conversion is usually only considered when discussing about phage typing but not for modern genotyping methods.

The presence of genes from converting bacteriophages should induce a more goal-directed variability in the genomes of *S. aureus* in comparison to spontaneous mutations such as insertions or deletions of single nucleotides. This might be the reason, why lysogenic or temperent stage of single phages can simulate a high number of different subtypes not only

for phage typing but also for genotyping methods. The “non-goal directed infections” of *S. aureus* through bacteriophages in interaction with environmental conditions determinate further the predominance of certain phenotypic characteristics. Changing conditions should prefer the selection of various strains with different characteristics and sensitivity for phages. The high prevalence of closely related endemic strains from different hosts simulate the strong relationship between the biotyping schemes, source of strains and genotyping by PFGE (Cluster A, C, E, H, I, L, M and N in Appendix Table 42). However the low number of strains usually cultured from the same side has to be the main reason why only single isolates belong to different clusters (Cluster B, D, H, G, J, K, and O in Appendix Table 42). Or why isolates cultured from food harbour different strains than endemic strains from the same host (Cluster D, F and K in Appendix Table 42). It further explains the low variation between different typing methods when investigating separately only single herds or isolates from a single outbreak and the considerable differences between strains from different herds or different outbreaks.

The phenomenon that the detection of different subtypes in one herd (Nyhan and Archer ,1967; Gedek, 1968; Giesecke et al., 1972 and herd D, F, A in Table 20) are based on the variation of the same phages, is very important for the understanding of both the variability of typing results and the importance of the sampling plan. The dominating *S. aureus* types in each herd determine obviously the sensitivity for a characteristic set of certain bacteriophages. This leads to the high diversity of individual subtypes respectively phage types in each single herd or outbreaks belonging to one PT, ST or one characteristic phage profile (Figure 2, Table 19, Appendix: Figure 25 and 27). Subtypes from one clonal complex are usually characterized by different characteristics indicating the close relationship between phenotypes, prophages and the change of *S. aureus* populations. Closely related phages in different numbers obviously lead to specific changes of single sequences in one gen region causing a high number of slight variations and simulate different subtypes. This might be the reason for the high number of different genotypes belonging to the same clonal complex (see Jørgensen et al., 2005; Ikawaty et al., 2009; results shown in Figure 2).

The low correspondence between sources of isolates and the biotyping sheme (Isigidi et al., 1990) demonstrates that more characteristics have to be considered to improve reliability of results. Two of five characteristics of the human biotype (production of β- and crystal-violet type; (Isigidi et al., 1990)) show a considerable variability indicating difficulties in classifying human strains. For single outbreaks strains biotyping shows the biggest variation of all typing methods tested (Tenover et al., 1994), especially when comparing strains from different

outbreaks (outbreaks strains I and III in Appendix: Table 44). Tenover et al. (1994) however used a high number of biochemical and phenotypic characteristics, which increase the possibility of methodical difficulties. The high variability of both strains from different hosts (Hajek and Marsalek, 1971) or bovine strains from different studies (Munch- Peterson, 1965) shows the low reliability of haemolysis for classifying *S. aureus* isolates (Appendix: Table 20 and 27). The strong relationship between phage types, crystal-violet type and coagulation of bovine plasma demonstrates the relevance of the HPS and BPS for biotyping *S. aureus* isolates (Appendix: Table 20 and 22). This relevance of HPS and BPS on the one hand might explain the low suitability of the simplified biotyping schemes of Isigidi et al. (1990). On the other hand a suitable phage set can for example replace the investigation of the crystal-violet type and coagulation of bovine plasma. The pigmentation is in comparison to the egg-yolk reaction and haemolysis not a confidential characteristic regarding the discrimination of different *S. aureus* types (Gedek, 1966). This was confirmed for single isolates during this study. Single bacteria colonies with a different pigmentation from frozen isolates had the same phage type or PT and were identical in all other characteristics (results not shown).

The low prevalence of phage type 119, 96 and 78 in the past is one possible explanation why α- haemolysis, egg yolk- and clumping factor reaction are not considered in traditional biotyping shemes of bovine strains. The low suitability of the antibiogram for bovine *S. aureus* strains was according to Gedek (1966) and Meyer (1966) as well mainly due to the low prevalence of penicillin resistant strains in comparison to human isolates. The same situation is observed when plasmid profiling is used for typing bovine strains. Plasmid profile analysis has a low discriminatory efficiency for typing bovine *S. aureus* isolates due to the large number of plasmid-free isolates (see Lange et al., 1999; Baumgartner et al., 1984 and Aarestrup et al., 1995b). The strong relationship between *S. aureus* types and penicillin resistance confirms both the chromosomal location of penicillin resistance and the high suitability for epidemiological investigations. The increasing penicillin resistance and high association between penicillin resistance and certain *S. aureus* types points at the relevance of antibiogram-based methods, especially when considering penicillin resistance for typing bovine strains (Vintov et al., 2003b). The antibiogram and plasmid profiling (Tenover et al., 1994) seem therefore not only appropriate for typing human strains from hospital outbreaks but also for bovine strains. Plasmid–encoded tetracycline resistant determinants in strains from bovine mastitis (Schwarz et al., 1998) are additionally an important hint regarding the potential of bovine strains, that in future single multi-resistant strains carrying plasmid will gain significance in bovine mastitis as well.

The use of restriction enzymes for many genotyping methods is based on the restriction of specific DNA sequences. Restriction enzymes lead to a randomized combination of DNA fragments respectively bands. The high specificity of restriction enzymes induces (Tenover et al., 1994; Gonano et al., 2009; Ikawaty et al., 2009 and the results shown in Figure 2) a high number of subtypes (Appendix: Tables 43, 44 and 52, Figure 28). The strong influence of restriction enzyme types is demonstrated by the results of Tenover et al. (1994). Tenover et al. (1994) observed only low differences when using *Sma* I *for* PFGE or FIGE in comparison to ribotyping when using *Cla* or *HindIII* (Appendix: Table 52). Systematic differences between pulso-and binary types by using *Sma* I (Zadoks et al., 2002) indicate that DNA sequences of macrorestrictions fragments can gain more importance than the profile of macrorestrictions fragments (Appendix: Table 49).

The criteria for interpreting PFGE patterns from outbreaks strains based on Tenover et al. (1995) are problematic particularly when considering the results of Aires-de-Sousa et al. (2007) and of Appendix: Tables 50 and 54. The study of Kerouanton et al. (2007) demonstrates that outbreak strains usually belong the same PT (Appendix: Table 42 and Figure 25). The distinctive disadvantage of genotyping methods using restriction enzymes refers to the problem of comparability across different studies. Comparisons of different macrorestrictions fragments patterns are in this context impossible. Not only the comparability of PT`s from different laboratories containing only MRSA from hospitals, even the grouping of strains in one investigation can make problems in spite of the use of a single library with the same software. Methodical difficulties of PFGE were still discussed (see chapter 4.5.3). For example the isolates belonging to phage type 119 in PFGE-profile 1 (Figure 2) had a low similarity in comparison to the other isolates of phage type 119 in PFGE-profile 28-34. The MLST type 484 in spite of the identical enterotoxin profile and clonal complex such as MLST type 30 belonged to the cluster 51 and not to cluster 10 (Jørgensen al., 2005). Furthermore the MLST type 97 from a different clonal complex was grouped with MLST type 30 to cluster 10 (Appendix: Figure 26 and Table 44).

The identification of the clonal complex is needed due to the instability of clusters based on genotyping methods with restriction enzymes. This is at least the reason for the necessity of genotyping methods based on sequencing such as MSLT or *spa* typing. Only sequencing allows a reliable classification of isolates. Spa *typing*, sequencing of the short sequence repeat (SSR) of the Protein A gene (spa) according to Frenay et al. (1996), combines many of the advantages of MLST, but is faster to perform. According to Koreen et al. (2004), sequencing of SSR is rapid, effective and discriminative, and compares favourably to other typing

schemes. The results of Ikawaty et al. (2009) however show that difficulties in classifying some strains still can exist when performing *spa*-typing and MLST. Methodological difficulties in clustering strains by PFGE and the heterogeneity especially of MLST type 71 and 97 in combination with different *spa*-types lead to difficulties in classifying these strains (Appendix: Figure 28). Furthermore Ikawaty et al. (2009) considered neither the antibiogram and phenotypic characteristics nor the allelic profile of MLST-types allowing the determination of the clonal complex from the corresponding isolates.

Highly conserved genes encoding for energy, lipid, nucleic acid or amino acid pathways (Heron et al., 2002) might explain a low discriminatory efficiency of MLST for certain *S. aureus* types (see Smith et al., 2005; Jørgensen, 2005 and Appendix: Figure 25, Table 42). Using PFGE many closely related strains seem to belong to different MLST types with only slight variations in each allelic sequence indicating the high prevalence of conserved metabolic pathway related genes in MLST. The results presented by Jørgensen (2005), Smith et al. (2006) and Ikawaty et al. (2009) suggest that MLST may not be required and that MLEE alone would be sufficient for the epidemiological analysis of strains obtained from bovine milk, which is in line with the findings of Kapur et al. (1995). Whole genome sequencing approaches may allow a solid foundation for future investigation of mechanisms of virulence and host specificity (Heron et al., 2002). The multiple conversions of various genes by bacteriophages is one result of these new methods (Kumagai et al., 2007). The more we know about phage conversion the more we know about the variation of phenotypic characteristics and intermediary phage- and genotypes. This justifies doubts if a whole genome sequencing approach would solve these problems.

In summary, we suggest that only modern genotyping methods in combination with phenotypic characteristics will in future allow a suitable classification of strains. One typing method alone such as PFGE is only suitable for investigations in one chain or for the identification of outbreak strains. Phage typing with specific phages leads similar to MLST, MLEE or *spa* typing to an efficient identification of the dominating clonal complexes o*f S. aureus* strains with fewer costs. The traditional selection criteria and the antibiogram with the enterotoxin profile together with the origin of isolates are not only highly related with important *S. aureus* types, but these characteristics are additionally in the centre of medical investigations.

6 Conclusion

The interdependence between the bacteriological assessment and the dynamics of subclinical mastitis is one of the two challenges when controlling *S. aureus* in dairy herds. Additionally the diversity of typing methods plays a critical role for defining biodiversity of *S. aureus* in the dairy chain. Our results demonstrate that the strengths and weaknesses of these typing techniques have to be carefully considered when generalizing any observation of *S. aureus* in dairy herds. However using typing methods extremely careful is not sufficient to control *S. aureus* in these herds. Population analysis also needs know-how about more traditional forms of bacteriological assessment and diagnostics, respectively. Culturing isolates is the first critical step, constituting the base of each following application of typing methods. Recommendations of considering specific phenotypic characteristics at this step have to be attended extremely careful due to the potential exclusion of single *S. aureus* types, which might be relevant for diagnostics. Furthermore not only veterinary specialized knowledge about critical influencing factor in management of cattle herds like breeding and feeding, but also the history of milk production and the introduction of specific measurements have to be taken into account when discussing the significance of *S. aureus* and bovine mastitis in the dairy chain.

A critical methodological problem for both assessment and control of *S. aureus* is on the one hand the volume of milk samples for bacteriological examinations on the other hand the number of cultivable colonies reflecting the range of different types in one sample. The shedding patterns of chronically infected cows with contagious *S. aureus* types is reflected by 100 CFU/ml in the foremilk supporting the common approach of investigating only 0.01 ml foremilk. The largest range of different *S. aureus* types in relation to the herd size are only detectable in sample volumes of one up to 1000 ml bulk tank milk. Methodological difficulties increase strongly, when bulk milk contains the milk of different dairy herds. The dynamic of mastitis is the main reason, why only the dairy chain in a single farm can be investigated.

The natural process of colonizing of the bovine teat duct and mammary gland during the first lactation leads to an increasing average SCC with each new lactation. The increasing level and number of different udder bacteria causes especially for older cows methodological difficulties during bacteriological examinations. High levels of contamination flora, insufficient distribution of bacteria on the agar plate, and the only use of traditional phenotypic characteristics on the corresponding medium underlie the common

underestimation of biodiversity and deficiencies in susceptibility assessments of *S. aureus*. Becker et al. (1987) suggested the missing inclusion of all suspected colonies as the main reason for defective results of *S. aureus* assessments. However, on the other hand the careful analysis of all suspected colonies is very time consuming and cost intensive particularly when assessing many cows or the whole herd within a sanitation program. Furthermore the investigation of 0.01 ml milk takes predominately contagious *S. aureus* into account that cause clinical mastitis or high SCC. Additionally in monitoring programs foremilk samples are not always available. In the veterinary practice samples are taken frequently after morning and before evening milking. This is a further element of uncertainty in the bacteriological diagnostics. The investigation of frozen foremilk and stripping samples confirm these observations (Godden et al., 2002; Appendix: Figure 14).

In many cases penicillin resistant or SEA producing human *S. aureus* biotypes are only detected in *low* numbers in main milk and/or stripping samples of cows without elevated SCC. Missing or weak haemolysis together with a strong egg-yolk reaction of many human isolates is another reason, why the isolates are detected only in dairy products but not in monitoring programs of dairy herds. Furthermore these strains are both more similar to environmental udder pathogens and more associated to the teat skin and teat canal infections than contagious *S. aureus* causing usually bovine mastitis. All these points together with phenotypic characteristics indicate that the bovine udder is colonized from two different biotypes of two different ecological niches: penicillin resistance and egg-yolk reaction reflects an adaptation to the skin similar to human *S. aureus* strains; β-haemolysis, variation of both coagulase and clumping factor reaction and the production of biofilms in the most bovine mastitis isolates reflect an adaptation to the mammary gland.

Antibiotic resistant or enterotoxigenic *S. aureus* of the skin do very likely not belong to pathogenic strains of bovine mastitis. However, these strains are involved in food poisoning outbreaks of raw milk products or responsible for a high prevalence of antibiotic resistant or SEA producing strains in dairy herds. The role of gene transfer through mobile elements exemplarily of phages or plasmids between biotypes of different ecological niches must be considered in this context. The high prevalence of isolates combining characteristics of different ecological niches, antibiotic resistance and enterotoxigeneity indicate the relevance of intermediary subtypes. Furthermore it demonstrates the interdependence between occasionally infections of different ecological niches and their different strategies in adapting on the specific corresponding conditions. The identification and classification of new *S.*

aureus types is the second purpose of population analysis. It is the base of understanding the shift and realistic exposure of pathogenity from unknown new types.

The biggest differences between nowadays detected *S. aureus* populations and that of 40 years before are observed for antibiotic sensitive bovine strains and their sensitivity to bovine phages. This is reflected by the rising importance of phages in phage group III which is based on the frequent use of antibiotic drugs; the use of these drugs was justified by the high prevalence of antibiotic sensitive bovine strains and human strains, which were found in the hospitals during the 1950`s. The strong shift of sensitivity to bovine phages demonstrates that the dry cow treatment reduces the typical bovine isolates in phage group IV, whereas the increasing prevalence of the antibiotic sensitive phage types 119 and 78 indicates still deficits in the control of contagious mastitis pathogens during the lactation period and clinical mastitis, especially of young cows. In this context it has been suggested that phage typing has to be used in susceptibility testing in national monitoring programs focused on the assessment of national policy of bovine mastitis.

The time period during the elimination of the dominating subpopulation and before another *S. aureus* types becomes finally established, is characterized by a fast and not goal-directed dynamic, allowing for the simple detection of many different types. The crucial problem of sanitation programs and related antibiotic treatments is the missing knowledge about the pathogenity of new established strains, which belong at the beginning only to the minority of isolates. This is a general phenomenon of medical population analysis or resistant monitoring programs from pathogenic bacteria. The medically relevant pathogenic strains belong usually to the minority of the investigated bacteria population. The MRSA populations are the best example in this context. They are normally only detected in hospitals or in the clinical area of veterinary medicine.

Furthermore it is usually not taken into account that one host accommodates different sites with different ecological niches, which are colonized from different types with different characteristics. Possibly the site specificity leads in some cases to the same differences, how as it was observed for strains from the same site of different hosts. Therefore the crucial question concerns which universe of the population of one host is investigated. A too low number of enterotoxigenic or antibiotic resistant strains in many studies, for example in Hajek and Marsalek (1973; Appendix: Table 21), is the reason, why information about the pathogenity of certain *S. aureus* subpopulations is insufficient. Only collections considering more enterotoxigenic or antibiotic resistant strains from different ecological niches

respectively sites give statistical evidence about the real distribution of the *S. aureus* subpopulations in one host.

Due to both its pathogenity and wide distribution in different hosts *S. aureus* became a "model bacteria" in microbiology, since it has been discovered at 1884 as "*Staphylococcus Rosenberg*". On the one hand this is the reason, why our knowledge for many important questions is very large going back for more than 100 years and concerning fields of applied and basic research in microbiology, medicine and agriculture. But on the other hand the large number of unrelated research activities and laboratories, respectively, working with *S. aureus* of different hosts and using very different typing methods for this purpose, came also up with a large body of inconsistent often confusing findings; this trend has been further increased by the use of new methods of genotyping or biochemical typing in the last 20 years.

This period to evaluate significant methodologies however was necessary, but it is now the timepoint for selecting the most suitable typing methods respectively for developing a standardized procedure in typing and classifying *S. aureus* isolates. The strong international effort during the 1960´s under the leadership of Davidson (1972) resulted in a large data set about the bovine *S. aureus* population 40 years ago that constitutes an excellent base to measure the shift of the *S. aureus* population during this period in the dairy chain. Nowadays a better network of collaborating laboratories might facilitate comparisons from results of *S. aureus* population analysis with different typing methods. It might further accelerate the process in standardizing all distinctive steps, which are essential for consistent population analyses of *S. aureus*. New software and commonly available data sets might represent new methods to realize these goals. The increasing numbers of strains with patterns of phenotypic characteristics very similar to *S. aureus* types, demonstrate the necessity of the additional registration of phenotypic characteristics, antibiogram and toxigenity in data banks.

The clumping factor, haemolysis, egg-yolk reaction and penicillin resistance are highly associated to specific *S. aureus* types. These characteristics give information about the suitability of corresponding media in culturing certain *S. aureus* types or indicate changing *S. aureus* populations because of changing environmental conditions or ineffective antibiotical treatments. The use of FTIR- technology, based on data banks, might be appropriate in this context. Additionally to the save identification of udder bacteria FTIR- Identity- test and FTIR- cluster analysis enable the discrimination of bacteria subpopulations. This method might be also suitable for the selection of specific strains from big scale screenings for a subsequent typing method with more consistent discriminatory efficiency.

The consequent use of commonly available data bases might be further critical for the discrimination of pathogenically relevant S. *aureus* subtypes. *S. aureus* strains with crucial differences in phenotypic characteristics - phage specificity, *spa* sequencing or MLEE type - could be further analyzed following recommendations of alternative phages or another typing method based on the shared data set. Statistical calculations based on these shared data sets are helpful for defining the specificity of phage reactions. The more all available results of different traditional bacteriological and typing methods are considered in a systematic data collection for the evaluation of population analysis, the more the discriminatory efficiency with respect to the dynamic of epidemiologic questionings will increase.

The control of *S. aureus* in the dairy chain is a still unsolved problem. According to Dodd (1980) mastitis has been investigated for over 100 years, but progress in control has been slow. According to Wendt et al. (1998) the safety of udder healthy could not keep pace with breeding cows of high milk yields. The increasing literature during the last decades is the best proof for this new situation and shows the increasing concern of environmental udder pathogens. Especially the prevalence of serve *E. coli* mastitis accompanied by systemic clinical signs is increased. This demonstrates that much more system relevant physiological parameters in a complex combination with other environmental influencing factors determine the healthy status of a dairy herd. Furthermore problems in control of subherds with high SCC rise with the herd size. Wendt et al. (1998) diagnosed that the herd management has to take into account these parameters in their preventive measurements to achieve any success of *S. aureus* control in dairy herds.

The influence of hygienic conditions is generally overestimated for this purpose but disinfection of milking machines, the most important preventive measurement in this context, is usually not included when discussing about milk hygienic. Alone the exponential growths of udder bacteria, which lead to a fast multiplication of one bacterium per ml milk during few hours, show the limited possibilities of hygienic measurements. The destruction of udder flora from the teat skin through teat dipping after milking increases the risk of coli mastitis in dairy herds (Deutz and Obritzhauser, 2003). The only udder bacterium, which is almost completely eradicated from dairy herds, is *Str. agalactiae.* This was mainly achieved to his sole occurrence in the mammary gland and high sensitivity to penicillin, but not with a higher standardisation of hygienic conditions. However the high heterogeneity regarding the occurrence and sensitivity to penicillin is the reason for the intricacy in the control of *S. aureus* populations in dairy herds. The success of sanitation programs is therefore limited for contagious *S. aureus* types with similar characteristics in comparison to *Str. agalactiae.*

Preventive management strategies in the first weeks after calving reduce first of all the occurrence of clinical mastitis, but are almost without any effect on the distribution of major pathogens in the herd. The 25 to 50 times greater level of subclinical mastitis in comparison to clinical mastitis was the main cause according to Dood (1980) for the screening of the SCC in dairy herds. The relevance of clinical mastitis combined with high prevalence of *S. aureus* however shows primary the low suitability of BMSCC. The limit of $7{,}5x10^5$ SCC/ml bulkmilk in the USA confirms further the low importance of the BMSCC for the processing to dairy products. The high risk of *S. aureus* shedding cows with about $1{,}5x10^5$ SCC/ml is the cause for the low suitability of BMSCC. Problems with the detection of all shedding cows at one timepoint of sampling and the fast dynamic of mastitis should be the reason, why Zadoks et al. (2001) did not consider the *S. aureus* prevalence in dairy herds and why Dood (1980) concluded that the new infection rate is ultimately the most important factor but not in the early stages of control. Zecconi et al. (1997) showed the importance of using 10 ml foremilk samples.

The main factors governing the success of a control system in its 1st year are according to Dood (1980) the proportion of cows infected at the start of sanitation programs and average duration of infections. This however is not relevant when the disinfection of milking machine was used (Zadoks et al., 2001; Appendix: Table 15 and Figure 23). Our results in the HCPS herds clearly demonstrate that the transmission of *S. aureus* during milking from older cows to younger cows is the crucial problem of transmission of subclinical mastitis. It is further the cause for difficulties in the definition of IMI and prevalence of *S. aureus* in the dairy chain. Therefore the primary aim in controlling contagious udder pathogens has to be to promote each method, which segregates chronic shedders to avoid the transmission during milking. The fast transmission through the colonization of the teat skin or teat canal and high resistance for antibiotic therapy of *S. aureus* is the reason, why mastitis control strategies of organic farming should be used for this pathogen. Only a quality management system for dairy herds would achieve a consequent transfer of these measurements in dairy farming.

Continuous bacteriological examination of mastitis milk and monitoring of bulk milk for *S. aureus* should be part of the strategy to maintain minimum levels of contagious mastitis pathogens in herds. The investigation based on shorter intervals of the SCC, bacteria-, protein-, fat- and urea content of the bulk milk might additionally produce valuable information about the feeding management of the herd. To set up a suitable mastitis recording system however it is necessary to be a member of a milk recording scheme for individual cow SCC with additional consideration of the protein-, fat- and urea content and probably daily

temperature. Electronic data set systems would allow the effective identification of cows with a repeated level of > 10^5 SCC/ml and cows with a negative energy balance. This information is necessary for segregated milking of cows in tie stalls. Milking parlour systems with automated disinfection systems for the milking machine and transponder chips of cow hyperlinked with electronic data banks might facilitate the control of *S. aureus* IMI in free stalls.

Routine bacteriological examination of mastitis cases in HCPS herds has to be established short after calving. When performing disinfection of the milking machine other bacteriological examinations are less relevant due to the reduced risk of mastitis transmission. The control of *S. aureus* in HCPS herds is only possible with the disinfection of the milking machine at least over once lactation period, because all older cows came back during this time from the dry off period in the herd again or they get chronic shedders. During the first year the disinfection of the milking machine has to be performed after each cow with exception of non shedding heifers with low SCC. After one year it might be sufficient when the disinfection of the milking machine is performed only after milking of cows with persistently mastitis as it is likely in LCPS herds. This procedure allows the limitation of treating shedding cows at the end of the lactation period or culling of cows without the transmission of udder bacteria during the lactation period. It might maintain the SCC level of saleable milk in a sustainable mode.

A solid balance of acid and non acid-producing bacteria in raw milk is the best prevention for food poisoning outbreaks in the dairy chain. The level of *E. coli* in raw milk products contains the most information about the acidification during the production process, when no data about the pH are available. A high level of *E. coli* is generally an indicator for inhibited acidification, whereas high *S. aureus* counts accompanied by low *E. coli*-level in raw milk products indicate a too high *S. aureus*-level in the bulk milk. The level of coliforms is less suitable, because the number of coliforms varies in relation to the hygienic conditions during milking and cheese making in higher extends than *E. coli*. Additionally coliforms can grow in contrast to *E. coli* in the storage tank until 4°C. That is the reason, why detailed information about the level of coliforms and the treatment of milk inclusive cheese making are necessary.

The balance of bacteria has also to be taken into account in bovine mastitis. Many older cows have frequently a sTable balance with udder bacteria and are chronic shedders because of the production of specific antibodies. A solid level of SCC and control about single older chronic shedders together with healthy cows in the 1st and 2nd lactation through disinfection of the milking machine or segregated milking is of strong interest for milk production. One reason

for the high prevalence of clinical mastitis in some herds with low BMSCC is the high number of young cows with no specific antibody production, possibly linked additionally with heifers of other herds. Furthermore the relevance of staphyloccoci in the udder flora in randomised selected herds (Appendix: Table 3) is replaced through *E. coli* in herds with mastitis problems, which is usually only associated with clinical mastitis. Only the traditional use of penicillin for treating cows with clinical *E. coli* mastitis can explain the high prevalence of penicillin resistant *S. aureus* isolates in monitoring programs of herds with mastitis problems. This also indicates deficits of milkers in discriminating between the pathogenesis of coli mastitis and other udder pathogens, which would be now necessary for the immediately use of the corresponding antibiotics.

Therefore it has to be analyzed, if the first therapy of clinical disease could not administer an extrinsical application of lysozyme. The unspecific killing of all bacteria cell walls through high concentrations of lysozyme is not only used in molecular biology, it is further part of the immune answer from the bovine mammary gland in low concentrations itself. A vaccination for phage type 78 is commended for small ruminants. This would protect the animals against serve mastitis; it is at the same time a preventive measurement for food poisoning outbreaks for this SEC producing *S. aureus* type. The only use of penicillin can be not commended, when antibiotics for clinical mastitis are finally necessary. Almost all common udder bacteria are sensitive according to the results of Krabisch et al. (1999) for the combination of gentamicin and ampicillin. Streptomycin has been widely used in combination with penicillin in the past. Previous studies have however found streptomycin to be among the most prevalent types of resistance detected among *S. aureus* in dairy herds (Vintov et al., 2003a, Werckenthin et al., 2001). The biggest differences between the antibiogram of herds from randomized sampling and herds with mastitis problems showed the *E. coli* isolates with an increasing tetracyclin and gentamicin resistance (Krabisch et al., 1999). These results demonstrate the need of more effective methods for investigating the antibiotic resistance of the most relevant udder pathogens of the whole herd in higher volumes.

This is from strong concern because at the moment the udder pathogens are only detected at 0.01 ml foremilk. Antibiotic resistant udder pathogens in this concentration however are usually still spread in the whole herd. The investigation of the bulk milk or mastitis milk after a failed antibiotic therapy with the most probable number technique (MPN), traditionally used only in food microbiology, would be the best method in this context. MPN is based on an enrichment of high volumes in tenfold dilutions such as 100, 10 or 1ml milk. The addition of the current administered antibiotics in herds to the enrichment for investigating their bulk tank

milk samples only allow the growth of the corresponding resistant udder pathogens. Antibiotic resistant strains could be detected with this method still in very low concentrations based on the dilution effect of each cow and without difficulties in considering all colonies with different antibiograms for susceptibility testing in one sample.

The use of MPN with suitable selective media for all major pathogens after the enrichment allows further a standardized microbiological determination. The additional investigation of 0.1 and 0.01 ml bulk milk on agar plates informs about the dominating udder bacteria in the herd. These results might be the distinct information for resistance monitoring programs. It would allow for a quantitative assessment of antibiotic resistant udder pathogens together with the shift of all udder bacteria in dairy herds related to the national policy for the use of antimicrobial agents. Furthermore it might enable a comprehensive picture about all multiresistant major pathogens.

The enterotoxigeneity of *S. aureus* is more complex because the enrichment for the relevant characteristics, as it is supposed to be for antibiotics, is not possible. When producing raw milk products it has to be avoided to eradicate harmless typical bovine *S. aureus* strains in dairy herds, which occupy the ecological niches in the mammary gland and have a protective function in cheese making. The strong reduction of about 4 times less samples from subclinical mastitis taken by udder-health-service extension workers in the Switzerland between 1987 and 1996 induced obviously a high prevalence of non ß-haemolytic, egg-yolk and clumping factor positive *S. aureus* types producing enterotoxin A or C without *tst* in spite of low BMSCC (Table 20 , Appendix: Table 1 and Table 25). Furthermore the clumping factor negative strains with *sec* and *tst* may cause frequently chronic or acute mastitis but are not detected in higher numbers in the bulk milk (Table 3, Appendix: Table 8 and Table 26).

The presence of dominating strains or persistently shedders producing SE however is the crucial question in raw milk cheese producing farms or dairies. The investigation of 1 ml raw milk and 0.01 ml enrichment of the same sample with the Real-Time PCR and specific targets (gene sonds) for enterotoxins and protein A allows for the detection of all relevant SE-producing *S. aureus* strains in one herd. The quantitative results of Real-Time PCR enable a fast risk assessment about the level of enterotoxin genes in dairy products. A higher level of enterotoxin genes in an enrichment of 0.01 ml bulk milk in comparison to 1 ml bulk milk is appropriate for the detection of chronic shedders producing SE and show the necessity of sanitation programs in corresponding herds.

7 References

Aarestrup RK, Wegener HC, Rosdahl VT. 1995a. Evaluation of phenotypic and genotypic methods for epidemiological typing of *Staphylococcus aureus* isolates from bovine mastitis in Denmark. *Vet. Microbiol.* 45:139-150.

Aarestrup RK, Wegener HC, Rosdahl VT. 1995b. A comparative study of *Staphylococcus aureus* strains isolated from bovine subclinical mastitis during 1952- 1956 and 1992. *Acta Vet. Scand.* 36:237-243.

Aarestrup EM, Wegener HC, Jensen NE, Jonsson O, Mylls V, Thorberg BM, Rosdahl VT. 1997. A study of phage and ribotype patterns of *Staphylococcus aureus* isolated from bovine mastitis in the Nordic countries. *Acta Vet. Scand.* 38:243-252.

Aarestrup EM, Jensen NE. 1998. Development of penicillin resistance among *Staphylococcus aureus* isolated from bovine mastitis in Denmark and other countries. *Microbial drug resistance.* 4: 237-256.

Abbar FM, Mohammed MT, Arslain SH. 1986. Selected biological properties of enterotoxigenic staphylococci isolated from milk. *J. Food Protection* 49:871-873.

Abo-Elnaga I, Kandler O. 1965a. Zur Charakterisierung der in Milch verbreiteten Staphylokokken. *Arch. Lebensmittel Hyg.* 6:132-137.

Abo-Elnaga I, Kandler O. 1965b. Zur Verbreitung der Staphylokokken in Milch. *Zbl. Bakt. I Orig. A,* 196:438-451.

Abo-Elnaga I, Kandler O. 1965c. Studies on the microflora of milking machines. *Milchwiss.* 20:660-662.

Adekeye JD, Adesiyun AA. 1984. Characteristics for *Staphylococcus aureus* isolated from bovine mastitis milk. Frequency of isolation of enterotoxigenic staphylococci from milk of nursing mothers in Kaduna, Nigeria. *J. Hyg. Camb.* 93:531-538.

Adesiyun AA. 1995. Characteristics for *Staphylococcus aureus* isolated from bovine mastitis milk. Bacteriophage and antimicrobial agent susceptibility and enterotoxigeneity. *J. Vet. Med.* 42:129-139.

Aires-de-Sousa M, Boye K, de Lencastre H, Deplano A, Enright MC, Etienne J, Friedrich A, Harmsen D, Holmes A, Huijsdens XW, Kearns AM, Mellmann A, Meugnier H, Rasheed JK, Spalburg E, Srommenger B, Struelens MJ, Tenover FC, Thomas J, Vogel U, Westh H, Xu J, Witte W. 2006. High interlaboratory reproducibility of DNA sequence-based typing of bacteria in a multicenter study. *J. Clin. Microbiol*. 44:619-621.

Ali-Vehmas T, Westphalen P, Mylls V, Sandholm M. 1997. Binding of *Staphylococcus aureus* to milk fat globules increases resistance to penicillin-G. *J. Dairy Res*. 64:253-260.

Ali-Vehmas T, Vikerpuur M, Pyorälä S, Atroshi F. 2001. Characterization of haemolytic activity of Staphylococcus aureus strains isolated from bovine mastitis milk. *Microbiological research* 155:399-344.

Almeida RA, Matthews KR, Cifrian E, Guidry AJ, Oliver SP. 1996. *Staphylococcus aureus* invasion of bovine mammary epithelial cells. *J. Dairy Sci*. 79:1021-1026.

Anderson JC. 1982. Progressive pathology of staphylococcal mastitis with a note on control, immunization and therapy. *Vet. Rec*. 110:372- 376.

Anonymus. 1992. Council directive. 92/46/EEC laying down the health rules for the production and placing on the market of raw milk, heat-treated milk and milk-based products.
Anonymus. 2005. Commission regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs.

Asheshov EH, Coe AW, Porthouse A. 1977. Properties of strains of *Staphylococcus aureus* in the 94-, 96-complex. *J. Med. Microbiol*. 10:171-178.

Baird-Parker AC. 1962. An improved diagnostic and selective medium for isolating coagulase positive staphylococci. *J. Appl. Bact*. 25:12-19.

Bajlosov D., Grigorova M. 1968. On the phage-typing of bovine pathogenic staphylococci. *Zbl. Bakt. Hyg. I Abt. Orig. A*. 209:478-482.

Barkema HW, Schukken YH, Lam TJGM, Beiboer ML, Wilmink H, Benedictus G, Brand A. 1998. Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. *J. Dairy. Sci*. 81:411-419.

Barkema HW, Schukken YH, Zadoks RN. 2006. Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *J. Dairy. Sci*. 89:1877-1895.

Barlett PC, Miller SY. 1993. Managerial risk factors for intra-mammary coagulase positive staphylococci in Ohio dairy herds. *Prev. Vet. Med*. 17:33-40.

Barlett PC, Agger JF , Houe H, Lawson LG. 2001. Managerial risk factors for intra-mammary coagulase positive staphylococci in Ohio dairy herds. Incidence of clinical mastitis in Danish dairy cattle and screening for non-reporting in a passively collected national surveillance system. *Prev. Vet. Med.* 48:73-83.

Batish VK, Nataraj B, Grover Brand S. 1989. Variation in the behaviour of enterotoxigenic *Staphylococcus aureus* after heat stress in milk. *J. Appl. Bac*. 66:27-35.

Baum C, Haslinger-Löffler B, Westh H, Boye K, Peters G, Neumann C, Kahl BC. 2009. Non-*spa*-typable clinical *Staphylococcus aureus* strains are naturally occurring protein A mutants. *J. Clin. Microbiol*. 47:3624-3629.

Baumgartner H, Kästli P, Hofer P. 1968. Das Verhalten von *Staphylococcus aureus* in Milch und bei der Herstellung von Emmentalerkäse. *Schweiz. Milchzeitung* 94. Wiss. Beilage Nr. 118:994-996.

Becker H, Zaadhof KJ, Terplan G. 1987. Zur Charakterisierung von *Staphylococcus aureus* Stämmen des Rindes unter besonderer Berücksichtigung des Klumpingfaktors. *Arch. Lebensmittel Hyg*. 38:1-32.

Beech JA. 1967. A study of the results obtained from bacteriological and cytological examinations of various portions of cow's milk. *Vet. Rec*. 80:667-672.

Beerda B., Ouweltjes W, Sebek LBJ, Windig JJ, Veerkamp RF. 2007. Effects of genotype by environment interactions on milk yield, energy balance and protein balance. *J. Dairy. Sci*. 90:219-228.

Beseth L. 1963. Untersuchungen über Staphylokokken als Mastitiserreger beim Rind in Trondelag (schwedisch). *Nord. Vet. Med*. 15:745-754.

Bergeys Manual of Systematic Bacteriology. 1984. Vol. 2: 1013-1035. Williams and Wilkins, Baltimore .

Birren BW, Lai E. 1993. Pulsed field gel electrophoresis: a practical guide. Academic Press, Inc., San Diego, Calif..

Blackburn PS. 1968. The cell count of cow`s milk and the microorganisms cultured from the milk. *J. Dairy Res*. 35:59-65.

Blair JE, Carr M. 1960. Distribution of phage groups of *Staphylococcus aureus* in the years 1927 through 1947. *Science*. 132:1247-1248.

Blair JE, Williams REO. 1961. Phage typing of staphylococci. *Bull. Wld. Hlth. Org*. 24:771-784.

Blobel H, Schließer T. 1979. Handbuch der bakteriellen Infektionen bei Tieren. Band II:22-115. Jena: Fischer Verlag.

Blouse L.E, Mauney CU, Marraro RV, Dupuy HJ. 1972. Apparent spontaneous induction of *Staphylococcus aureus* isolated from clinical sources. *Appl. Microbiol*. 23:1023-1024.

Blouse LE, Brockett RM, Steele NP, Ward N. 1979. Apparent spontaneous induction of *Staphylococcus aureus* isolated from clinical scources. *J. Clin. Microbiol*. 10:604-606.

Bonin W, Blobel, H. 1967. Typisierung boviner Staphylokokken. *Zbl. Bakt. I Abt. Orig A.* 205:309-318.

Bramley AJ, King JS, Higgs TM, Neave FK. 1979. Colonization of the bovine teat duct following inoculation with *Staphylococcus aureus* and *Escherichia coli*. *Br. Vet. J.* 135:149-162.

Bramley A, Dodd FH. 1984. Reviews on the progress in dairy science: Mastitis control-progress and prospects. *J. Dairy. Res*. 51:481-512.

Brolund L. 1985. Cell counts in bovine milk. *Acta Vet. Scand. Suppl*. 80:1-123.

Brown RW, Shuman RD, Nord N, McDonald J.S.Mc, Rose DL. 1974. Staphylococcal alpha antitoxin and antibodies to extracellular antigens of Staphylococci and Micrococci in the serums of dairy cows. *Cornell Vet*. 64:559-572.

Bruckmayer RM, Weiss D, Wiedemann M, Schmitz S, Wendl G. 2004. Changes of physiochemical indicators during mastitis and the effects of milk ejection on their sensitivity. *J. Dairy Res.* 71:316-321.

Brun Y, Bes M, Boeufgras JM, Monget D, Fleurette J, Auckenthaler R, Devriese LA, Kocur M, Marples RR, Piemont Y. 1990. International collaborative evaluation of the ATB 32 staph gallery for identification of the Staphylococcus species. *Zbl. Bakt. I Abt. Orig A*. 273:319-326.

Burnet FM, Lush D. 1935. The staphylococcal bacteriophages. *J. Path. Bact.* 40:455-469.
Busato A, Trachsel P, Schällibaum M, Blum JW. 2000. Udder health and risk factors for subclinical mastitis in organic farms in Switzerland. *Prev. Vet. med.* 44:205-220.

Caroll PJ, Francis PG. 1985. The basic phage set for typing bovine staphylococci. *J. Hyg. (London*) 95:665-669.

Caroll JD, Cafferkey MT, Coleman DC. 1993. Serotype F double-and triple-converting phage insertionally inactivate the *Staphylococcus aureus* beta-toxin determinant by a common molecular mechanism. *FEMS Microbiol. Lett*. 106:147-155.

Cha JO, Lee JK, Jung YH, Yoo JI, Park YK, Kim BS, Lee YS. 2006. Molecular analysis of *Staphylococcus aureus* isolates with staphylococcal food poisoning in South Korea. *J. Appl. Microbiol.* 101:864-871.

Chung M, de Lencastre H, Matthews P, Tomasz A, Adamsson I, Aries de Sousa M, Camou T, Cocuzza C, Corso A, Couto I, Dominguez A, Gniadkowski M, Goering R, Gomes A, Kikuchi K, Marchese A, Mato R, Melter O, Oliveira D, Palacio R, Sa-Leao R, Santos Sanches I, Song J, Tassios PT, Villari P. 2000. Molecular typing of methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis: comparison of results obtained in a multilaboratory effort using identical protocols and MRSA strains. *Microb. Drug Resist*. 6:189-198.

CLSI. 2005. Performance standards for antimicrobial susceptibility testing. CLSI approved standard M100-S15. Wayne, PA: CLSI. 2005.

Coleman DC, Sullivan DJ, Russell R, Arbuthnott JP, Carey BF, Pomeroy HF. 1989. *Staphylococcus aureus* bacteriophages mediating the simulaneous lysogenic conversion of beta-lysin, staphylokinase and enterotoxin A: molecular mechanism of triple conversion. *J. Gen. Microbiol*. 135:1679-1697.

Coles EH, Eisenstark A. 1959a. Staphylococcic phages II. The use of human typing phages and adapted human typing phages in the typing of *Staphylococcus aureus* of animal origin. *Am. J. Vet. Res*. 20:835-837.

Coles EH, Eisenstark A. 1959b. Staphylococcic phages III. Typing of *Staphylococcus aureus* cultures isolated from the bovine udder. *Am. J. Vet. Res*. 20:838-840.

Cullen GA, Herbert CN. 1967. Some ecological observations on micro-organisms in habitating bovine skin, teat canals and milk. *Br. Vet. J.* 123:14-25.

Daley MJ, Oldham ER, Williams TJ, Coyle PA. 1991. Quantitative and qualitative properties of host polymorphonuclear cells during experimentally induced *Staphylococcus aureus* in cows. *Am. J. Vet. Res.* 52:474-479.

Davidson I, Slavin G. 1958. The disinfection of milking units in the control of mastitis due to *Streptococcus agalacticae*. *Vet. Rec*. 70:893-898.
Davidson I. 1961a. A set of bacteriophages for typing bovine staphylococci. *Res. Vet. Sci*. 2:396- 407.

Davidson I. 1961a. A set of bacteriophages for typing bovine staphylococci. *Res. Vet. Sci*. 2:

396- 407.

Davidson I. 1961b. Observations on the pathogenic staphylococci in a dairy herd during a period of six years. *Res. Vet. Sci*. 2:22-40.

Davidson I. 1963. Experiments on controlling staphylococcal mastitis. *Res. Vet. Sci*. 4:64-76.

Davidson I. 1966. Phage typing of bovine staphylococci. In: Subcommittee on Phage Typing. 1967.

Davidson I. 1972. A collaborative investigation of phages for typing bovine staphylococci. *Bull. Wld. Hlth. Org*. 46:81-98.

Degre M. 1967. Reproducibility of staphylococcal bacteriophage types by use of different concentrations of bacteriophage. *J. Appl. Microbiol.* 15:907-911.

De Buyser ML, Dilasser F, Hummel R, Bergdoll MS. 1987. Enterotoxin C and toxic shock syndrome toxin-1 production by staphylococci isolated from goat`s milk. *Int. J. Food Microbiol.* 5:301-309.

De Oliveira AP, Watts JL, Salmon SA, Aarestrup FM. 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. *J. Dairy Sci*. 83:855-862.

Devrise LA, Oeding P. 1976. Characteristics of *Staphylococcus aureus* strains isolated from different animal species. *Res. Vet. Sci*. 21:284-291.

Devrise LA. 1979. Identification of clumping factor-negative staphylococci isolated from cow's udders. *Res. Vet. Sci*. 27:313-320.

Dinsmore RP, English PB. Gonzalez PB, Sears PM, Schulte PM. 1991. Evaluation of methods for the diagnosis of *Streptococcus agalactiae* intramammary infections in dairy cattle. *J. Dairy Sci.* 74:1521-1526.

Dodd FH, Neave FK. 1970. Mastitis control. National Institute for Research in Dairying. *Biennial Reviews*. N. I. R. D. Paper No. 3559.

Dodd FH, Westgarth DR, Griffin TK. 1977. Strategy of mastitis control. JAVMA. 170:1124-1128.

Dodd FH .1980. Mastitis control. In: Mastitis control and herd management. Technical Bulletin 4, National Institute for Research in Dairying, Reading, England. Pp 11- 23.

Deutz A, Obritzhauser W. (2003). Eutergesundheit und Milchqualität. Leopold Stocker Verlag Graz, ISBN 3-7020-09876.

Edwards SJ, Rippon JE. 1957. The characters and distribution of certain staphylococci pathogenic for the bovine udder dairy cow characteristics related to *Staphylococcus aureus* isolation from quarter samples. *J. comp. Path.* 67:111-125.

Enevoldsen C, Gröhn YT, Thysen L. 1995. Dairy cow characteristics related to *Staphylococcus aureus* isolation from quarter samples. *J. Dairy Res.* 62:69-81.

Farah IO, Pedersen E, Halgaard C, Bruhn K. 1988. Comparative characterization and biotyping of *Staphylococcus aureus* isolates from human and bovine sources. *Acta Vet. Scand.* 29:303-310.

Fitzgerald JR, Hartigan PJ, Meaney WJ, Smyth CY. 2000. Molecular population and virulence factor analysis of *Staphylococcus aureus* from bovine intramammary infections. *J. Appl. Microbiol*. 8:1028-1037.

Fitzgerald JR, Hartigan PJ, Meaney WJ, Smyth CJ. 2001. Characterization of a putative pathogenicity Island from bovine *Staphylococcus aureus* encoding multiple superantigens. *J. Bact*. 183:63-70.

Forster I, Grand M, Glättli H. 1987. Mikrobiologische Qualität der Rohmilch in verschiedenen Regionen der Schweiz. FAM Info Februar 1988/164 p/W. Sonderdruck aus *Milch. Forschung* 16:79-83.

Fox LK, Gershman M, Hancock DD, Hutton CD. 1991. Fomites and reservoirs of *Staphylococcus aureus* causing intramammary infections as determined by phage typing: The effect of milking time hygiene practices. *Cornell Vet*. 81:183-193.

Fox LK, Gaskins CT, Hancock DD, Newkirk D, Hutton CT. 1992. Comparison of media to isolate *Staphylococcus aureus* from teat skin and milking unit liners. *Cornell Vet.* 82:225-231.

Fox LK, Gay JM. 1993. Contagious mastitis in update on bovine mastitis. In: Anderson KL, (Ed.), Veterinary clinics of North America: Food Animal Practice 9:475-487.

Fox LK, Zadoks RN, Gaskins CT. 2005. Biofilm production by *Staphylococcus aureus* associated with intramammary infection. *Vet. Microbiol*. 107:295-299.

Frost AJ. 1967. Phage typing of *Staphylococcus aureus* from dairy cattle in Australia. J. Hyg. Camb. 65:311-319.

Frenay HM, Bunschoten AE, Schools LM, van Leeuwen WJ, Vandenbroucke-Grauls CM, Verhoef J, Mooi FR. 1996. Molecular typing of methicillin-resistant *Staphylococcus* aureus on the basis of protein A gene polymorphism. *Eur. J. Clin. Microbiol. Infect. Dis.* 15:60-64.

Frost AJ, Bradshaw E. 1980. The role of lysogeny in the modification of phage typing patterns of *Staphylococcus aureus* isolated from dairy cows. *J. Hyg. Camb*. 85:301-307.

Garcia ML, Moreno B, Bergdoll MS. 1980. Characterization of staphylococci isolated from mastitis cows in Spain. *Appl. and environ. Microbiol*. 39:548-553.

Gedek W. 1966a. Ein Bakteriophagensatz zur Typisierung boviner Staphylokokken. *Berl. Münch. tierärztl. Wschr.* 79:292-295.

Gedek W. 1966b. Zur Lysotopie in Milch vorkommender Staphylokokken. *Arch. Lebensm. Hyg.* 17:265-269.

Gedek W. 1967. Koagulase positive Staphylokokken aus Kuhmilch. I. Charakteristika. *Arch. Lebensm. Hyg.* 18:247-250.

Gedek W, Kopp U. 1967. Koagulase positive Staphylokokken aus Kuhmilch. II. Antibiotika resistente Stämme. *Arch. Lebensm. Hyg.* 18:21-254.

Gedek W. 1972. Die Staphylokokken-Mastitis bei Haustieren. VEB Gustav Fischer Verlag Jena.

Gedek W. 1978. Penicllinase- Bildung und minimale Hemstoffkonzentrationen von Betalaktam-Antibiotika bei euterpathogenen Staphylokokken. *Berl. Tierärtztl. Wschr.* 91:367-369.

Giesecke WH, Heever LW, du Toit IJ. 1972. Staphylococcal mastitis: Phage types and patterns of *Staphylococcus aureus*. *Onderstepoort J. Vet. Res.* 39:87-96.

Giesecke WH, Viljoen TB. 1974. The diagnosis of subclinical mastitis in lactating cows: A comparison of cytological methods and monovalent radial immunodiffusion test. *Onderstepoort J. Vet. Res*. 41:51-74.

Godden SM, Jansen JT, Leslie KE, Smart NL, Kelton DF. 2002. The effect of sampling time and sample handling on the detection of *Staphylococcus aureus* in milk from quarters with subclinical mastitis. *Can. Vet. J.* 43:38- 42.

Goerke C, Gressinger M, Endler K, Breitkopf C, Wardecki K, Stern M, Wolz C, Kahl BC. 2007. High phenotypic diversity in infecting but not in colonizing *Staphylococcus aureus* populations. *Environ. Microbiol.* 9:3134- 3142.

Gomez-Lucia E, Goyache J, Orden JA, Domenech A, Hernandez FJ, Quiteria JAR, Lopez B, Blanco JL, Suarez G. 1992. Growth of *Staphylococcus aureus* and synthesis of enterotoxin during ripening of experimental Manchego-type cheese. *J. Dairy Sci*. 75:19- 26.

Gonzalez RN, Jasper DE, Bushnell RB, Farver TB. 1986. Relationship between mastitis pathogen numbers in bulk milk and bovine udder infections in California dairy herds. *JAVMA*. 189:442-445.

Gonano M, Hein I, Zangerl P, Rammelmayr A, Wagner M. 2009. Phenotypic and molecular characterization of *Staphylococcus aureus* strains of veterinary, dairy and human origin. *Epidemiol. and Infect.* 137:688-699.

Griffin TK, Dodd FH, Neave FK, Westgarth DR, Kingwill RG. 1977. A method of diagnosing intramammary infections in dairy cows for large experiments. *J. Dairy. Res.* 44:25-45.

Grigorova M, Bajlosov D. 1972. Investigations on bovine staphylococci of varying geographical origin. *Zbl. Bakt. Hyg. I Abt. Orig. A.* 219:151-155.

Grindal RJ, Hillerton JE. 1991. Influence of milk flow rate on new intramammary infection in dairy cows. *J. Dairy. Res.* 58:263-268.

Gröhn YT, Erb HN, Mcculloch CE, Saloniemi HS. 1990. Epidemiology of mammary gland disorders in multiparous Finnish Ayrshire cows. *Prev. Vet. Med*. 8:241-252

Gröschel D. 1968. The present role of staphylococci of the phage complex 52, 52A, 80, 81 in hospital infections in an American hospital California dairy herds. *Zbl. Bakt. Hyg. I Abt. Orig. A*. 207:283-289.

Grün L. 1968. Zur Bestimmung der Standortvarianten der Staphylokokken humanr und boviner Herkunft. *Milchwissen*. 23:604-608.

Gudding R. 1980. Staphylococcal nuclease in udder secretions of cows with acute mastitis. *Acta Vet. Scand*. 21:79-95.

Gutser K. 2001. Kältetolerante und mesophile Bakterienflora hocherhitzter Milch. Diplomarbeit. TUM. Wissenschaftszentrum Weihenstephan. 2001

Hajek V, Marsalek E. 1971. The differentiation of pathogenic staphylococci and a suggestion for their taxonomic classification. *Zbl. Bakt. I Orig*. A. 217:176-182.

Hajek V, Marsalek E. 1973. The occurrence of enterotoxigenic *Staphylococcus aureus* strains in hosts of different animal species. *Zbl. Bakt. I Orig. A*. 276:487-492.

Hajek V. 1976. *Staphylococcus intermedius*, a new species isolated from animals. *Int. J. Syst. Bact*. 26:401-408.

Hajek V, Horak V. 1978. A comparison of the lytic activity of poultry, human and bovine phages with staphylococci of different origin. *Zbl. Bakt. I Abt. Orig A*. 242:446-455.

Harvey J, Gilmour A. 1985. Application of current methods for isolation and identification of staphylococci in raw bovine milk. *J. Appl. Bact*. 59:207-221.

Heeschen W, Tolle A, Zeidler H. 1968. Die selektive Züchtung und Anreicherung Koagulase-positiver Staphylokokken und euterpathogener Streptokokken in flüssigen Nährmedien. *Arch. Lebensmittelhyg*. 8:184-189.

Hein I, Jørgensen HJ, Loncarevic S, Wagner W. 2005. Quantification of *Staphylococcus aureus* in unpasteurised bovine and caprine milk by real-time PCR. *Res. in Microbiol*. 156:554-563.

Helm D. 1992. Untersuchungen zur Klassifizierung von Bakterien mittels ihrer FTIR Spektren unter besonderer Berücksichtigung des Genus Staphylococcus Rosenbach, 1884; Diss. FB Biologie; Freie Univeristät Berlin.

Hennekinne JA, Kerounanton A, Brisabois A, De Buyser ML. 2003. Discrimination of *Staphylococcus aureus* biotypes by pulsed-field gel electrophoresis of DNA macrorestriction fragments. *J Appl Microbiol.* 94 (2): 321-329.

Herron LL, Chakravarty R, Dwam C, Fitzgerald JR, Musser JM, Retzel E, Kapur V. 2002. Genome sequence survey identifies unique sequences and key virulence genes with unusual rates of amino acid substitution in bovine *Staphylcococcus aureus. Infect. and Immun.* 70:3978-3981.

Hess E, Meyer B. 1978. Ergebnisse einer neuzeitlichen Mastitisdiagnostik. Schweiz. *Arch. Tierheilk.* 120:163-170.

Hillerton JE, Shearn MFH, Teverson RM, Langridge S, Booth JM. 1993. Effect of premilking teat dipping on clinical mastitis on dairy farms in England. *J. Dairy Res.* 60:31-41.

Hillerton JE, Bramley J, Straker RT, Mckinnon CM. 1995. Patterns of intramammary infection and clinical mastitis over 5 year period in a closely monitored herd applying mastitis control measures. *J. Dairy Res.* 62:39-50.

Hillerton JE. 1999. Redefining mastitis based on somatic cell count. Bull. Int. Dairy Fed. 345:4-6.

Hoblet KH, Bailey JS, Prichard DE. 1988. Coagulase-positive staphylococcal mastitis in a herd with low somatic cell count. *J. Am. Vet. Med. Assoc*. 192:777-780.

Hogan JS, Smith KL, Hoblet KH, Schoenberger PS, Todhunter DA, Hueston WD, Prichard DE, Bowman GL, Heider LE, Brockett BL, Conrad HR. 1989. Field survey of clinical mastitis in low somatic cell count herds. *J. Dairy Sci*. 72:1547-1566.

Honkanen-Buzalski T, Sandolm M. 1981. Association of bovine secretory immunoglobulins with milk fat globule membrans. *Comp. Immun. Microbiol. Infect. Dis.* 4:329-342.

Holmberg O. 1975. Phage typing of *Staphylococcus aureus* strains isolated in Sweden from bovine milk. *Acta Vet. Scand.* 16:487-492.

Huber E. 1970. Zellzahlbestimmung und Zelldifferenzierung in der Milch aufeinanderfolgender Gemelksfraktionen. *Schweiz. landw. Forsch*. 9:121-151.

Hüfner J. 1988. Überwachung der Produktionshygiene bei der Herstellung von Weichkäse mit Hilfe der coliformen Keime - vor allem im Hinblick auf das Vorkommen von *Listeria*. Teil I. *Deutsche Molkerei-Zeitung.* Heft 1:14-22.

Hüfner J. 1988. Überwachung der Produktionshygiene bei der Herstellung von Weichkäse mit Hilfe der coliformen Keime - vor allem im Hinblick auf das Vorkommen von *Listeria.* Teil II. *Deutsche Molkerei-Zeitung.* Heft 2:48-57.

Hummel R, Devriese LA, Lehmann G. 1992. Characteristics of bovine *Staphylococus aureus* with special regard to clumping factor activity. *Zbl. Bakt. I Abt. Orig A*. 276:487-492.

Hughes DL. 1960. Some epidemiological patterns in bovine mastitis. *Vet. Rec.* 72:485-497.

Hueston WD, Heider LE. 1987. The use of high somatic cell count prevalence in epidemiologic investigations of mastitis control practices. *Prev. Vet. Med*. 4:447- 461.

Hutton CT, Fox LK, Hancock DD. 1990. Mastitis control practices: Differences between herds with high and low milk somatic cell counts. *J. Dairy Sci*. 72:1135-1143.

IDF-Bulletin. 2001. Mastitis Newsletter N°24. Bulletin of the international dairy federation. 367/2001.

IDF-Bulletin. 2006. *Staphylococcus aureus* intramammary infections. Bulletin of the international dairy federation. 408/2006.

Ikawaty R, Brouwer EC, Jansen MD, van Duijkeren E, Mevius D, Verhoef J, Fluit AC. 2009. Characterization of Dutch *Staphylococcus aureus* from bovine mastitis using a Multiple Locus Variable Number Tandem Repeat Analysis. Vet. Microbiol. 136:277-284.

ISO/FDIS 6888 1/1. 1999. DAM 1. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) Part 1: Technique using rabbit plasma fibrinogen agar medium. Bulletin of the international dairy federation. ISO/TC 34/SC 9.

ISO/FDIS 6888 1/2. 1999. DAM 1. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) Part 2: Technique using Baird- Parker agar medium. Bulletin of the international dairy federation. ISO/TC 34/SC 9.

ISO/FDIS 6888-3. 2001. Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) Part 3: Detection and MPN technique for low numbers. Bulletin of the international dairy federation.

Jevons MJ, Parker MT. 1966. The evolution of new hospital strains of *Staphylococcus aureus*. *J. Clin. Path*. 17:243-250.

Jevons MJ, John M, Parker MT. 1966. Cultural characters of a newly recognized group of hospital staphylococci. *J. Clin. Path*. 19:305-312.

Jørgensen HJ, Mørk T, Caugant DA, Kearns A, Rørvik LM. 2005. Genetic variation among *Staphylococcus aureus* from Norwegian bulk milk. *Appl. and Environ. Microbiol*. 71:8352-8361.

Jørgensen HJ. 2005. *Staphylococcus aureus* from Norwegian bulk milk: Prevalence, molecular epidemiology and enterotoxigenic potential. Thesis Norwegian school of veterinary science.

Kaiser R, Gottschalk G. 1972. Elementare Tests zur Beurteilung von Messdaten. Bibliographisches Institut AG, Mannheim.

Kapur V, Sischo WM, Greer RS, Whittam TS, Musser JM. 1995. Molecular population genetic analysis of *Staphylococcus aureus* recovered of cows. *J. Clin. Microbiol.* 33:376- 380.

Kawano J, Shimizu A, Kimura S. 1986. Coagulase typing of *Staphylococus aureus* isoalted from animals. *Zbl. Bakt, Hyg. I Orig. A*. 261:407-410.

Kerouanton A, Hennekinne JA, Letertre C, Petit L, Chesneau O, Brisabois A, De Buyser ML. 2007. Characterization of *Staphylococcus aureus* stains associated with food poisoning outbreaks in France. *Int. J. Food Microbiol.* 115:359-375.

Klima H. 1972. Comparative studies on staphylococcal strains from milk samples with increased cell content. *Milchwissen*. 27:87- 89.

Knight CH, Hirst D, Dewhurst RJ. 1994. Milk accumulation and distribution in the bovine udder during the interval between milkings. *J. Dairy Res*. 61:167-177.

Koreen L, Ramasawamy SV, Graviss EA, Naidich S, Musser JM, Kreiswirth BN. 2004. *Spa* typing method for discriminating among *Staphylococus aureus* isolates: Implications for use of a single marker to detect genetic micro-and macrovariation. *J. Clin. Microbiol*. 42:792-799.

Krabisch P, Gangl A, Wittkowski G, Fehlings J. 1999. Prävalenz der Antibiotika-Resistenz in Milchvieherden bei Infektionserregern mit human-medizinischer Bedeutung. *Chemother. J*. 6 (8):210-218.

Kümmerle M. 1998. Identifikation von Hefen durch Fourier- Transformation Infrarotspektroskopie. Diss., Institut für Mikrobiologie am FML Weihenstephan. TU-München.

Kumagai R, Nakatani K, Ikeya N, Kit Y, Kaidoh T, Takeuchi S. 2007. Quadruple or quintruple conversion of *hlb*, *sak*, *sea* (or *sep*), *scn*, and *hcp* genes by bacteriophages in non-β--producing bovine isoaltes of *Staphylococus aureus*. *Vet. Microbiol.* 122:190-195.

Kurosihi T, Komine K, Kai K, Itagaki M, Kobayashi J, Ohta M, Kamata S, Kumagai K. 2003. Concentrations and specific antibodies of staphylococcal enterotoxin C and toxic shock syndrome toxin-1 in bovine mammary gland secretions, and inflammatory response to the intramammary inoculation of these toxins. *J. Vet. Med. Sci.* 65:899-906.

Kurzhals P, Klima H, Manz D. 1985. Relations between cell count, cell pattern and bacteriological findings in bovine subclinical mastitis. *Milchwissen*. 40:6-9.

Kurzweil R, Busse M. 1973. Keimgehalt und Florazusammensetzung der frisch ermolkenen Milch. *Milchwissen*. 28:427-431.

Lange C, Cardoso M, Senczek D, Schwarz S. 1999. Molecular subtyping of *Staphylococcus aureus* isolates from cases of bovine mastitis in Brazil. *Vet. Microbiol*. 67:127-141.

Larsen HD, Sloth KH, Elsberg C, Enevoldsen C, Pedersen LH, Eriksen NHR, Aerestrup FM, Jensen NE. 2000. The dynamics of *Staphylococcus aureus* intramammary infection in nine Danish dairy herds. *Vet. Microbiol*. 71: 89-101.

Leitner G, Yadlin B, Glickman M, Chaffer M, Saran A. 2000. Systemic and local immune response of cows to intramammary infection with *Staphylococcus aureus*. *Res. Vet. Sci.* 69:181-184.

Loeffler DA, Norcross NL. 1987. Use of enzyme- linked immunosorbent assay to measure bovine milk and serum antibodies to alpha toxin, beta toxin and capsular antigens of *Staphylococcus aureus*. *Vet. Immun. and Immunopath.* 141:145-156.

Lombai G, Janosi L, Katona F, Mayor P, Milch H, Ormay L, Takacs J. 1976. Eigenschaften und lebensmittelhygienische Bedeutung von aus Eutern isolierten *Staphylococcus aureus*-Stämmen. *Arch. Lebensm. Hyg.* 31:206-209.

Loncarevic S, Jørgensen HJ, Lovseth A, Mathisen T, Rorvik LM. 2005. Diversity of *Staphylococcus aureus* enterotoxin types within single samples of raw milk and raw milk products. *J. Appl. Microbiol.* 98:344-350.

Lorbacher H, Blobel H. 1968. Isolierung von Phagen für die Lysotropie „boviner Staphylokokken“. *Zbl. Vet. Med. B* 15:555-563.

Lukasova J, Vavrova H. 1974. Lipolytic activity of staphylococci, isolated from milk products. *Zbl. Bakt. Abt. II.* 129:278-285.

Mackie DP, Pollock DP, Rodgers SP, Logan EF. 1987. Phage typing of *Staphylococcus aureus* associated with subclinical bovine mastitis. *J. Dairy Res.* 54:1-5.

Mairques E, Ubeda C, Tormo MA, Ferrer MD, Lasa I, Novick RP, Penades JR. 2007. Role of Staphylococcal phage and SAPI integrase in intra- and interspecies SAPI transfer. *Journal of Bacteriology.* 15, 5608-5616.

Mattila T, Pyörälä S, Sandholm M. 1985. Comparison of milk antitrypsin, albumin, N-acetyl-b-D-glucosaminidase, somatic cells and bacteriological analysis as indicators of bovine subclinical mastitis. *Vet. Res. Commun.* 10:113-124.

Matsunaga T, Kamata S, Kakiichi N, Uchida K. 1993. Characteristics of *Staphylococcus aureus* isolated from peracute, acute and chronic bovine mastitis. *J. Vet. Med. Sci.* 55:297-300.

Mavrogianni VS, Cripps PJ, Tzora A, Skoufos I, Fthenakis GC. 2006. Effects of hand milking on the bacterial flora of mammary gland and teat duct of ewes. *J. Dairy Res.* 73:353-356.

Mayer S. 1975. Eigenschaften von aus Kuhmilch isolierten Staphylokokken im Hinblick auf die Beurteilung von Milch. *Milchwissen*. 30:607-608.

McLauchlin J, Audurier A, Frommelt A, Gerner-Smidt P, Jacquet C, Loessner MJ, van der Mee-Marquet, Rocourt J, Shah S, Wilhelms D. 1996. WHO study on subtyping Listeria monocytogenes: Results of phage-typing. *Int. J. Food Microbiol.* 32:289-299.

McLauchlin J, Narayanan GL, Mithani V, O`Neill G. 2000. The detection of enterotoxins and toxic shock genes in *Staphylococcus aureus* by polymerase chain reaction. *J. Food Protection.* 63:479- 88.

Mehrotra M, Wang G, Johnson WM. 2000. Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1 and methilicin resistance. *J. Clin. Microb.* 38:1032-1035.

Meyer W. 1966a. Schema zur Auslese potentiell pyogener Staphylococcus aureus-Stämme. *Med. Microbiol. and Immun*. 152:332-341.

Meyer W. 1966b. Differenzierungsschema für Standortvarianten von *Staphylococcus aureus*. *Zbl. Bakt. I Orig.A*. 201:465-481.

Meyer W. 1967. *Staphylococcus aureus* strains of phage group IV. *J. Hyg. Camb*. 65:439-447.

Meyer W, Rische H. 1984. Staphylokokken und Staphylokokken-Erkrankungen. VEB *Gustav Fischer Verlag Jena*.

Miltenburg JD, De Lange D, Crauwels APP, Bongers JH, Tielen MJM, Schukken YH, Elbers ARW. 1996. Incidence of clinical mastitis in a random sample of dairy herds in the southern Netherlands. *Vet. Rec.* 31:204-207.

Mijnen E, Jaartsveld FHJ, Verstegen MWA, Tielen MJM. 1982. The value of cell count, lactose content, pH and conductivity of milk for mastitis detection in individual cows. *Neth. Milk Dairy J*. 36:65-77.

Milchprüfring Bayern E.V. 2001. Tätigkeitsbericht.

Mochmann H, Richter U, Karsch W, Witte W, Meyer W. 1976. Untersuchungen über die Enterotoxin-Bildung von *Staphylococcus aureus*-Stämmen unterschiedlicher Herkunft. *Zbl. Bakt. I Orig. A*. 234:434- 449.

Monday SR, Bohach GA. 1999. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. *J. Clin. Microbiol.* 37:3411-3414.

Monday SR, Bohach GA. 2007. Use of multiplex PCR to detect classical and newly described pyrogenic toxin genes in staphylococcal isolates. *J. Clin. Microbiol.* 37:3411-3414.

Morandi S, Brasca M, Lodi R, Cremonsi P, Castiglioni B. 2007. Detection of classical enterotoxins and identification of enterotoxin genes in *Staphylococcus aureus* from milk and dairy products. *Vet. Microbiol.* 124:66-72.

Morse D, DeLorenzo MA, Wilcox CL, Collier RL, Natzke RP, Bray DR. 1988. Climatic effects on occurrence of clinical mastitis. *J. Dairy Sci.* 71:848-853.

Munch-Petersen E. 1963. Staphylococci in food and food intoxication a review and an appraisal of phage typing results. *J. Food Sci.* 28:692-710.

Munch-Petersen E, Gardiner MR. 1965. Staphylococci in secretions from the bovine udder in Western Australia. *Aust. Vet. J.* 41:5-13.

Munch-Petersen E. 1970. A comparison of four culture media for the demonstration of staphylococci in milk. *Milchwissen.* 25:36-39.

Munch-Petersen E. 1972. Spread of udder infections in dairy herds. *Milchwissen.* 27:89-98

Murphy JM. 1943. The use of strict foremilk in the study and diagnosis of chronic bovine mastitis. *Cornell Vet*. 33:52-56.

Mylls B, Honkanen T, Huovinen P, Sandholm M, Nurmi E. 1994. Association of changes in the bacterial ecology of bovine mastitis with changes in the use of milking machine and antibacterial drugs. *Acta Vet. Scand*. 35:363-369.

Nakagawa M. 1958. Studies on staphylococci from the bovine udder. I. Biological characteristics of staphylococci and some observations on the pathogenic strains. *Jap. J. Vet. Res.* 6:19-33.

Nakagawa M. 1960. Studies on bacteriophage typing of staphylococci isolated from bovine milk. Typing by means of a new phage set. *Jap. J. Vet. Res.* 8:331-342.

Naumann D, Helm D, Labischinski H. 1991. Microbiological characterizations by FTIR spectroscopy. *Nature* 351:81-82.

Newbould FHS, Neave FK. 1965a. The recovery of small numbers of *Staphylococcus aureus* infused into the bovine teat cistern. *J. Dairy Res.* 32:157-161.

Newbould FHS, Neave FK. 1965b. The effect of inoculating the bovine teat duct with small numbers of *Staphylococcus aureus. J. Dairy Res.* 32:163-170.

Nickerson SC. 1989. Immunological aspects of mammary involution. *J. Dairy Sci.* 72:1665-1678.

Niskanen A, Koiranen S. 1977. Correlation of enterotoxin and thermonuclease production with some physiological and biochemical properties of staphylococcal strains isolated from different sources. *J. Food Protection* 40:543-548.

Niskanen A, Koiranen L, Roine K. 1978. Staphylococcal enterotoxin and thermonuclease production during induced bovine mastitis and clinical reaction of enterotoxin in udders. *Infect. and Immun*. 19:493-498.

Nooitgedagt AJ, Hartog BJ. 1988. A survey of microbiological quality of Brie and Camembert cheese. *Neth. Milk Dairy. J*. 42:57-72.

Novick RP. 2003. Mobile genetic elements and bacterial toxinoses: The superantigen-encoding pathogenicity islands of *Staphylococcus aureus*. *Plasmid*. 49:93-105.

Nyhan JF, Archer GT. 1967. Phage types of *Staphylococcus aureus* in herd bulk milk and quarter samples. *Veterinary Record.* 61:202-205.

Oberreuter H, Brodbeck A, von Stetten S, Goerges S, Scherer S. 2003. Fourier-transform infrared (FT-IR) spectroscopy is a tool for monitoring the population dynamics of microorganisms in food stuff. *Eur. Food Res. Technol.* 216:434-439.

Østeras O, Sandvik L, Aursjo GG, Gjul GG, Jorstad A. 1991. Assessment of strategy in selective dry cow therapy for mastitis control. *J. Vet. Med.* 38:513-522.

Østeras O, Edge VL, Marin SW. 1999. Determinants of success or failure in the elimination of major mastitis pathogens in selective dry cow therapy. *J. Dairy Sci.* 82:1221-1231.

Olson JC, Casman EP, Baer EF, Stone JE. 1970. Enterotoxigenicity of *Staphylococcus aureus* cultures isolated from acute cases of bovine mastitis. *J. Applied Microbiology* 20:605-607.

Oxoid. Handbuch. 2003. 6. aktualisierte deutsche Auflage.

Paape MJ, Pearson RE, Schultze WD. 1978. Variation among cows in the ability to support phagocytosis and in the abilitiy of polymorphonuclear leucocytes to phagocytose *Staphylococcus aureus*. *Am. J. Vet. Res*. 39:1907-1911.

Parker MT, Lapage SP. 1957. Penicillinase production by *Staphylococcus aureus* strains from outbreaks of food poisoning. *J. Clin. Path.* 10:313-317.

Parker MT. 1983. The significance of phage typing in *Staphylococcus aureus*. In: Eastmore CFS, Allam C. (Eds.). Staphylococci and staphylococcal infections. Academic Press London. 58:33-62.

Pearson JKL, Mackie DP. 1979. Factors associated with the occurrence, cause and outcome of clinical mastitis in dairy cattle. *Vet. Rec.* 105:456-463.

Parisi JT, Baldwin JN. 1963. The incidence and persistence of certain strains of *Staphylococcus aureus* in dairy herds. *Am. J. Vet. Res.* 24:551-556.

Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ. 2007. Novel alternatives to antibiotics: Bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *J. Appl. Microbiol*. 104:1-13.

Peeler EJ, Green MJ, Fitzpatrick JL, Green LE. 2002. Study of clinical mastitis in British dairy herds with bulk milk somatic cell counts less than 150,000 cells/ml. *Vet. Rec.* 10:170-176.

Peles F, Wagner M, Varga L, Hein I, Rieck P, Gutser K, Kereszturi P, Kardos G, Turcsanyi I, Beri B, Szabo A. 2007. Characterization of *Staphylococcus aureus* isolated from bovine milk in Hungary. *Int. J. Food Microbiol.* 118:186-193.

Pol M, Ruegg PL. 2007. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. *J. Dairy Sci.* 90:262-273.

Poutrel B, Sarradin P. 1992. Diagnosis of *Staphylococcus aureus* intramammary infections by detecting in milk with an ELISA kit. Lait. 72:321-325.

Pulverer G. 1966. Vergleich pathogener Staphylokokken von Mensch und Tier. *Zbl. Bakt. I Orig. A*. 201:27- 41.

Rainard P, Poutrel B. 1982. Dynamics of nonclinical bovine intramammary infections with major and minor pathogens. *Am. J. Vet. Res*. 43:1296-1299.

Rataja-Schulz PJ, Smith KL, Wolter M, Hogan JS, Love BC. 2004. Antimicrobial susceptibility of mastitis pathogens from first lactation and older cows. *Vet. Microbiol.* 102 33-42.

Rebuffo-Scheer CA, Schmitt J, Scherer S. 2007. Differentiation of *Listeria monocytogenes* Serovars by Using Artificial Neural Network Analysis of Fourier- Transformed Infrared Spectra. *Appl. and Environ. Microbiol*. 73 (3): 1036-1040.

Reichmuth J. 1975. Somatic cell counting – Interpretation of results. *Proc. Int. Dairy Fed. Ann. Bull*. 85(2):26-30.

Reid WB, Wilson JB. 1959. A Study of the staphylococci associated with the bovine udder. *Am. J. Vet. Res*. 10:825-831.

Reneau JK. 1986. Effective use of Dairy Herd Improvement somatic cell counts in mastitis. *J. Dairy Sci. 69*:1708-1713.

Richardson JF, Rosdahl VT, van Leuwen WJ, Vickery AM, Vindel A, Witte W. 1999. Phages for methicillin-resistant *Staphylococcus aureus*: An international trial. *Epidemiol. Infect*. 122:227-233.

Roberson JR, Fox LK, Hancock DD, Gay CC, Besser TE. 1994a. Coagulase-positive *Staphylococcus aureus* intramammary infections in primiparous dairy cows. *J. Dairy Sci. 77*:958-969.

Roberson JR, Fox LK, Hancock DD, Gay JM. 1994b. Ecology of *Staphylococcus aureus* isolated from various sites on dairy farms. *J. Dairy Sci*. 77:3354-3364.

Rupp R, Beaudeau F, Boichard D. 2000. Relationship between somatic-cell counts in the first lactation and clinical mastitis occurrence in the second lactation of French Holstein cows. *Prev. Vet. Med.* 46:99-111.

Ruys AC, Borst J. 1959. A new method of the reporting of phage patterns of *Staphylococcus aureus*. *J. Microbiol. Serol.* 25:247-240.

Sabolic M, Wonka W, Gerlach R. 1980. Zum Vorkommen penicillinasebildender Staphylokokken und Mikrokokken im Euter bei Milchvieherden. *Tierärztl. Umschau* 10:671-674.

Sarikaya H, Werner-Misof C, Atzkern M, Bruckmaier RM. 2005. Distribution of leucocyte populations, and milk composition, in milk fractions of healthy quarters in dairy cows. *J. Dairy Res.* 72:486-492.

Sato K, Bartlett PC, Erskine RJ, Kaneene JB. 2005. A comparison of production and management between Wisconsin organic and conventional dairy herds. *Livestock Prod. Sci.* 93:105-115.

Schalm OW, Gladys M, Woods MA. 1952. Effect of massive doses of Penicillin and Dihydrostreptomycin, employed singly or in combination on *Staphylococcus pyogenes* mammmary infections. *Am. J. Vet. Res.* 13:26-30.

Schalm OW, Gladys M, Woods MA. 1953. *Micrococcus pyogenes* in bovine milk II. Relationship of shedding characteristics to occurrence of clinical mastitis. *Am. J. Vet. Res.* 14:534-538.

Schalm OW, Lasmanis J, Caroll EJ. 1964. Pathogenesis of experimental coliform *(Aerobacter aerogenes)* mastitis in cattle. *Am. J. Vet. Res.* 25:83-89.

Schlichting C, Branger C, Fournier JM, Witte W, Boutonnier A, Wolz C. 1993. Typing of *Staphylococcus aureus* by pulse-field gel electrophoresis, zymotyping, capsular typing and phage typing: Resolution of clonal relationships. *J. Clin. Microbiol*. 31:227-232.

Schmidt J. 1962. Untersuchungen über Staphylokokken-Doppelinfektionen. *Zbl. Bakt. I Orig. A*. 41:41-46.

Schwarz, S, Roberts MC, Werckenthin C, Pang Y, Lange C. 1998. Tetracycline resistance in *Staphylococcus spp*. from domestic animals. *Vet. Microbiol*. 31:227-232.

Sears PM, Smith BS, English PS, Herer PS, Gonzalez RN. 1990. Shedding pattern of *Staphylococcus aureus* from bovine intramammary infections. *J. Dairy Sci.* 73:2785-2789.

Sears PM, Wilson DJ, Gonzalez RN, Hancock DD. 1991. Microbiological results from milk samples obtained premilking and postmilking for the diagnosis of bovine intramammary infections. *J. Dairy Sci.* 74:4183-4188.

Seelemann M. 1941. Die wichtigsten Seuchen der Milchkühe. Milchwirtschafliche Lehrbuchreihe. Verlag der Molkerei-Zeitung, Hildesheim.

Seelemann M, Obiger G, Rackow HG, Welz W. 1963. Staphylokokkenbefunde und ihre Beurteilung bei der Überwachung der Vorzugsmilch. *Kieler Milchwirtschaftliche Forschungsberichte* 15:89-102.

Seelemann M. 1964. Über den Zellgehalt der Milch, seine Feststellung, Bedeutung und Beurteilung. *Milchwissen*. 19:182-194.

Seto JT, Wilson JB. 1958. Bacteriophage typing of micrcococci of bovine origin. *Am. J. Vet. Res.* 19:241-246.

Sheldrake RF, Hoare RJT, McGregor GC. 1983. Lactation stage, parity, and infection affecting somatic cells, electrical conductivity, and serum albumin in milk. *J. Dairy Sci*. 66:542-547.

Shimizu A. 1977. Establishment of a new bacteriophage set for typing Avian staphylococci. *Am. J. Vet. Res*. 38:1601-1605.

Skalka B, Smola J, Pillich J. 1979. A simple method of detecting staphylococcal . *Zentralblatt für Bakteriologie I Orig.A.* 245:283-286.

Slanetz LW, Jawetz E. 1941. Isolation and characteristics of bacteriophages from staphylococci of bovine mastitis. *J. Bact.* 41:447-455.

Smith BA, Baird-Parker AC. 1964. The use of sulphamezathine for inhibiting Proteus spp. on Baird-Parker`s medium for *Staphylococcus aureus*. *J. appl. Bact*. 27:78-82.

Smith JW, Schulze WD. 1967. Variation in cell content of milk associated with time of sample collection. I. Diurnal variation. *J. Dairy Sci*. 50:1083-1087.

Smith HW. 1948a. Investigations on the typing of staphylococci by means of bacteriophage. I. The origin and nature of lysogenic strains. *J. Hyg. Camb*. 46:74-81.

Smith HW. 1948b. The typing of staphylococci of animal origin by the bacteriophage method. *J. comp. Path.* 58:179-188.

Smith KL, Todhunter DA, Schoeneberger PS. 1985. Environmental mastitis: Cause, prevalence, prevention. *J. Dairy Sci.* 68:1531-1553.

Smith EM, Green LE, Medley GF, Bird HE , Fox LK, Schukken YH, Kruze JV, Bradley AJ, Zadoks RN , Dowson CG. 2005. Multilocus Sequence Typing of Intercontinental Bovine *Staphylococcus aureus* Isolates. *J. Clin. Microbiol*. 43:4737-4743.

Sobiraj A, Kron A, Schollmeyer U, Failing K. 1997. Bundesweite Untersuchung zur Errregerverteilung und In-vitro-Resistenz euterpathogener Bakterien in der Milch von Kühen mit subklinischer Mastitis. *Tierärztl. Praxis* 25:108-115.

Sommerhäuser J, Kloppert B, Wolter W, Zschöck M, Sobiraj A, Failing, K. 2003. The epidemiology of *Staphylococcus aureus* infections from subclinical mastitis in dairy cows during a control programme. *Vet. Microbiol.* 96:91-102.

Sordillo LM, Doymaz MZ, Oliver SP. 1989. Morphological study of chronic *Staphylococcus aureus* mastitis in the lactating bovine mammary gland. *Res. Vet. Sci*. 47:247-252.

Sperber WH, Tatini SR. 1975. Interpretation of the tube coagulase test for identifcation of *Staphylococcus aureus*. *J. Appl. Microb.* 29:502-505.

Stamatin N, Tacu A, Marcia D. 1949. L`activite haemolytique et coagulante des Staphylococcus isoles a partir des infections humaines ou animales. *Annals Inst. Pasteur, Paris* 76:178-180.

Stadhouders J, Hassing F, van Aalst-van Maren NO. 1976. A pour-plate method for the detection and enumeration of coagulase positive *Staphylococcus aureus* in the Baird-Parker medium without egg yolk. *Neth. milk dairy J.* 30:222-229.

Steck W. 1920. Beiträge zur Kenntnis der Bakterienansiedlung in normalen Kuheutern. Schweiz. *Arch. Tierheilk*. 62:525-534.

Steinwidder A, Wurm K. 2005. Milchviehfütterung Tier und Leistung gerecht. Leopold Stocker Verlag Graz, ISBN 3-7020-1101-3.

Stephan R, Annemüller C, Hassan AA, Lämmler C. 2001. Characterization of enterotoxigenic *Staphylococcus aureus* strains isolated from bovine mastitis in north east Switzerland. *Vet. Microbiol.* 78:373-382.

Stephan R, Buehler K, Lutz C. 2002. Prevalence of genes encoding enterotoxins, exfoliative toxins and toxic shock syndrome toxin 1 in *Staphylococcus aureus* strains isolated from bulk tank milk samples in Switzerland. *Milchwissen.* 57:502-504.

Studer E, Schaeren W, Naskova J, Pfaeffli H, Kaufmann T, Kirchhofer M, Steiner A, Grabner HU. 2008. A longitudinal field study to evaluate the diagnostic properties of a quantitative real-time polymerase chain reaction-based assay to detect *Staphylococcus aureus* mastitis in milk. *J. Dairy Sci.* 91:1893-1902.

Subcommittee (Int. Committee) on Phage Typing. 1959. Report on Phage Typing of Staphylococci. *Int. Bull. Bact. Nomencl. Taxon*. 9: 115-118.

Subcommittee on Phage Typing. 1967. Report on Phage Typing of Staphylococci (Moscow 1966). *Int. J. syst. Bact.* 17: 113-125.

Subcommittee on Phage Typing. 1971. Report on Phage Typing of Staphylococci (1966-1970). *Int. J. syst. Bact.* 21: 167-170.

Subcommittee on Phage Typing. 1975. Report on Phage Typing of Staphylococci (1970-1974). *Int. J. syst. Bact.* 25: 241-242.

Suriyasathaporn W, Heuer C, Noordhuizen-Stassen EN, Schukken,YH. 2000. Hyperketonemia and the impairment of udder defense: A rewiew. *Vet. Res.* 31:397-412.

Swartz R, Jooste PJ, Novello JC. 1985. Bacteriophage typing of *Staphylococcus aureus* strains isolated from Bloemfontein dairy herds. *J. S. Afr. Vet. Assoc*. 56:69-73.

Takahashi I, Johns CK. 1959. *Staphylococcus aureus* in cheddar cheese. *J. Dairy Sci.* 42 1032-1039.

Tatini SR, Jezeski JJ, Olson JJ, Cashman JC, Casman EP. 1971a. Factors influencing the production of enterotoxin A in milk. *J. Dairy Sci.* 54:312-320.

Tatini SR, Jezeski JJ, Morris HA, Olson JJ, Casman EP. 1971b. Producing staphylococcal enterotoxin A in cheddar and colby cheeses. *J. Dairy Sci*. 54:815-825.

Tatini, SR, Jezeski JJ, Morris HA, Olson JJ, Casman EP. 1973. Production of staphylococcal enterotoxin A in Blue, Brick, Mozzarella and Swiss cheeses. *J. Dairy Sci*. 56:429-435.

Tenhagen BA, Köster G, Wallmann J, Heuwieser W. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J. Dairy Sci.* 89:2542-2551.

Tenover FC, Arbeit R, Archer G, Biddle J, Bryne S, Goering S, Hancock G, Herbert CA, Hill B, Hollis R, Jarvis WR, Kreiswirth B, Eisner W, Maslow J, McDougal LK, Miller JM, Mulligan M, Pfaller MA. 1994. Comparison of traditional and molecular methods of typing isolates of *Staphylococcus aureus*. *J. Clin. Microbiol*. 32:407-415.

Tenover FC, Arbeit R, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J. Clin. Microbiol.* 33:2233-2239.

Tolle A, Zeidler H, Heeschen W. 1966. Ein Verfahren zur elektronischen Zählung von Milchzellen. *Milchwissen*. 21:93-98.

Tolle A, Heeschen W, Hamann J. 1977. Grundlagen einer systematischen Bekämpfung der subklinischen Mastitis des Rindes. *Kieler Milchwirtschaftlicher Forschungsberichte* 29:3-75.

Untermann F, Kusch D, Lupke H. 1973. Zur Bedeutung der Mastitis- Staphylokokken als Ursache von Lebensmittelvergiftungen. *Milchwissen*. 28:686-688.

Van Belkum G, van Leeuwen W, Kaufmann ME, Cookson B, Foey F, Etienne J, Goering R, Tenover F, Steward C, O'Brien F, Grubb W, Tassios P, Legakis N, Morvan A, Névine El Solh N, de Ryck R, Struelens M, Salmenlinna S, Vuopio-Varkila J, Kooistra M, Talens A, Witte W, Verbrugh H. 1998. Assessment of Resolution and Intercenter Reproducibility of Results of Genotyping *Staphylococcus aureus* by Pulsed-Field Gel Electrophoresis of *Sma*I Macrorestriction Fragments: a Multicenter Study. *J. Clin. Microbiol.* 36:1653-1659.

Van Schaik G, Lotem M, Schukken YH. 2002. Trends in somatic cell counts, bacterial counts and antibiotic residue violations in New York state during 1999-2000. *J. Dairy Sci.* 85:782-789.

Van Wamel JR, Hartigan PJ, Meaney WJ, Smyth CJ. 2006. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on β--converting bacteriophages. *J. Bact.* 188:1310-1315.

Vautor E, Abadie G, Guibert JM, Huart C, Pepin M. 2003. Genotyping of *Staphylococcus aureus* isolated from various sites on farms with dairy sheep using pulsed-filed gel electrophoresis. *Vet. Microbiol*. 96:69-79.

Vesterholm-Nielsen M, Olholm Larsen M, Olsen JE, Aarestrup FM. 1999. Occurrence of the blaZ gene in penicillin resistant *Staphylococcus aureus* isolated from bovine mastitis in Denmark. *Acta Vet. Scand.* 40:279-286.

Vintov J, Aarestrup FM, Zinn CE, Olsen JE. 2003a. Phage types and antimicrobial resistance among Danish bovine *Staphylococcus aureus* isolates since 1950s. *Vet. Microbiol*. 97:63-72.

Vintov J, Aarestrup FM, Zinn CE, Olsen JE. 2003b. Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. *Vet. Microbiol*. 95:133-147.

Vries T. 1976. Primary infection of milk 2. Variation of bacterial and cell counts in milk during the course of milking. *Neth. milk and Dairy J*. 30:9-16.

Vorbach C, Capecchi MR, Penninger JM. 2006. Evolution of the mammary gland from the innate immune system. *BioEssays*. 28:606-616.

Wilson CD. 1961. The treatment of staphylococcal mastitis. *Vet. Res*. 73:1019-1024.

Wallmann, J, Schröter K, Wieler LH, Kroker R. 2003. National antibiotic resistance monitoring in veterinary pathogens from sick food-producing animals: the German programme and results from the 2001 pilot study. *Int. J. Antimicrob. Agents* 22:420–428.

Wallmark G, Thörne H. 1958. Bacteriophage-types and sensitivity to penicillin of staphylococci isolated from bovine mastitis. *Nord. Vet. Med.* 19:76-81

Wendt K, Lotthammer KH, Fehlings K, Spohr M. 1998. Handbuch Mastitis. Kamlage Verlag Osnabrück, ISBN 3-7020-09876.

Wenworth B. 1963. Bacteriophage typing of the staphylococci. *Bact. Rev*. 27:253-272.

Werckenthin C, Cardoso M, Martel JL, Schwarz S. 2001. Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcrine *Staphylococcus hyicus* and canine *Staphylococcus intermedius. Vet. Res.* 32:341-362.

Williams REO, Rippon JE. 1952. Bacteriophage typing of *Staphylococcus aureus*. *J. Hyg (London)*. 50: 320-353.

Williams REO, Jevons MP. 1961. Lyotypen von *Staphylococcus aureus*. verschiedener Herkunft. *Zbl. Bakt. I Orig. A*. 181: 349-358.

Wilson CD, Davidson J. 1961. The control of staphylococcal mastitis. *Vet. Rec.* 73:321-323.

Witte W. 2004. Personally advisory information. (Institute Robert Koch).

Witte W, Grigorova M, Bayloyosov D, Hummel R. 1988. Nachweis des Verklumpungsfaktors mit stabilisiertem Fibrinogen für die schnelle Diagnostik von *Staphylococcus aureus*. *Z. Klin. Med.* 43:1333-1335.

Zangerl P. 1999a. Nachweis, Vorkommen und Entwicklung von *Staphylococcus aureus* im Käse. Diss. Institut für Milchforschung und Bakteriologie der Universität für Bodenkultur, Wien.

Zangerl P. 1999b. Vergleich von Baird-Parker Agar und Kaninchenplasma-Fibrinogen Medium zum Nachweis von *Staphylococcus aureus* in Rohmilch und Rohmilchprodukten. *Arch. Lebensmit. Hyg*. 50:4-9.

Zadoks RN, Allore HG, Barkema HW, Sampimon OC, Wellenberg GJ, Gröhn YT, Schukken YH. 2001. Cow and quarter-level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis. *J. Dairy Sci.* 84:2649-2663.

Zadoks RN, van Lweeuwen WB, Kreft D, Fox LK, Barkema HW, Schukken YH, van Belkum A. 2002. Comparison of *Staphylococcus aureus* isolates from bovine and human skin, milking equipment, and bovine milk by phage typing, pulsed-field gel electrophoresis and binary typing. *J. Clin. Microbiol.*. 40:3894-3902.

Zadoks RN, Gonzalez RN, Boor KJ, Schukken YH. 2004. Mastitis-causing Streptococci are important contributors to bacterial counts in raw bulk tank milk. *J. Food Protection* 67:2644-2650.

Zecconi A, Piccinini R, Zepponi A, Ruffo G. 1997. Recovery of *Staphylococcus aureus* from centrifuged quarter milk samples. *J. Dairy Sci*. 80:3058-3063.

Zecconi A, Piccinini R, Fox K. 2003. Epidemiologic study of intramammary infections with *Staphylococcus aureus* during a control program in nine commercial dairy herds. *JAVMA* 50:684-688.
Zecconi A, Cesaris L, Liandris E, Dapra V, Piccinini R. 2006. Role of several *Staphylococcus aureus* virulence factors on the inflammatory response in bovine mammary gland. *Microbial Pathogenesis* 40:177-183.

Zehren VC, Zehren VD. 1968. Relation of acid development during cheesemaking to development of staphylococcal enterotoxin A. *J. Dairy Sci*. 51:645-549.

Zeidler H, Tolle A, Heeschen W. 1968. Zur Beurteilung zytologisch-bakteriologischer Untersuchungsbefunde im Rahmen der Mastitisdiagnostik. *Michwissen.* 23:674-677.

Zeidler H, Tolle A, Reichmuth J, Heeschen W. 1969. Zur Beurteilung zytologisch-bakteriologischer Untersuchungsbefunde im Rahmen der Mastitisdiagnostik. *Arch. Lebensmittel. Hyg.* 20:193-198.

Zierdt CH, Robertson EA, Williams RL, Maclowry JD. 1980. Computer analysis of *Staphylococcus aureus* typing data from 1957 to 1975, citing epidemiological trends and natural evolution within the phage typing system. *Appl. and environ. Microbiol.* 29:623-669.

Zschöck M, Kloppert B, Wolter W, Hamann HP, Lämmler C. 2005. Pattern of enterotoxin genes *seg*, *she, sei, sej,* positive *Staphylococcus aureus* from bovine mastitis. *Vet. Microbiol.* 108:243-249.

Zorah KT, Daniel CW, Frost AJ. 1993. Detection of bacterial antigens in milk samples from clinical cases of bovine mastitis in which culture is negative. *Vet. Rec.* 132:208-210.

8 Kurzfassung zur Dissertation

„Biodiversität und Kontrolle von *Staphylococcus aureus* in der Milchwirtschaft “

von Klaus Mathias Gutser

8.1 Einführung und Problemstellung

S. aureus gehört als Verursacher von subklinischen Mastitiden zur natürlichen Flora von Rohmilch. Ein gehäuftes Auftreten von *S. aureus* Mastitiden wurde mit der Einführung der Melkmaschine festgestellt. Nicht zuletzt wegen seiner hohen Persistenz über die Trockenstellperiode hinaus wurde *S. aureus* neben *Str. agalactiae* als einziger kontaktöser Mastitiserreger in der Milchproduktion eine immer größer werdende Bedeutung beigemessen. Die Zellzahl der Tankmilch führt wegen ihrer geringen Aussagekraft zu einer starken Unterschätzung der Prävalenz von *S. aureus* im Tierbestand, wodurch in den meisten anderen Betrieben keine Maßnahmen ergriffen werden und nur sporadische Behandlungen von Kühen mit hohen Zellzahlen von >400.000 /ml erfolgen. Vor allem seit Einführung des Grenzwerts von 10.000 *S. aureus*/g für Rohmilchprodukte, aufgrund vereinzelter Lebensmittelvergiftungen durch *S. aureus*, stand dieser Erreger im Mittelpunkt bei Rohmilch verarbeitenden Unternehmen. Erst der Versuch gezielter Sanierungsmaßnahmen in Milchviehherden einzelner Rohmilch verarbeitender Betriebe zeigte die Schwierigkeiten, *S. aureus* in Tierbeständen zu kontrollieren, vollständig auf. Die Möglichkeit der Kontamintaion von *S. aureus* während der Milchverarbeitung rückte dabei immer mehr in den Hintergrund. Da der Grenzwert von 10.000 *S. aureus*/g unter normalen Produktionsbedingungen dauerhaft nahezu nicht zu garantieren war, wurde dieser 2004 auf 100.000 *S. aureus*/g erhöht.

Bereits vor über 60 Jahren konnten phänotypische Unterschiede von *S. aureus*- Isolaten verschiedener Herkunft zwischen Tier und Mensch nachgewiesen werden, welche schließlich vor 40 Jahren ihre Fortsetzung in einer Klassifizierung des Erregers je nach Herkunft fand. In diesen Zeitraum fallen auch nahezu alle Spezialisierungen in der angewandten Grundlagenforschung, welche ihren Anfang zwischen 1930-1940 genommen haben. Bis heute sind sie in folgenden interdisziplinären Bereichen von ausschlaggebender Bedeutung:

- Entwicklung von Selektivnährmedien in der klassischen Mikrobiologie zur Isolierung von *S. aureus* in der Veterinär- und Humanmedizin
- Immunologie in der Milchdrüse mit der ersten wissenschaftlichen Definition verschiedener Mastitisformen und Entwicklung eines Verfahrens zur vollautomatischen Erfassung somatischer Zellen in Milch
- Verbesserung der Phagentypisierung vor Einführung der Genotypisierung als wichtigste Typisierungsmethode der Epidemiologie bis vor 20 Jahren

Die vorliegende Arbeit will ein umfassendes Bild der Biodiversistät von *S. aureus* und der Kontrolle dieses Erregers in der Milchwirtschaft vermitteln. Aufgrund der starken Abhängigkeit der Fragestellungen

- von der Probenahmetechnik,
- von international verwendeten Isolierungs- und Typisierungsmethoden,
- von der Dynamik der *S. aureus*- Populationen über längere Zeiträume,
- vom Antibiotikaeinsatz mit seinen Auswirkungen auf die *S. aureus*- Population einer Herde (wenn auch zunächst nur begrenzt auf wenige Typen)
- sowie von gesetzlichen Vorgaben wie der Begrenzung der somatischen Zellzahl,

mussten die Auswirkungen und Fehlerquellen dieser Einflussgrößen untersucht und diskutiert werden, bevor generelle Rückschlüsse für die Gesamtsituation gezogen werden können.

Die stete Weiterentwicklung der verschiedenen Methoden in den einzelnen Teilbereichen meist unabhängig voneinander und parallel dazu die sich stetig verändernden Anforderungen in der Milcherzeugung sind die Hauptursachen, warum viele methodische Probleme und entscheidende Veränderungen in der *S. aureus*- Problematik bisher unerkannt geblieben sind. An erster Stelle sind hierbei die Veränderungen der *S. aureus*- Population in den Kuhbeständen zu nennen. Das Hauptproblem für diese Fragestellung liegt in der mangelnden Kontinuität und Disziplin bezüglich der Verwendung epidemiologischer Methoden in der veterinärmedizinischen Forschung. Dies ist deshalb problematisch, weil die entscheidenden Veränderungen in der bovinen *S. aureus*- Population noch vor der Einführung der Genotypisierung auftraten. Ähnliche Probleme sind in der Isolierung von *S. aureus* für die Mastitisdiagnostik und Lebensmitteluntersuchung festzustellen. Zudem stellt sich die Frage, ob in der Mastitisdiagnostik, wo meist nur das Vorgemelk berücksichtigt wird, alle *S. aureus* - Typen und nicht nur vorzugsweise die Mastitis relevanten Erreger isoliert werden, die zu einer Erhöhung der Zellzahl der Kuh führen. Das Erfassen aller *S. aureus*- Ausscheider ist insbesondere bei der Verarbeitung von Rohmilch von Interesse, weil hier allen Kühen, die *S. aureus* in die Tankmilch abgeben, eine Bedeutung zukommt. Somit ist die Kontrolle des

Erregers im Tierbestand die zweite entscheidende Fragestellung, die in diesem Zusammenhang diskutiert werden muss.

Um die Diskussion auf eine stabile Grundlage stellen zu können, muss ein breites Spektrum der Literatur der vergangenen 50 Jahre in die Interpretation der Ergebnisse miteinbezogen werden. Die für das Verständnis wichtigsten Ergebnisse des Literaturstudiums für die vorliegende Arbeit wurden in einem getrennten Anhang „Tabellen und Abbildungen" zusammenfassend ausgewertet. Diese Ergebnisse dokumentieren die große Bandbreite des bearbeiteten Forschungsgebietes, ermöglichen eine Langzeitbetrachtung der bovinen *S. aureus*- Population im Tierbestand und lassen so auch eine Bewertung der derzeit aufgefundenen Situation zu. Nur eine Gegenüberstellung alter und neuer Ergebnisse erlaubt Rückschlüsse auf methodische Fehleinschätzungen bzw. Veränderungen der *S. aureus*-Population im Kuhbestand. Aufgrund der Vielzahl des Datenmaterials werden in dieser Arbeit nur die wichtigsten Ergebnisse ausführlicher diskutiert.

8.2 Methodik zur Bestimmung der Biodiversität von *S. aureus*

Zuerst wurde die Rolle der traditionellen elektiv Kriterien von *S. aureus* aus boviner Mastitismilch von international verwendeten Nährböden und die methodischen Probleme in der Epidemiologie in Zusammenhang mit Antibiotikaresistenz und Enterotoxinbildung überprüft. Dazu wurden 213 *S. aureus*- Stämme vom nationalen Monitoringprogramm (Sammlung A: BGVV, 2002), 72 Stämme aus einem EGD- Labor in Ostdeutschland (Sammlung B: Güstrow, 2001) und 68 multi- oder Penicillin resistente Stämme aus einem bayerischen Monitoringprogramm in Problembetrieben (Sammlung C: EGD Grub, 1999) untersucht.

Es konnte eine hohe Korrelation zwischen phänotypischen Eigenschaften, Antibiotikaresistenz und Enterotoxinbildung nachgewiesen werden. 10 bis 27 % der Stämme zeigten keine oder nur eine schwache Hämolyse und mehr als 50 % dieser Isolate waren Eigelb positiv und zu über 75% Penicillin resistent. *S. aureus* aus Sammlung B und C hatten einen signifikant höheren Prozentsatz an Stämmen mit keiner und schwacher Hämolyse (P= 0.057). Die Mehrheit boviner Stämme aus der Mastitisdiagnostik wird jedoch traditionell von Isolaten dominiert, wie sie beispielsweise in der Sammlung A für Stämme aus dem nationalen Monitoringprogramm vorgefunden wurden. 80% sind β- hämolytisch, 53% und weniger weisen eine Eigelbreaktion auf und nur 24 % sind Penicillin sensibel. Alle Clumpingfaktor negativen Stämme zeigten bis auf einzelne Isolate eine β- Hämolyse, waren ausnahmslos Penicillin sensibel und häufig Enterotoxin C und Toxic-shock Syndrombildner.

Die negative Koagulasereaktion einzelner *S. aureus* Isolate im Röhrchen wurde durch einen fehlenden Trübungshof auf Baird Parker mit Plasma Fibrinogen bestätigt. Die Clumpingfaktor negativen Stämme waren auf diesem Nährboden die Hauptursache einer sehr schwachen, verzögernden oder fehlenden Ausbildung eines Trübungshofs. Dieser Sachverhalt belegt ebenso wie die schlecht ausgeprägte Pigmentierung und Koloniemorphologie nach Reduktion von Tellurit die methodische Schwäche von Baird Parker mit und ohne Plasma Fibrinogen. Das Medium dieser Methode findet ausschließlich in der Untersuchung von Lebensmitteln und Rohmilch Verwendung.

Die meisten Stämme der vier wichtigsten Phagentypen 116, 96 ,78 und 119 konnten mit Hilfe verschiedener Kombinationen von Eigelbreaktion, Hämolyse, Clumpingfaktor bzw. Koagulaseaktivität und Penicillinresistenz unterschieden werden. Dies unterstreicht nicht nur die Notwendigkeit der Einbeziehung sämtlicher phänotypischer Merkmale für die Isolierung des Erregers. Entsprechend zeigt es auch die Schwächen der *S. aureus* Untersuchung im milchwirtschaflichen Bereich auf. Die Verwendung von Blutagar in der Mastitisdiagnostik unter gesonderter Berücksichtigung klar ausgeprägter Hämolyse steht dem Baird-Parker mit der Gewichtung einer positiven Eigelbreaktion gegenüber.

Ähnliche methodische Unstimmigkeiten stellen die Qualität vieler epidemiologischer Untersuchungen in Frage. Ursache hierfür ist ein Mangel an Standardisierung in den Typisierungsmethoden sowohl der 1. Generation (Phagentypisierung) als auch 2. Generation (Genotypisierung). Die Verwendung verschiedener internationaler Phagen unterschiedlicher Herkunft von Mensch und Tier in Kombination mit dem Einsatz von Phagensuspensionen in verschiedenen Konzentrationen stellen immer noch das ungelöste Problem der Typisierungsmethoden der 1. Generation dar.

Obwohl die Notwendigkeit boviner Phagen zur Typisierung von *S. aureus* aus Mastitiden von Rindern seit den 1960er Jahren international bewiesen ist, findet häufig nur der humane Phagensatz in der Charakterisierung boviner *S. aureus* Isolate seine Anwendung. Die Verwendung ausschließlich humaner Phagen führt zu stark divergierenden Ergebnissen. Der Anteil typisierbarer Isolate sinkt von 4 und 1% auf 36 und 35 % in Sammlung A bzw. B. Weitaus deutlicher fällt der Unterschied aus, wenn nur Phagenreaktionen mit semi-confluenter und confluenter Lysis unter Verwendung der 100×RTD Berücksichtigung finden. Damit würde die Typisierbarkeit mit dem humanen Phagensatz in Sammlung A und B von 68 bzw. 70 % auf 9 bzw. 13 % zurückgehen. Die bessere Eignung des humanen Phagensatzes zur Typisierung Penicillin- oder multiresistenter boviner Stämme wurde durch die Resultate der Sammlung C nachgewiesen. Hier zeigte nur ein Anteil von 33 % keine ausgeprägte Lysis

gegenüber humanen Phagen. Neben der hohen Sensitivität der Phagen aus der Lyogruppe III waren hier besonders die Phagen verstärkt von Bedeutung, die keine Zugehörigkeit zu einer Lyogruppe aufweisen, wie die Phagen 81, 812, 96 und 187.

Die falsche Eingruppierung vieler Isolate ist das zweite grundlegende Problem bei Verzicht auf bovine Phagen. Eine Mehrheit von etwa 25 % der Stämme, die mit dem bovinen Phagensatz dem Phagentyp 119 angehören, werden mit dem humanen Phagensatz in die Lyogruppe I bzw. dem 80-Komplex zugeordnet. Dies gilt in ähnlicher Weise für die Isolate in Lyogruppe III. Die überwiegende Mehrheit dieser Isolate reagieren bei Verwendung boviner Phagen fast ausschließlich zusätzlich mit Phagen der Lyogruppe IV. Nur mit der sofortigen Anwendung der höher konzentrierten Phagensuspensionen in der 100×RTD werden zumeist die spezifischen Reaktionen von 20-100 Plaques der humanen Phagen in Lyogruppe I und III realisiert. Meist waren nur die Phagen mit semiconfluenter oder confluenter Lysis (> 1000 Plaques) bei einer Verwendung der 100×RTD ausschlaggebend, um eine gute Übereinstimmung mit den phänotypischen Merkmalen zu erhalten. Die Spezifität schwacher Reaktionen von 20-100 Plaques bei einer Konzentration der Phagensuspension in der 100×RTD wurde häufig durch phänotypische Merkmale, Genotyping mit PFGE oder FTIR bestätigt.

Anhand der übereinstimmenden Typisierungsergebnisse von Sammlung A und B konnte die starke Populationsverschiebung von *S. aureus* in deutschen Kuhbeständen eindeutig nachgewiesen werden. Vor 40 Jahren gehörten 50 - 65 % der Stämme in Deutschland der Lyogruppe IV an. Derzeit sind es nicht mehr als 17 %. Dafür sind jetzt 3, 7, 17 und etwa 24 % der Mastitiserreger den Phagentypen 116, 96 (bzw. AC1), 78 und 119 zuzuordnen. Dies sind jedoch genau die Phagen, die in dem internationalen Projekt zur Entwicklung eines bovinen Phagensatzes vor 40 Jahren von den deutschen kooperierenden Labors nicht weiterempfohlen wurden, da sie zusammen nur einen Anteil von < 2 % der bovinen Stämme lysierten. Die Stämme, die noch der Lyogruppe IV zuzuordnen waren, reagieren zudem im Gegensatz zu früher häufig nur noch mit einzelnen Phagen dieser Gruppe. Die Isolate in Lyogruppe IV sind jetzt nur noch zu etwa 65 % β- hämolytisch mit meist fehlender Pigmentierung, weisen aber zu über 50 % eine Penicillinresistenz und zu etwa 44 % eine positive Eigelbreaktion auf. Die überwiegende Mehrheit der jetzt hohen Anzahl von etwa 30 % an Clumpingfaktor negativen Stämmen gehören dem Phagentyp 119, der restliche Teil meist dem Phagentyp 96 an.

Stellt eine zeitraumbezogene Grundgesamtheit und Dynamik einer Erregerpopulation die Erstellung eines Phagensatzes immer wieder vor neue Herausforderungen, so ist das Hauptproblem der Typisierungsmethoden der 2. Generation die unterschiedliche Anzahl an verschiede-

nen Genotypen in Abhängigkeit der jeweilig verwendeten Methode. Die neue Literatur bestätigt in Übereinstimmung mit unseren Ergebnissen die zu hohe Sensitivität der PFGE, welche gerade in der richtigen Zuordnung der vorherrschenden Typen einer Population Schwierigkeiten bereiten kann. Die genaue Interpretation unserer Ergebnisse ist gut mit neuen Erkenntnissen bezüglich der regulierenden Funktion der Phagen im Genom von *S. aureus* in Einklang zu bringen. Dies würde nicht nur die mangelnde Übereinstimmung in den relevanten phänotypischen Eigenschaften bis hin zur Enterotoxinbildung einzelner Isolate, welche bestimmten *S. aureus*- Typen angehören, erklären. Es wäre vielmehr auch ein wichtiger Erklärungsansatz für die Vielzahl an verschiedenen Subtypen eines Klons bzw. für methodische Schwierigkeiten bei der Phagentypisierung, wenn keine oder viele schwache Reaktionen bei hohen Phagenkonzentrationen nachweisbar sind. Die entscheidende Frage für die Genotypisierung hierbei wird sein, inwieweit das im Bakterium integrierte Genom temperenter Phagen die Ergebnisse molekularbiologischer Methoden beeinflussen. Die Blockierung der Lysis von Phagen des Phagensatzes durch vorhandene temperente Phagen wilder Stämme wurde für die Phagentypisierung, die Methode der 1. Generation, bereits nachgewiesen.

8.2.1 Kontrolle von *S. aureus*- Mastitiden in Milchviehherden

Die Mastitisdiagnostik bzw. Erfassung sämtlicher *S. aureus*- Typen zu einem Probenahmezeitpunkt auf Einzeltierebene erwies sich bei der Kontrolle von *S. aureus* auf verschiedenen Produktionsstufen, dem zweiten Themenkomplex dieser Arbeit, als entscheidende Fehlerquelle neben dem falschen Einsatz von Kulturen bei der Herstellung von Rohmilchprodukten. Die Ermittlung aller *S. aureus* positiven Kühe einer Herde in Kombination mit der schnellen „Infektionsdynamik“ ist die Hauptursache, warum der *S. aureus*-Gehalt 10.000/g nicht zuverlässig eingehalten werden kann. Die schnelle Infektionsdynamik steht einer unterschiedlich effektiven Phagozytoserate der Michkühe gegenüber. Dies ist letztlich auch die Hauptursache, warum die Prävalenz von *S. aureus* im Tierbesand keine reele Größe darstellt bzw. häufig nur einen kurzen Zeitbezug hat.

Um die genauen Ursachen bezüglich der methodischen Probleme in der Kontrolle von *S. aureus* in den Tierbeständen zu ermitteln, wurden in jeweils sechs kooperierenden Rohmilch verarbeitenden Betrieben mit einer hohen und niedrigen *S. aureus*- Prävalenz über einen Zeitraum bis zu 14 Monate genaue Untersuchungen durchgeführt. Diese Betriebe produzierten alle bereits über mehrere Jahre Rohmilchkäse; somit waren genauere Daten über den *S. aureus*- Gehalt der Rohmilch und der Produkte als auch Ergebnisse von Bestandsunter-

suchungen durch den Eutergesundheitsdienst vor und während unserer Studie vorhanden. In Anlehnung an Literaturangaben wurden die Herden mit weniger als 10 % positive Vorgemelksproben in einer Bestandsuntersuchung als Betriebe mit niedriger *S. aureus* Prävalenz eingestuft. Dies war in guter Übereinstimmung mit einem hohen Anteil an Tankmilchproben mit <100 *S. aureus* KBE/ml bzw. Käseproben <10.000 *S. aureus* KBE/g. Die Betriebe mit hoher und niedriger *S. aureus* -Prävalenz hatten die gleiche Verteilung bezüglich der unterschiedlichen Haltungsformen, Kuhrassen und Milchleistung. Der Laktationsdurchschnitt unterschied sich in beiden Betriebsgruppierungen nicht.

Die ausschließliche Verwendung von Vorgemelk und die bakteriologische Untersuchung von nur 0.01 ml Milch auf Einzeltierebene stellen bei der Stufenkontrolle (Tier, Tankmilch, Produkt) in den verschiedenen Produktionsabschnitten das Hauptproblem dar. Aus pragmatischen Gründen wird weiterhin die Milch in Routineuntersuchungen nur mit einer Öse auf der Platte verteilt anstatt gespatelt, was eine gezielte Auswahl der Isolate nach Kolonie morphologischen Kriterien bei dichtem Aufwuchs auf Agarplatten unmöglich macht. Die erste Untersuchung verdeutlichte, dass ohne eine zusätzliche Probenahme des Hauptgemelks - diese findet nur in der monatlichen Leistungskontrolle statt- die *S. aureus* an die Tankmilch abgebenden Kühe nur unzureichend erfasst werden. Ähnliches wurde für das Nachgemelk festgestellt.

Kühe, die nur im Hauptgemelk *S. aureus* positiv waren, wurden nahezu ausschließlich in Kuhbeständen nach offensichtlichen Fütterungsfehlern ermittelt, so zum Beispiel unmittelbar nach Futterumstellungen mit Eiweißüberschuss oder Energiemangel und in einem Fall nach Wassermangel (Hitzeperiode im Sommer 2003). Durch Zuhilfenahme der Ergebnisse der Phagentypisierung, Melkreihenfolge und Mitberücksichtigung der hohen Korrelation zwischen der Ausscheidungsrate und Milchmenge einzelner Kühe in Hauptgemelk und Tankmilch (r^2=0.95) konnte Kontamination als Ursache weitgehend ausgeschlossen werden. Entsprechend alter Literaturstellen ist davon auszugehen, dass eine Spülung von Melkzeug und Probenahmegerät (TRU-Tester) mit heißem oder sogar nur kaltem Wasser ausreichen, um eine Kontamination während der Probenahme für eine Untersuchung von 0.1 ml Milch nahezu völlig ausschließen zu können.

Die Bedeutung des Fütterungsmanagements für das Mastitisgeschehen wird nur erkannt, wenn neben der Untersuchung von Zellzahl und bakteriologischen Parametern zudem auch der Eiweiß-, Fett- und Harnstoffgehalt erfasst wird. Die Probenahme des Hauptgemelks zeitgleich mit der Tierleistungsprüfung hat sich hierbei bewährt. Der *S. aureus*- Gehalt im Hauptgemelk war positiv korreliert (r^2=0.84) mit der Zellzahlklasse (<50, 50-99, 100-199,

200-399, >400 Zellen in 1000/ml) und nahm durchschnittlich mit 125 KBE/ml zu. Diese sichere Beziehung zwischen *S. aureus*- Gehalt im Hauptgemelk und Zellzahl ist jedoch nur gegeben, wenn Kühe mit einer mangelhaften Energie- und Eiweißversorgung, ermittelt über das Eiweiß/Fett- Verhältnis und dem Harnstoffgehalt, von den Berechnungen ausgeschlossen werden. Alle Arten von Stress, so eine ungünstige Zusammensetzung der Ration oder unzureichende Futteraufnahme (inklusive von Wasser) beeinflussen deutlich die Ausscheidungsrate von *S. aureus* im Hauptgemelk. Dies erklärt nicht nur die täglichen Schwankungen des *S. aureus* -Gehalts der Tankmilch (meist um 20 bis 200 KBE/ml), sondern auch die Schwierigkeiten bei der Ermittlung der in einer Herde vorhandenen Ausscheider oder der Verursacher für hohe tägliche Peaks an *S. aureus* in der Tankmilch.

Bei der letzten Probenahme wurde in allen Tierbeständen Vor-, Haupt- und Nachgemelk auf Einzeltierebene untersucht. Grundsätzlich waren positive Gemelke in Herden mit einer hohen oder niedrigen *S. aureus*- Prävalenz unterschiedlich verteilt (P=0.004). Kühe, die in allen drei Gemelken (Vor-, Nach- und Hauptgemelk) *S. aureus* positiv waren, unterschieden sich sehr deutlich in Abhängigkeit der Prävalenz. Positiv getestet wurden in Herden mit hoher Prävalenz 39.5 %, in Herden mit niedriger Prävalenz aber nur 1.9 % der Kühe. Der Anteil in zwei Gemelken positiv getesteter Kühe betrug je nach Prävalenz 17.8 bzw. 7. 4 %. Die Anteile der in nur einem Gemelk positiv getesten Kühen unterschied sich mit 16.8 bzw. 18.1 % nur noch unwesentlich. Kühe aus Herden mit hoher Prävalenz und nur positivem Hauptgemelk waren bis auf wenige Ausnahmen alle in der 1. oder 2. Laktation (P= 0.044). Positive Nachgemelke wurden in diesen Beständen nur bei älteren Kühen (P= 0.020) ermittelt.

Der in den 12 Herden vorliegende Anteil positiver Vorgemelksproben korrelierte gut mit dem Anteil positiver Proben im Nachgemelk (r^2=0.84), chronischer Mastitiden (r^2=0.80) und aller *S. aureus* positiver Kühe (r^2=0.81). Ohne der Herde mit Wassermangel im August 2003 (siehe oben) stieg der Regressionskoeffizient (r^2) für das Hauptgemelk in den noch verbleibenden 11 Betrieben von 0.60 auf 0.73 an.

Die Verbreitung von *S. aureus* in Milchviehherden, identisch zu den Befunden der Zellzahl auf Einzeltierebene, steht in enger Beziehung zur Prävalenz des Erregers und dem Alter der Kühe. Der Anteil an positiven Kühen (mit *S. aureus* in Vor-, Haupt-, oder Nachgemelk) in Herden mit niedriger Prävalenz lag in der 1. Laktation bei durchschnittlich 18 %, in der 3. Laktation auf dem höchsten Wert von 36% und nahm bei älteren Kühen bis auf 24 % ab. Ein umgekehrtes Bild ergab sich für Tierbestände mit einer hohen Prävalenz (71, 53 und 69 %). Diese Unterschiede waren die Folge der hohen Dynamik von subklinischen *S. aureus*

Mastitiden in Herden mit hoher *S. aureus*- Prävalenz. Nur in Herden mit einer niedrigen Prävalenz ist es möglich, den Infektionsdruck von *S. aureus* einzugrenzen. Dies geschah durch die schnelle Identifizierung von einzelnen zumeist älteren Kühen mit chronischer Mastitis und deren Behandlung bzw. Eliminierung.

Die Dynamik subklinischer Mastitiden wird entscheidend durch die Anzahl der Tiere mit hoher Ausscheidungsrate von *S. aureus* im Nachgemelk beeinflusst. Herden mit hoher Prävalenz hatten eine durchschnittliche Ausscheidungsrate von $3{,}2\times10^3$ KBE/ml, Herden mit niedriger *S. aureus*- Prävalenz hingegen von nur $2{,}5\times10^2$ KBE/ml. Der Unterschied war signifikant ($p= 0{,}026$). Da der *S. aureus*- Gehalt des Nachgemelks weitgehend gleichzusetzen ist mit der *S. aureus*- Belastung der Zitzenbecher nach dem Melken und die Höhe der Ausscheidungsrate im Nachgemelk eng mit der Höhe der Prävalenz der Betriebe korreliert, darf gefolgert werden, dass der Melkmaschine bei der Verbreitung von *S. aureus* eine zentrale und damit überragende Bedeutung in der Milchproduktion zukommt.

Diese Schlussfolgerung wurde insbesondere bestätigt durch vorhandene Langzeituntersuchungen an der bereits wiederholt angesprochenen Herde (Wassermangel im August 2003) mit hohen Ausscheidungsraten ausschließlich im Hauptgemelk. Nur die Rinder mit mehr als einer Laktation waren zu diesem Zeitpunkt *S. aureus* positiv und alle für den Phagentyp 78. 14 Monate zuvor wurden Ausscheider für Phagentyp 78 geschlachtet. Offensichtlích wurden damals bereits alle laktierenden Kühe, welche 14 Monate zuvor in der Herde waren, über das Melkzeug mit diesem Typ unbemerkt infiziert. Dies zeigt die große Rolle einer dauerhaften Besiedelung der Zitzen und Strichkanäle für das Mastitisgeschehen auf und erklärt zudem das Auftreten weit verbreiteter spontaner Infektionen wie den Verlauf von Umweltmastitiden. Ohne einen längeren Betrachtungszeitraum, differenzierte Untersuchung der Gemelke und ohne Kenntnisse über das Herdenmanagement werden die engen Beziehungen zwischen chronischen Mastitiden und Infektionen des Strichkanals nicht ersichtlich.

8.2.2 Pathogenese und Biodiversität von *S. aureus* im Tierbestand

Häufig wird die Bedeutung der Zellzahl insbesondere für den Nachweis von Staphylokokken Mastitiden überschätzt. Als Ursachen hierfür sind die ausschließliche Untersuchung des Vorgemelks und die Vorselektion der Viertelgemelke durch den häufig angewendeten, aber wenig sensitiven Schalmtest anzuführen. Eine Infektion beginnt in der Regel in einem Euterviertel. Der dann von den drei anderen Vierteln mit weniger als 40.000 Zellen/ml bewirkte Verdünnungseffekt muss Berücksichtigung finden. Daraus wäre zu folgern, dass für die Zellzahlen einer Kuh ab 100.000/ml bereits der Verdacht für chronische Mastitis vorliegt. Der wichtigste Einflussfaktor für die Zellzahl ist das Alter, d. h. die Anzahl der Laktationen einer Kuh. Die Zellgehalte *S. aureus* negativer Kühe stiegen mit jeder Laktation durchschnittlich um 53.000 Zellen/ml an (r^2=0.81), gut übereinstimmend mit den Ergebnissen der nationalen Tierleistungsprüfung. Positive Kühe hatten in Herden mit hoher Prävalenz die höchsten Zellzahlen bereits in den ersten beiden Laktationen im Vergleich zu älteren Tieren mit dem Höchstwert von 395.000 Zellen/ml in der 2. Laktation (P= 0.087). In Herden mit niedriger Prävalenz erreichten positive Kühe den Höchstwert von 220.000 Zellen/ml erst in der 3. Laktation (P= 0.072).

Um *S. aureus* ausscheidende Tiere gezielter erfassen zu können, wäre eine eindeutige Beziehung zwischen Mastitisform und Zellzahl hilfreich. Diese Abhängigkeit war aus den vorhandenen Ergebnissen jedoch schwer ableitbar. Nur positive Kühe mit Zellzahlen unter 25.000/ml waren durchwegs frei von chronischer *S. aureus* Mastitis. Diese Tiere standen bis auf ein Tier in der 1. Laktation, so dass die geringe Zellzahl mit der einsetzenden Kolonisation des Euters junger Rinder durch Bakterien erklärt werden kann. Nur 2 von 42 Kühen (4.7%) mit chronischer *S. aureus* Mastitis hatten Zellzahlen unter 50.000/ml. Bereits ab Zellzahlen von >50.000 beginnt der kritische Bereich für chronische *S. aureus* Mastitiden; 52% der Kühe mit persistierender Mastitis fallen in die Gruppierung 150.000 Zellen/ml.

Alle Kühe mit positiven Befunden in Vor-, Haupt- und Nachgemelk waren chronische Ausscheider. Die Tatsache, dass zusätzlich nur einzelne Kühe mit positivem Befund im Nachgemelk, Vor- und Nachgemelk oder Haupt- und Nachgemelk wiederholt als *S. aureus* Ausscheider identifiziert wurden, bestätigt für persistierende Mastitiden die große Bedeutung positiver Befunde im Nachgemelk. Kühe mit positivem Vor- und Hauptgemelk sowie positivem Haupt- und Nachgemelk wiesen den höchsten Zellgehalt auf (Median: ≤190.000 Zellen/ml). Kühe mit zwei positiven Gemelken wiesen im ersten Gemelk einen mittleren Gehalt bis 8300 KBE/ml auf (Median: bis 380 KBE/ml). Der Mittelwert des zweiten positiven Gemelks lag mit nur 520 KBE/ml (Median: bis 60 KBE/ml) deutlich darunter. In positiven

Proben bis 200.000 Zellen/ml korrelierte der *S. aureus*- Gehalt am besten mit den Befunden des Vorgemelks, bei höheren Zellzahlen mit denen des Nachgemelks.

Um die stark streuende Dynamik der Zellgehalte besser zu verstehen, wird nachfolgend auf einige wichtige, die Zellzahl regulierende Vorgänge hingewiesen. Offensichtlich induziert ein hoher Gehalt von über 100 *S. aureus* KBE/ml im Nachgemelk eine verstärkte Ausschüttung von Leukozyten, um das Wachstum des in der Euterzisterne etablierten *S. aureus* über eine höhere Phagozytoserate einzugrenzen. Dies erklärt für Kühe mit >400.000 Zellen/ml die häufig festgestellten relativ niedrigen *S. aureus*- Gehalte (<100 KBE/ml) in den Vorgemelken. Hohe Phagozytoseleistungen der unteren Euterzisterne wirken sich überwiegend auf das Vorgemelk aus und erklären den häufig positiven Befund ausschließlich in Haupt- oder Nachgemelk. Neben der Phagozytose kommt im Haupt- und Nachgemelk zusätzlich ein Verdünnungseffekt durch die in die obere Euterzisterne steril einschießende Alveolarmilch für die Abnahme des *S. aureus*- Gehalte hinzu. Dieser zusätzliche Verdünnungseffekt während des Melkens ist gerade für *S. aureus*- Gehalte bis zu etwa 10 KBE/ml im Nachgemelk relevant; dieser verbleibende *S. aureus*- Gehalt dürfte älteren Literaturangaben zufolge zwischen zwei Melkzeiten in der Euterzisterne normalerweise phagozytiert werden. Meist nur bei chronischen Mastitiden über 10 *S. aureus* KBE/ml im Nachgemelk ist eine Vermehrung der Erreger in der Euterzisterne möglich, die dann in der folgenden Melkzeit im Vorgemelk mit 0.01 ml Milch in der Regel nachweisbar ist.

Ergebnisse mit einer ähnlichen oder höheren *S. aureus* Biodiversität einzelner Milchvieherden wurden in der Vergangenheit nur in Langzeitstudien erzielt. Besonders in Herden mit hoher *S. aureus*- Prävalenz wurde eine hohe Anzahl verschiedener Typen festgestellt. In diesen Tierbeständen erschwerte der verursachende Leitkeim für chronische Mastitis den Nachweis zusätzlich enthaltener Stämme und verdeutlicht die methodischen Probleme bei der Bestimmung der Biodiversität dieses Erregers in einer Herde. Es konnte zudem aufgezeigt werden, dass Sanierungsmaßnahmen in den Tierbeständen über die selektive Veränderung des Typen-Musters die Untersuchung auf *S. aureus* erschwert. Mit der Untersuchung von nur 0.01 ml Milch aus dem Vorgemelk werden in der Regel im Antibiogramm nur Erreger erfasst, welche zum Zeitpunkt der Probenahme Verursacher von chronischer Mastitis sind. Erst die selektive Untersuchung von Vor-, Haupt- und Nachgemelk unter Mitberücksichtigung einer möglichst hohen Anzahl phänotypisch unterschiedlicher Isolate erlaubt eine ungefähre Abschätzung der zum gewählten Probenahmezeitpunkt in der Kuhherde präsenten *S. aureus*-Typen.

Damit wird auch der extrem hohe wirtschaftliche Aufwand ersichtlich, der für die Sanierung von Betrieben mit hoher *S. aureus*- Prävalenz notwendig wird. So konnten 547 Isolate aus 10 Betrieben 30 verschiedenen Phagenprofilen mit nur leicht abweichenden Lysisbildern und nahezu identischen Phänotypen mit nur leicht divergierenden Ergebnissen bezüglich der Enterotoxingene zugeordnet werden. Nur fünf der 30 Profile wurden in mehreren Tierbeständen nachgewiesen. Wenn diese als verschiedene Typen einer Herde angesehen und den 30 Phagenprofilen zugefügt werden, wurden 41 verschiedene Phagentypen isoliert. Nur 38 % davon bildeten eine β–Hämolyse, 20 % waren α–hämolytisch, 33 % zeigten eine schwache Hämolyse und bei 9% der *S. aureus*- Typen konnte keine Hämolyse nachgewiesen werden. Jedoch 80 % der Typen waren Eigelb positiv und nur 2 % Clumpingfaktor negativ. 40% wiesen eine Resistenz für Penicillin auf und 15 % der 41 unterschiedlichen Typen waren *sea* positiv.

Der Vergleich mit den Ergebnissen der Typisierung aus herkömmlichen Monitoring-Programmen zeigte eindeutig eine Verschiebung der *S. aureus*- Population in Rohmilchbetrieben weg von den typischen Erregern chronischer Mastitiden und hin zu Typen mehr mit Eigenschaften humaner Stämme. Nur Isolate einzelner Kühe gehörten dem Phagentypen 119 und der Phagengruppe IV an, kein Isolat dem Lysisbild 116. Das Lysisbild 78 wurde nur in einer Herde nachgewiesen. Am weitesten verbreitet von den dominierenden Erregern aus Monitoring-Programmen waren Isolate mit einer hohen Sensitivität für Phage 96. Diese wurden zudem häufig von Kühen mit chronischen Mastitiden isoliert und waren aber alle Clumpingfaktor positiv. Die hohe Affinität bestimmter Stämme als Verursacher spontaner Infektionen - und dies anfänglich nur in geringen Konzentrationen - ähnlich den Erregern von Umweltmastitiden und deren hohe Verbreitung, wurde vor allem durch den Nachweis von Phagentyp 95 in vier von 10 Betrieben aufgezeigt. Entsprechend muss auch die hohe Anzahl von Isolaten mit Eigelb und Clumpingfaktor positiven Stämmen gewertet werden, welche häufig Enterotoxin, aber nicht *sec* und *tst* positiv sind und keine oder nur schwache Hämolyse aufzeigen.

8.3 Schlussfolgerungen

Die Bestimmung der Biodiversität und Kontrolle von *S. aureus* im milchwirtschaftlichen Bereich wird ganz entscheidend von dem Verständnis für die verfügbaren Methoden bezüglich Aussagekraft, Eignung und Anwendung beeinflusst. Zudem müssen die *S. aureus*-Prävalenz sowie die Historie von Sanierungsmaßnahmen mit in die Bewertung von Populationsanalysen und in Strategien für zukünftige Kontrollmaßnahmen einfließen.

Darauf abgestimmt ist eine Ziel führende Beprobungsstragie zu entwickeln. Die Ergebnisse der vorliegenden Arbeit sind ein Beleg für die Richtigkeit dieser Vorgehensweise.

Im überregionalen oder internationalen Vergleich von Populationsstudien finden weiterhin der Laktationsdurchschnitt, das Leistungsniveau und die Melkbarkeit der Kühe als auch das Aufstallungssystem, die Tierbestandsgröße, das Herdenmanagement und die klimatische Standortbedingung zu wenig Berücksichtigung. Viele aufwendige Arbeiten, die sich mit diesen Kenngrößen in Zusammenhang mit Mastitis beschäftigen, enthalten ihrerseits keine epidemiologischen Untersuchungen. Resistenzmonitoring- Programme in Milchviehherden berücksichtigen in der Regel weder die Kenngrößen in den zu beprobenden Tierbeständen, noch werden die Isolate zumeist typisiert.

Seit Einführung der Genotypisierung wurde zunehmend die Bedeutung phänotypischer Charakteristika in Frage gestellt. Die hohe Übereinstimmung zwischen phänotypischer Charakteristika und Genotypsierungsmethoden wurde in den letzten Jahre von neueren Arbeiten eindrucksvoll bestätigt. Die Ergebnisse der vorliegenden Arbeit kommen daher eindeutig zum Schluss, dass unbedingt alle verfügbaren phänotypischen Eigenschaften Eingang in Populationsanalysen finden müssen. Die Notwendigkeit dieses Vorgehens resultiert unter anderem auch in der Vergleichbarkeit der Ergebnisse, wenn verschiedene Isolierungs- oder Typisierungsmethoden verwendet werden. Da die klassischen phänotypischen Merkmale von *S. aureus* ausnahmslos Virulenzfaktoren darstellen, sind Veränderungen in diesen Eigenschaften mit einer Anpassung bzw. Populationsverschiebung durch Ausbreitung von *S. aureus* aus anderen Körperregionen gleichzusetzen. Dies ist insbesondere bei Mastitis von Interesse, wo auf engstem Raum zwei unterschiedliche ökologische Nischen anzutreffen sind. Eine befindet sich im oberen Strichkanal in Richtung Euterzisterne, die andere ist die Zitzen- und Euteroberfläche. Die Adaption von *S. aureus* an diese Umweltbedingungen stellt deshalb auch unterschiedliche Anforderungen an die Untersuchungsmethodik wie z. B. Anpassung der Nährmedien und Phagentypisierung.

Das Baird-Parker Medium und der erste internationale Phagensatz wurden überwiegend für die Isolierung und Typisierung der gewöhnlich Eigelb- positiven Stämme aus dem Hospital entwickelt. Der Blutagar hingegen wurde von Anfang an zur Isolierung kontaktöser Mastitis-erreger wie *Str. agalactiae* verwendet, welche in der Regel eine deutliche Hämolyse aufzeigen. Im Falle von *S. aureus* haben diese häufig nur eine hohe Sensitivität gegenüber Phagen, die aus Mastitismilch isoliert wurden. Dadurch ist der bovine Phagensatz stark anwendungsorientiert und nicht ausreichend diskriminativ für *S. aureus* von Körperober-flächen. Gerade bei Bestandsanierungen, welche in erster Linie zu einer Reduktion von *S.*

aureus aus chronischen Mastitiden führen, ist bereits in kürzeren Zeiträumen mit einem gehäuften Auftreten von *S. aureus* von der Zitzen- und Euteroberfläche zu rechnen. Ergebnisse neuerer epidemiologischer Arbeiten bestätigen, dass womöglich der Körperregion, in der *S. aureus* isoliert wurde, eine mindest ebenso große Bedeutung zukommt wie der Wirtsspezifität. Bovine *S. aureus* von der Zitzen- und Euteroberfläche können beispielsweise mittels PFGE nicht von *S. aureus* menschlicher Haut unterschieden werden.

Ausreichend diskriminierenden Typisierungsmethoden kommt daher eine ebenso große Bedeutung zu wie einer verbesserten und einheitlicheren Probenahme und bakteriologischen Diagnostik, um alle unterschiedlichen Typen korrekt erfassen und in einen epidemiologischen Kontext stellen zu können. Wenn auffallende Divergenzen zwischen den Ergebnissen einer epidemiologischen Untersuchung und den phänotypischen Eigenschaften wie Hämolyse, Clumpingfaktor- Aktivität, Eigelbreaktion und Antibiotikaresistenz bestehen, sollte eine zweite diskriminative Typisierungsmethode zusätzlich zum Einsatz kommen. Nur ein international normiertes Vorgehen, basierend auf einer elektronischen Datenbank, würde eine systematische Zuordnung der Stämme aus human- und veterinärmedizinischen Untersuchungen erlauben und mögliche phylogenetische Zusammenhänge erkennen lassen. Neben den traditionellen phänotypischen Eigenschaften und wichtigen Antibiotikaresistenzen erscheint als Typisierungsmethode der Wahl die *Spa*- Sequenzierung von Protein A am geeignetsten. Ausschließlich die Phagentypisierung aufgrund groß angelegter Untersuchungen in der Vergangenheit gibt Aufschluss über die Populationsverschiebung in den Kuhherden der letzten 40 Jahre. Nicht nur deshalb sollte die Phagentypisierung Bestandteil der Datenbank sein. Die Typisierung mit Phagen ist nach wie vor die praktikabelste, schnellste und günstigste Methode speziell für eine Untersuchung großer Probenmengen mit meist guter Korrelation zu den Genotypen verschiedener moderner Methoden der 2. Generation.

Die Kontrolle von *S. aureus* im Tierbestand bzw. einer unkontrollierten Verbreitung von Erregern, welche chronische Mastitis verursachen, kann nachhaltig nur durch präventive Maßnahmen erreicht werden. Dazu muss zuerst garantiert sein, dass eine Übertragung der Erreger während der Laktation über das Melkzeug unterbunden wird.

Eine vollautomatische Zwischendesinfektion der Melkmaschine durch heißes Wasser wäre aus arbeitswirtschaftlichen Gründen über eine Erfassung aller Daten der Milchleistungsprüfung in einer elektronischen Datenbank am besten geeignet. Gerade in Betrieben mit Melkständen und größeren Kuhherden ist ein separates Melken aufwendig. Nur wenn eine Übertragung während der Laktation verhindert wird, kann eine gezielte Bekämpfung der Erreger kurz vor oder während der Trockenstellperiode erfolgen, ohne dass der Keim bereits

in der ganzen Herde verschleppt wurde. Das Immunsystem der Kühe behält seine maximale Leistungsfähigkeit für die Phagozytose nur bei optimaler Versorgung mit Energie, Eiweiß und Wasser. Wegen dieser Einflussnahme auf die Ausbreitung der Mastitis kommt der Fütterung im Herdenmanagement eine besondere Bedeutung zu.

Für die Kontrolle von Mastitispathogenen im Tierbestand, gleichermaßen für die Anwendung von Antibiotika und das Resistenzmonitoring, wird die bakteriologische Untersuchung von Tankmilch zwischen 10 und 0.01 ml Milch empfohlen. Mastitispathogene, die in 0.1 und 0.01 ml Tankmilch nachweisbar sind, stammen überwiegend aus chronischen Euterentzündungen und stellen die Leitkeime im Mastitisgeschehen der Herde dar. Eine Anreicherung von 10 ml Milch mit und ohne Zugabe von Antibiotika der Wahl und unter Zuhilfenahme der MPN-Technik lassen vor allem auch eine Quantifizierung von antibiotikaresistenten Mastitis-erregern in geringen Konzentrationen zu, welche bei einer möglichen Applikation von Antibiotika gefördert werden können. Dies wäre wichtig, um durch eine geeignete Auswahl von Antibiotikas das Aufkommen resistenter Erreger in einer Herde zu verhindern. Dies würde aber zudem neben einer verbesserten bakteriologischen Diagnostik einen entscheidenden Fortschritt im Resistenzmonitoring bringen, weil neben der Verschiebung der Populationen auch die Tendenz im Aufkommen resistenter Erreger, bevor sie sich zu Leitkeimen entwickelt haben, frühzeitig erkannt werden können.

Die MPN-Methode kann auch zum Nachweis Enterotoxin positiver *S. aureus* in Rohmilch verarbeitenden Betrieben dienen. Durch spezifische Gensonden können mittels Realtime-PCR die relevanten Enterotoxinbildner in der Tankmilch nachgewiesen und deren Herden entsprechend ihrem Risikopotenzial eingestuft werden. Es ist aus Gründen der Lebensmittel-sicherheit nicht sinnvoll, einen *S. aureus*, der Leitkeim und Enterotoxin negativ ist, aus der Herde zu verdrängen, da womöglich dadurch erst die problematischen *S. aureus*- Erreger sich in den Beständen ausbreiten können. Viel wichtiger jedoch ist es, in den Rohmilch verarbeitenden Betrieben einerseits mehr auf die Käsereitauglichkeit bzw. Säuerungseigenschaften der Rohmilch zu achten und andererseits die eingesetzten Kulturen genau auf den Temperaturverlauf der Käseherstellung abzustimmen. Hierfür würde sich ein Programm für das Qualitätsmanagement in landwirtschaftlichen Betrieben anbieten.

9. Appendix:

Important results from the literature

List of Tables and Figures:

Table 1: Mastitis pathogens isolated in Switzerland 1987 and 1996 according to Schallibaum[1] (IDF 367/2001) in comparison to samples of German dairy herds according to Sobiray et al. (1997)

	Subclinical mastitis		Clinical mastitis		Sobiray et al.[2]
	1987 (n= 54 487)	**1996** (n= 13 443)	**1987** (n= 40 108)	**1996** (n= 55 296)	**1997 (n= 1242)**
Staphylococcus aureus	41.2	39.8	33.1	26.1	35.3
Coagulase- negative staphylococci	16.6	18.8	15.9	17.3	21.6
Streptococcus agalactiae	7.3	2.2	2.6	1.3	4.9
Other streptococci (+ enterococci!)	27.6	23.4	27.6	31.1	26.9
Coliforms	xxx	xxx	11.7	9.9	3.3
Actinomyces pyogenes	xxx	xxx	2.8	1.8	
Yeasts	xxx	xxx	1.3	1.7	
Miscellaneous *(Nocardias spp., Pseudomonas spp., Bacillus ssp., C. bovis)*	xxx	xxx	5.0	10.8	
Miscellaneous (Coliforms, *A. Pyogenes, C. bovis)*	7.3	15.8	xxx	xxx	
Negative samples	24.5	9.4	21.8	11.6	24.5

[1] in the Switzerland: samples from subclinical mastitis were taken by udder-health-service extension workers; samples from clinical mastitis were taken by veterinarians

[2] From 63 veterinary practices all over Germany

Figure 1: Estimated cumulative risk of a cow having mastitis during one year for different classes of mastitis 1974/1975 through 2000 according to Ósteras (BIDF 367/2001)

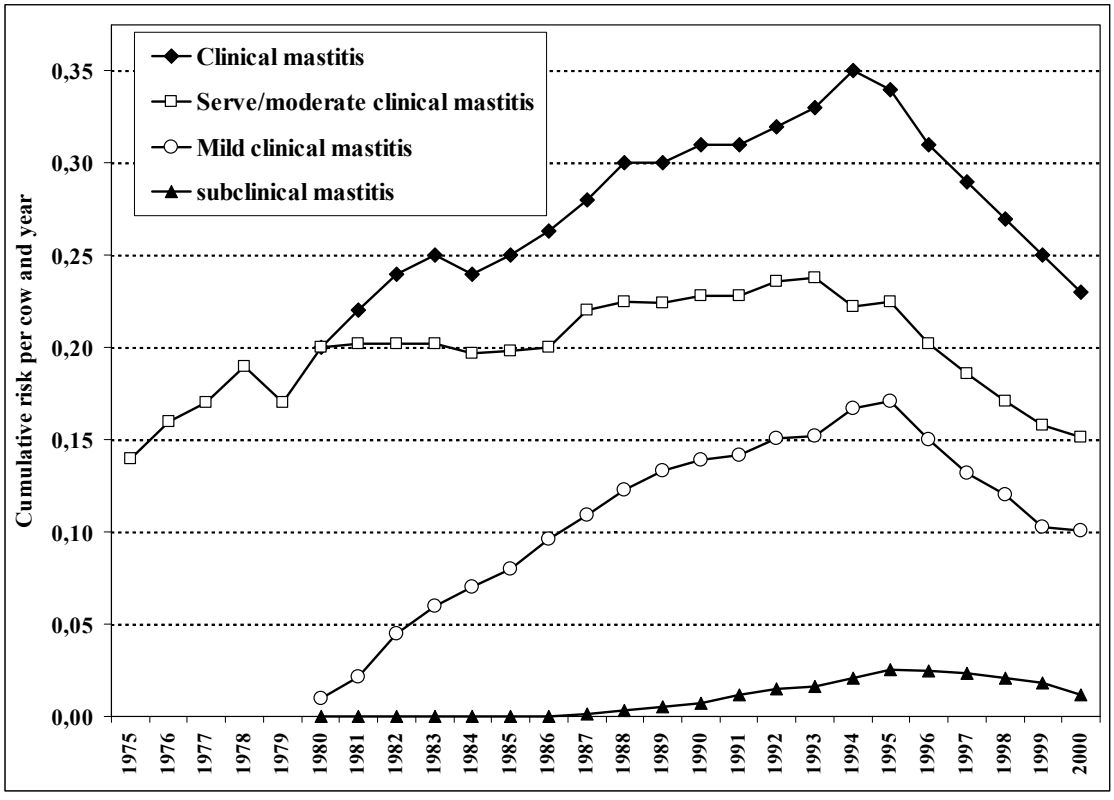


Table 2: Distribution of clinical cases within mastitis severity codes[1] by bacteriological status of quarters in herds with low SCC according to Hogan et al. (1989)

Bacteriological status	Severity code (prevalence in %)			Total (n=646)
	3 (n=248)[a]	4 (n=303)	5 (n= 95)	
Streptococcus agalactiae	0	0	0	0
Staphylococcus species (CNS)	6,0	5,3	4,2	5,4
Staphylococcus aureus	0,8	3,0	0,0	1,7
Environmental streptococci	28,2	26,0	15,8	25,4
Coliforms	21,4	27,3	58,9	29,7
Pseudomonas spp.	2,8	10,9	2,1	6,5
Corynebacterium bovis	4,4	0,3	0,0	1,7
Other microbes	1,6	2,0	1,0	1,7
Contaminated samples	0,4	1,0	0,0	0,6
Bacteriologically negative	34,3	24,3	17,9	27,2

[1] **Mastitis severity codes:** 3= abnormal milk, no quarter swelling, (subacute mastitis)
4= normal or abnormal milk and quarter swelling
5= abnormal milk, quarter swelling and systemic signs

[a] Number of clinical cases

Table 3: Distribution of udder pathogens in 685 random selected herds and 295 herds with mastitis problems and a frequent use of drugs (Krabisch et al., 1999)

	Herds of random selection (1869 isolates)	**Herds with mastitis problems (675 isolates)**
Staphylococcus aureus	**31.9**	**24.7**
Coagulase-negative staphylococci	**46.0**	7.4
Streptococcus agalactiae	3.3	8.4
Enterococci	13.9	7.4
Coliforms	0.6	3.4
Escherichia coli	4.2	**48.6**

Table 4: Correlation between SCC-values (in thousand /ml) and the strength of the CMT-reaction according to Seelemann (1964)

SCC-Level (SCC´000/ml)	**Samples** (n)	**Strength of CMT-reaction** (Distribution%)			
		−	+	++	+++
0-200	75	80.0	20.0	0	0
201-500	131	42.7	45.8	10.7	0.8
501-1000	76	10.5	31.6	55.3	2.6
1001-2000	46	0	0	10.9	89.1
> 2000	46	0	0	5.2	80.4

Table 5: Mean-value and median-value of the SCC in thousand/ml from 11292 quarter samples in relation to the findings of bacteriological examinations (Zeidler et al., 1968)

	Prevalence (%) (n= 2755)	Mean (SCC´000/ml)	Median (SCC`000/ml)
Staphlococcus aureus	34.8	**1616**	**449**
Streptococcus agalactiae	5.2	1965	793
Streptococcus dysagalactiae	5.2	1358	477
Streptococcus uberis (E)	3.2	1125	578
Streptococcus uberis	2.9	880	270
Enterococci	37.3	591	239
Other microbes	11.3	556	187
Negative Samples (n= 8537)	(75.6)	494,000	174,000

Figure 2: Influence of the number of lactations on the SCC-value (in thousand/ml) and bacteriological findings in quarter samples according to the results of Blackburn (1968)

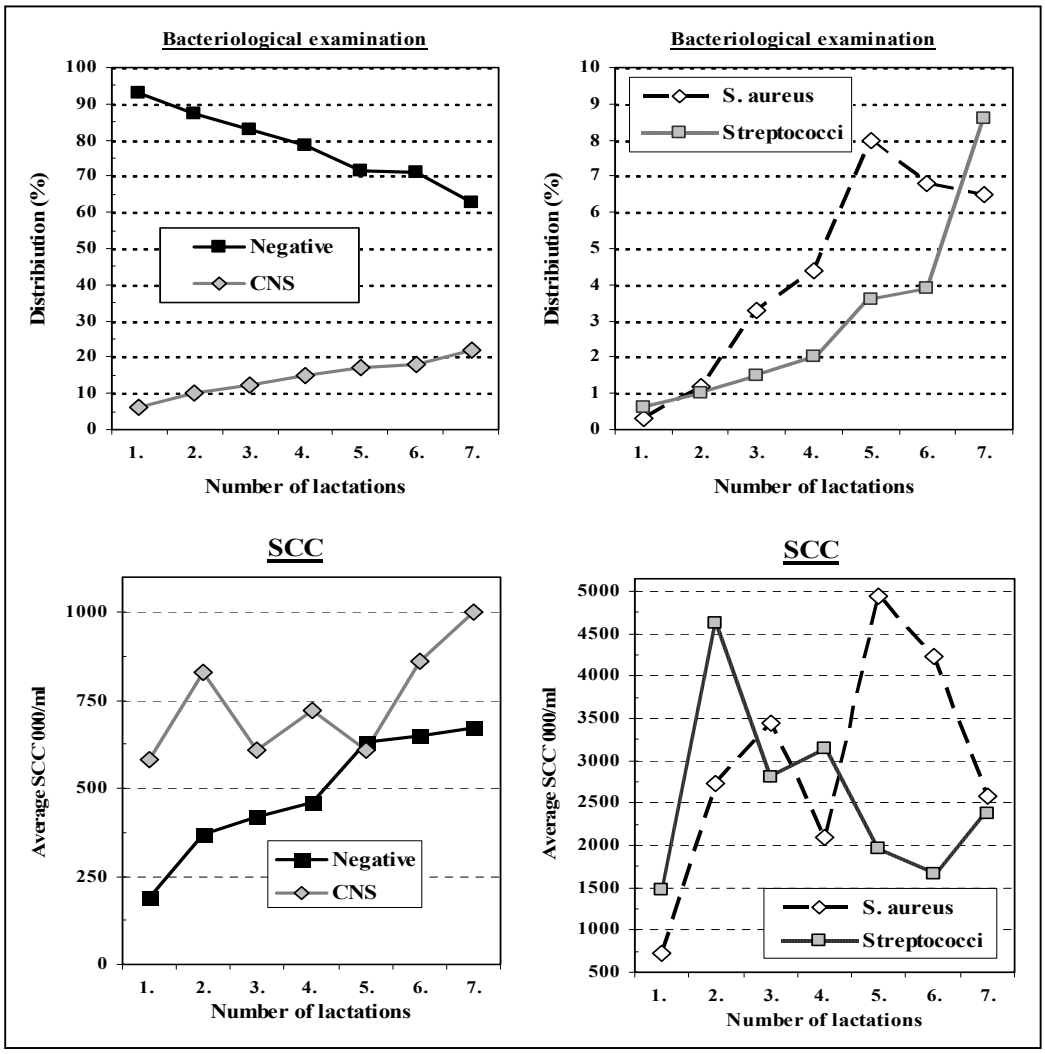


Figure 3: Influence of the lactation month and number of lactations on the SCC-value (x_G) according to the results of Zeidler et al. (1969) and Milchprüfring Bavaria (2001)

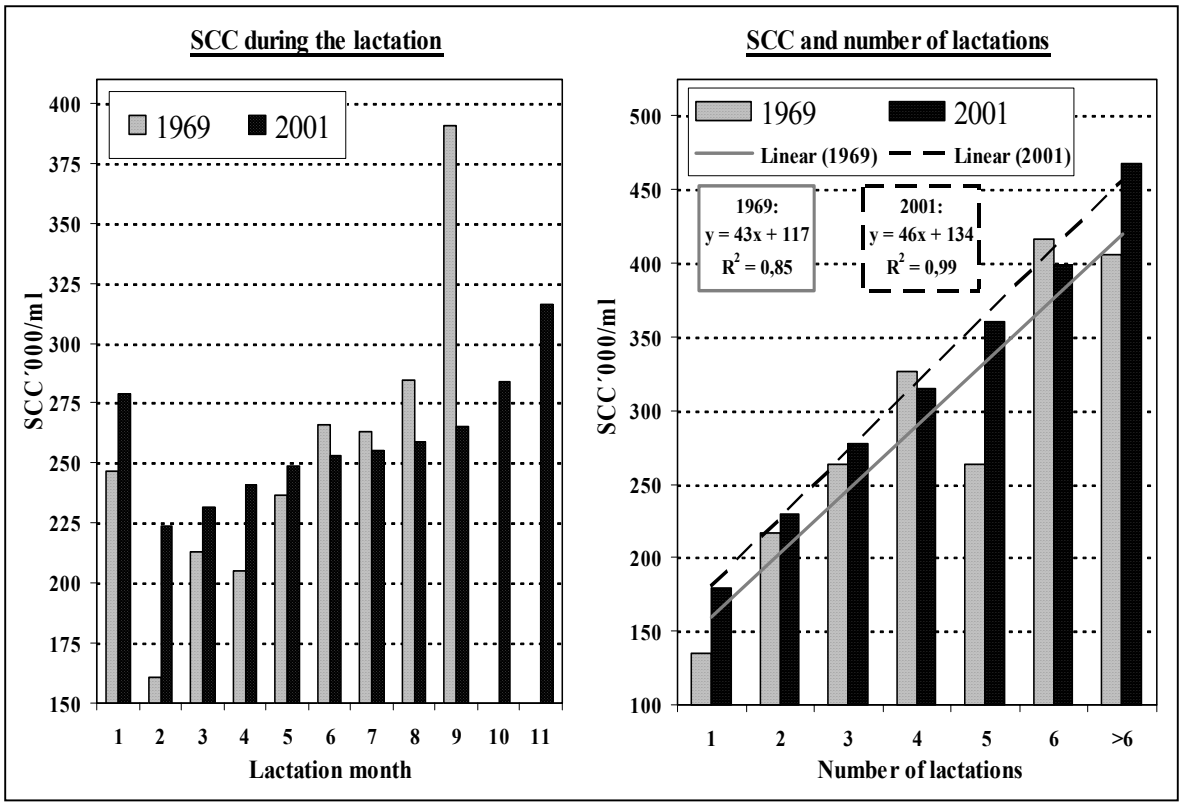


Figure 4: Distribution (%) in certain main milk (UTM) SCC-groups in thousand/ml(< 50; < 100; 100-250, 251 -500, >500) of Swedish cows for the Swedish red white breed (SRB) and Swedish Friesian breed (SLB) according to Brolund (1985)

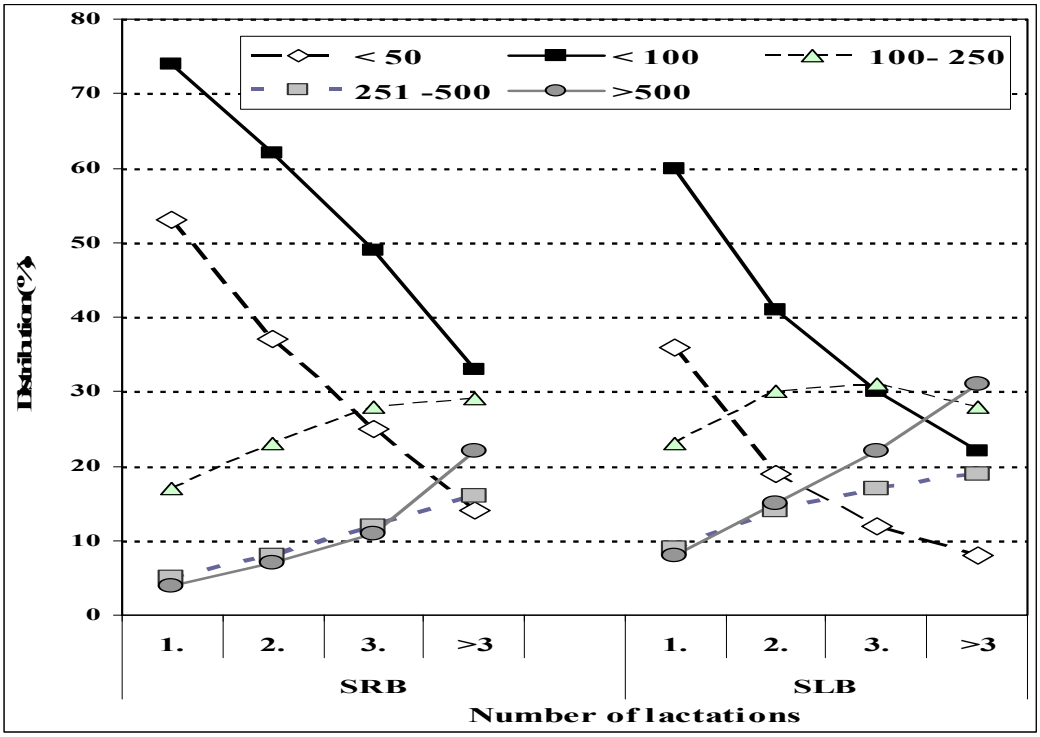


Figure 5: SCC-value in relation to the milk yield of a cow and breed per lactation (Milchprüfring Bavaria, 2001)

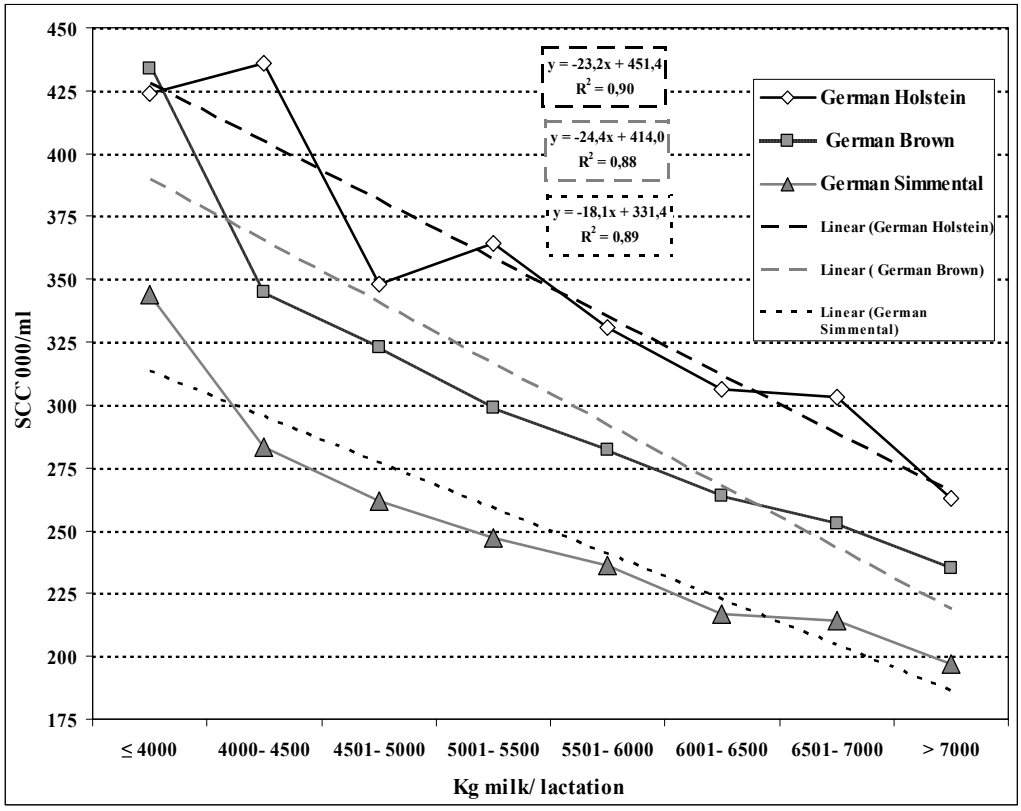


Figure 6: SCC-value during the year (Milchprüfring Bavaria, 2001)

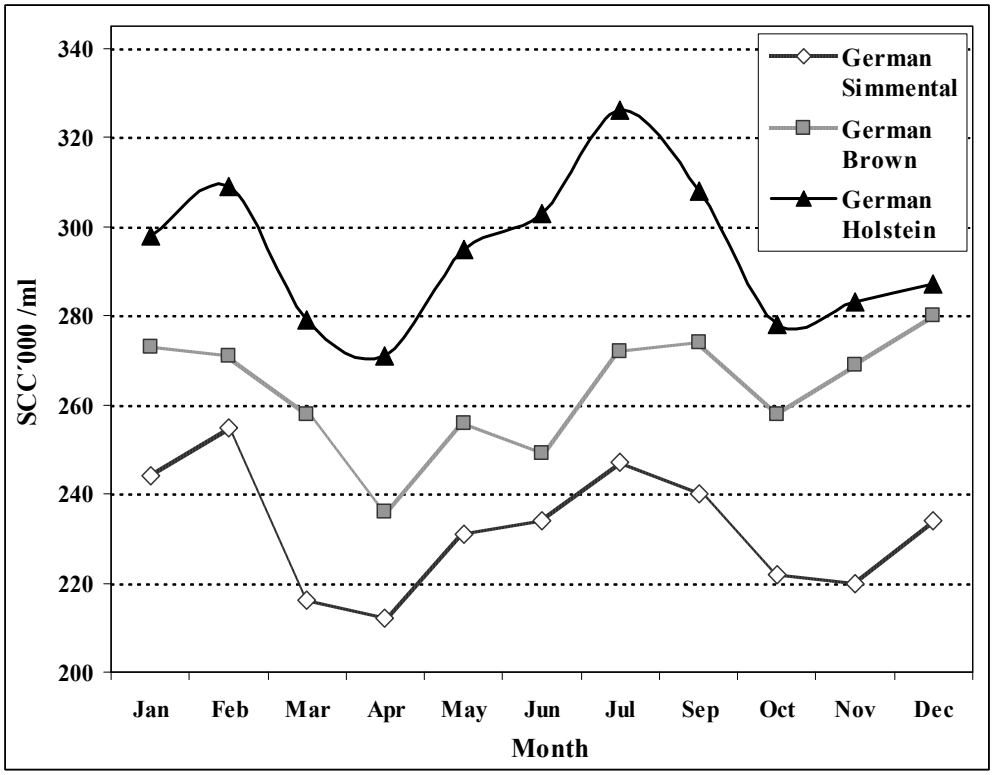


Figure 7: The risk (in quarter cases per 10000 days at risk) of clinical mastitis increase in relation to the number of lactations (Barkema et al., 1998)

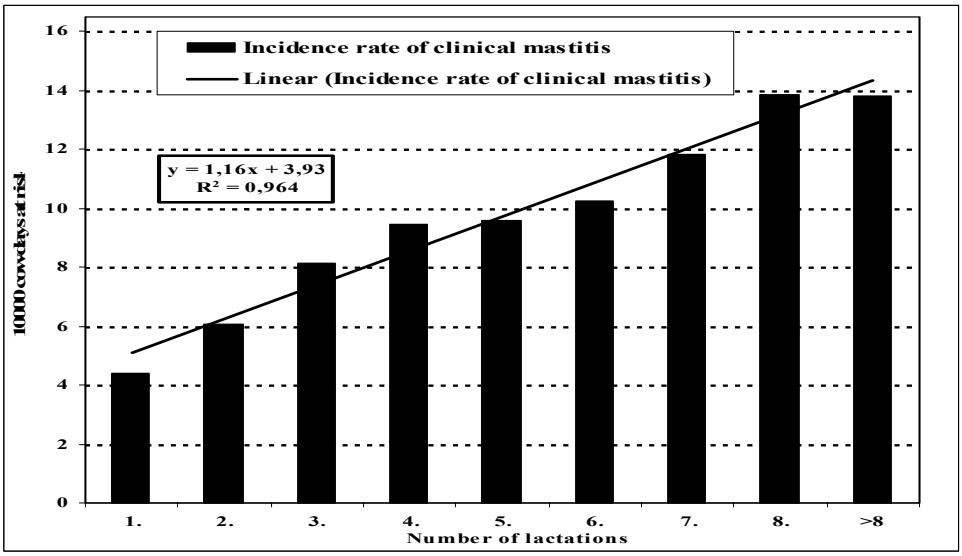


Figure 8: Distribution of 1071 cases of clinical mastitis by lactation month according to Miltenburg et al. (1996)

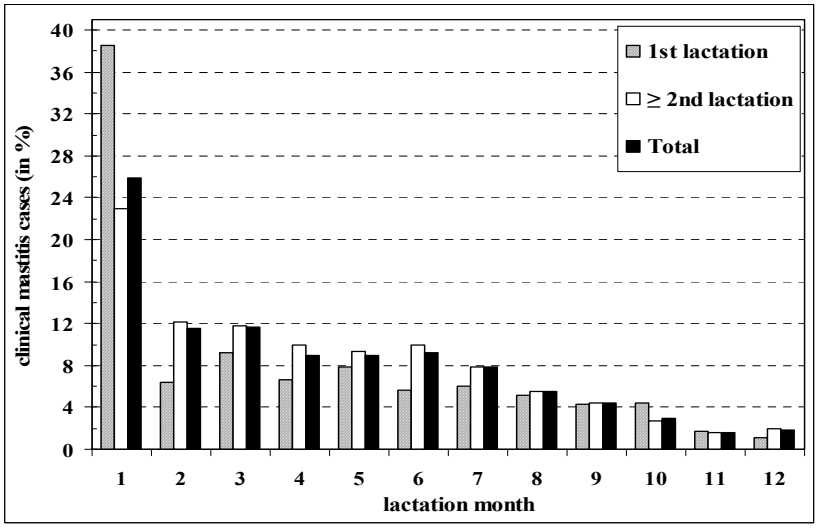


Figure 9: Distribution of the incidence rate of clinical mastitis (IRCM) of 274 herds stratified per bulk milk SCC (BMSCC) according to Barkema et al. (1998)

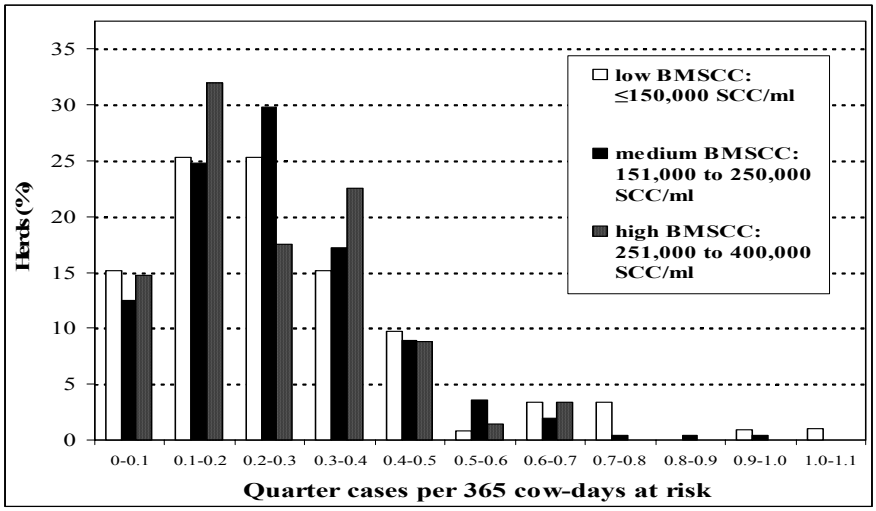


Figure 10: Geometric SCC of the bulk milk and prevalence of herds with a high geometric mean over 400,000 BMSCC/ml in one months (respectively herds with a minus of the payment because of elevated SCC) in relation to the amount of the daily bulk milk according to Milchprüfring Bavaria (2001)

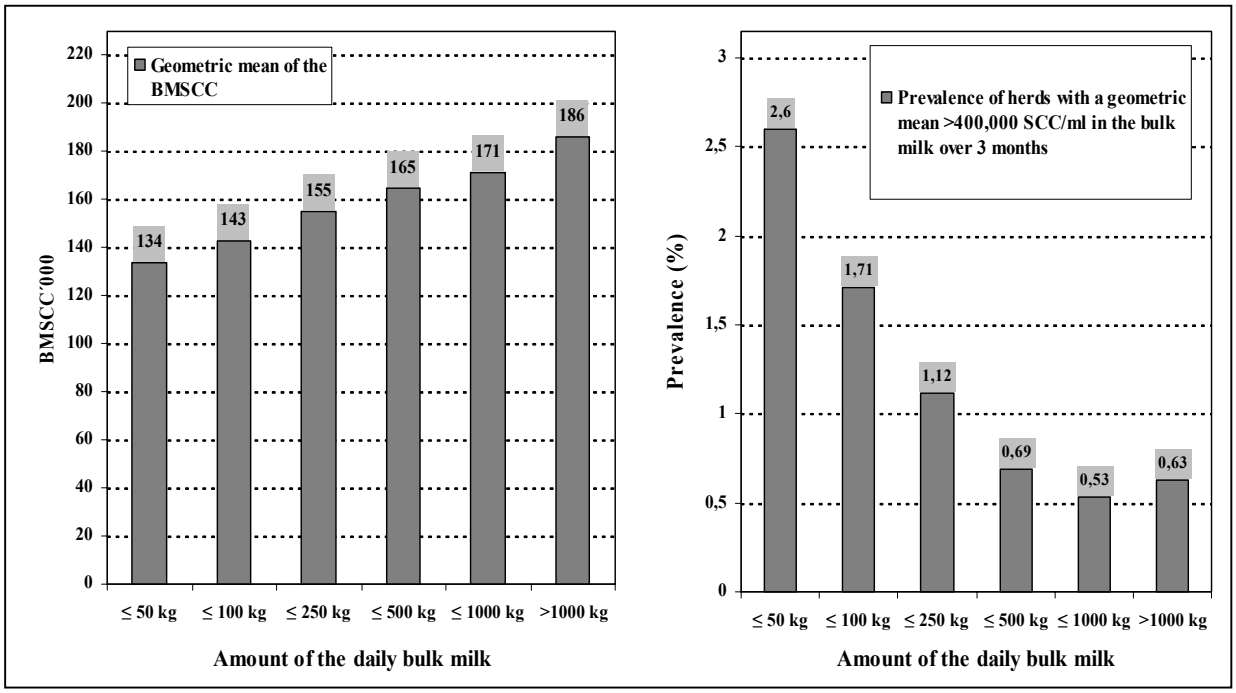
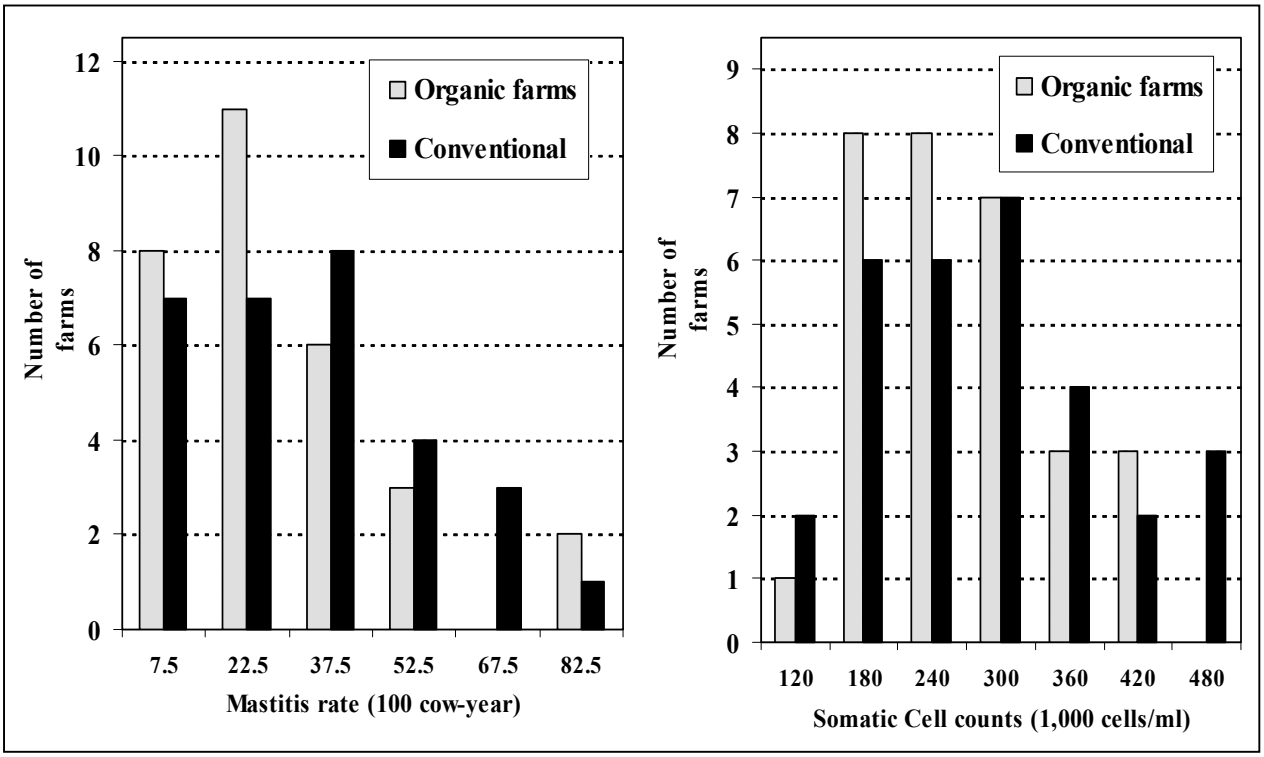


Figure 11: Frequency distribution of mastitis rates[1] and arithmetic mean of BMSCC[1] of organic farms (n=30) without using antibiotics and conventional farms (n=30) according to Sato et al. (2005)



[1]No statistical differences were observed (P > 0.3).

Figure 12 A: Number of cows, average milk yield and number of milking machines / 1000 cows in Finland from 1939 to 1990 according to Mylls et al. (1994)

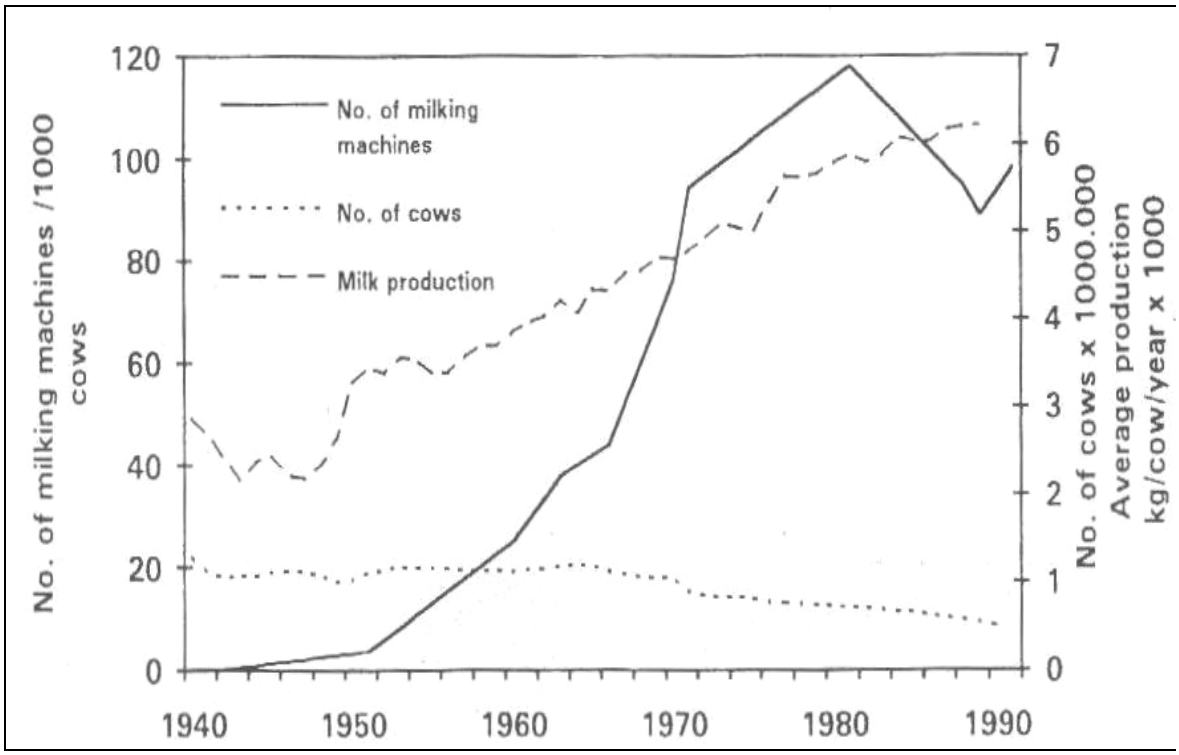


Figure 12 B: Percentage of *Streptococcus agalacticae*, *Staphylococcus aureus* and coagulase negative staphylococci (CNS) in all bovine milk samples with positive bacterial outcome, isolated between 1939 and 1990 at the national veterinary and Food Research Institute in Finland (total no. of quarter milk samples 1.15 million). Arrows indicate the first year when a repreSwentative of each antimicrobial group was advertised for the first time int Fininish Veterinary Journal (Mylls et al., 1994)

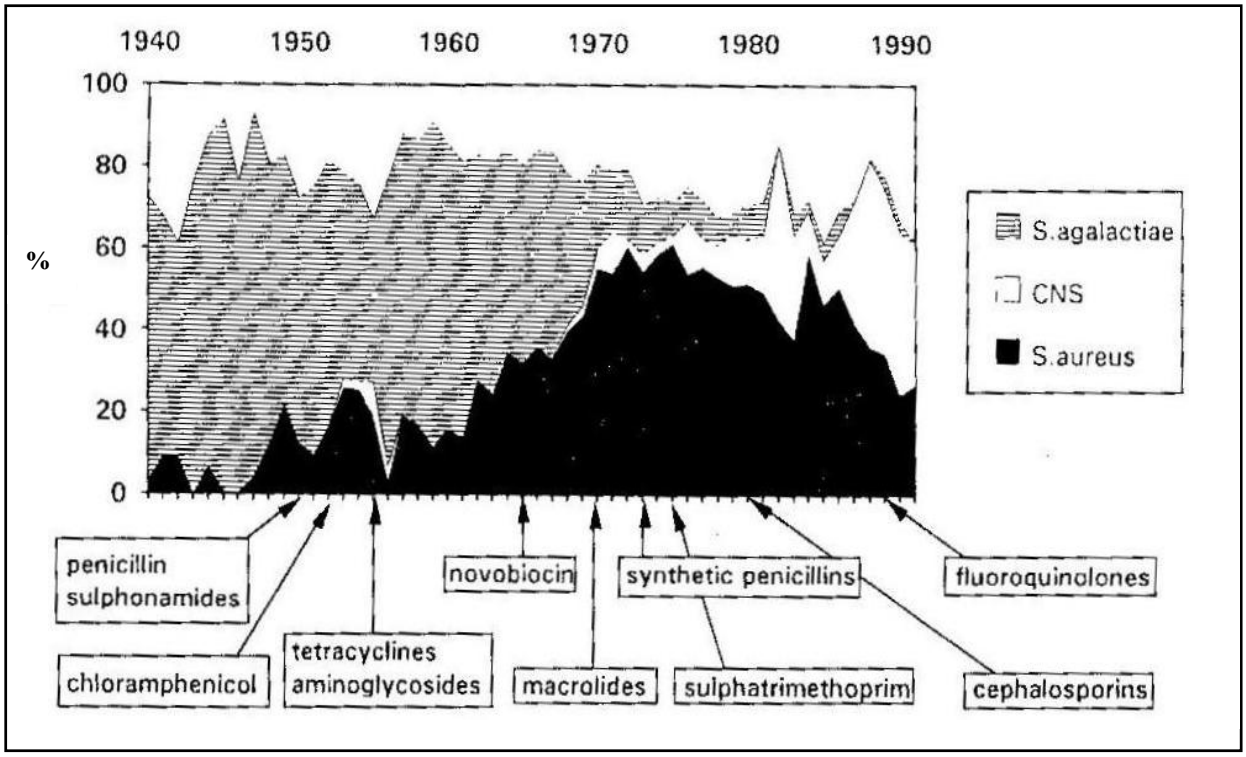


Table 6: Distribution of penicillin resistant staphylococci in herds with *Str. agalactiae* and herds free of *Str. agalactiae* according to Sabolic et al. (1980)

Distribution of herds with penicillin resistant staphylococci	Herds with *Str. agalactiae* (n=100)	Herds free of *Str. agalactiae* (n= 100)
No cow	21	56
Single cows	34	23
More cows	35	18
All cows	10	3

Table 7: Correlations between the kind of milking and results of electronic bacteria counts and SCC measurements per ml bulk milk or inhibitory tests according to Zeidler et al. (1969)

Kind of milking	**Number of herds**	**Arithmetic mean**		**Inhibitory substance positive** (%)
		Bacteria count (per ml bulk milk)	**SCC** (per ml bulk milk)	
Hand milking	1183	2,105,000	287,000	1,01
Machine milking	3926	1,367,000	326,000	2,24

Figure 13: Production of Enterotoxin A (+/- in Fig 4and Fig 5) in milk according to Tatini et al. (1971)

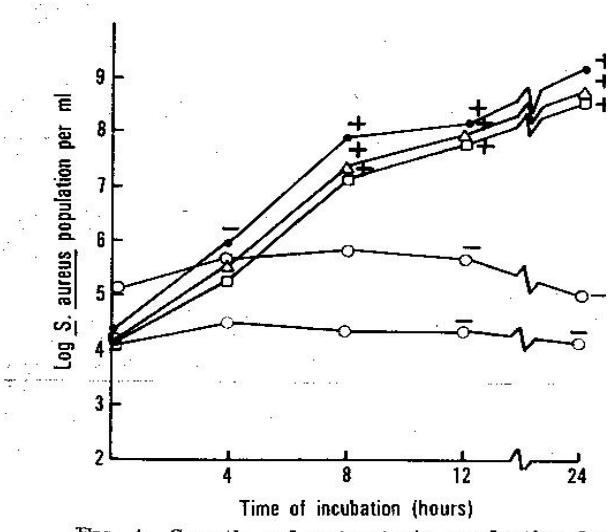


FIG. 4. Growth and enterotoxin production by *S. aureus* (F265) in raw and heated commercial whole milk at 32 C. ○—○ Raw milk (6.5 million/ml SPC); △—△ Milk heated at 62.8 C for 30 minutes (1,300/ml SPC); □—□ Milk heated 72 C for 16 seconds (1,000/ml SPC); ●—● Milk steamed for 30 minutes (<30/ml SPC).

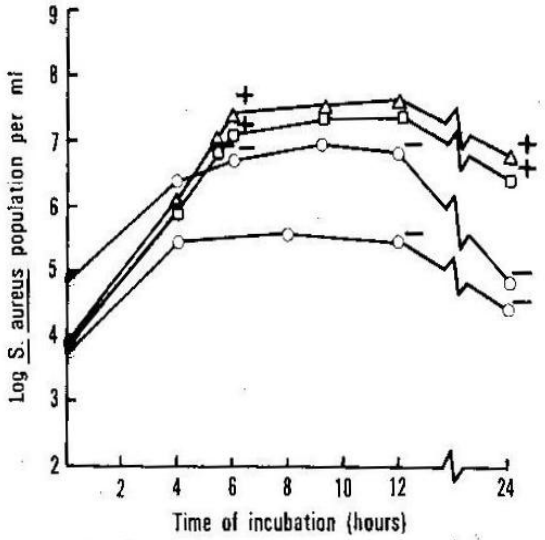


FIG. 5. Growth and enterotoxin production by *S. aureus* (196E) in raw and heated commercial whole milk at 37 C. ○—○ Raw milk (150,000/ml SPC); □—□ Milk heated at 65.6 C for 16 seconds (16,000/ml SPC); △—△ Milk heated at 72 C for 16 seconds (5,300/ml SPC).

Table 8: Correlation between *S. aureus*-values and SCC-values of 80 bulk tank milk samples from different farms producing raw milk products all over Germany (MLF Wangen, 1998; unpublished results; detection limit 0.1 ml milk)

BMSCC-Group	Number of samples	Samples with corresponding *S. aureus* -values in cfu/ml:			
		10-99	100-500	501-2000	> 2000[a]
< 50,000 SCC/ml	7	1	1	0	0
Positive samples			1 (28.6)		
50 -100,000 SCC/ml	16	2	3	1	3
Positive samples			9 (56.3)		
101-200,000 SCC/ml	18	2	5	3	1
Positive samples			11 (61.1)		
201- 400,000 SCC/ml	26	1	7	6	2
Positive samples			16 (61.5)		
>400,000 SCC/ml	15	1	5	1	1
Positive samples			8 (53.3)		
All samples	80	7 (8.8)	21 (26.3)	11 (13.8)	7 (8.8)
All positive			46 (57.5)		
Phenotypic characteristics (n=46)[1]:					
Egg yolk positive	30 (65.2)	5 (71.4)	13 (61.9)	7 (63.6)	5 (71.4)
No haemolysis[2]	16 (34.8)	3 (42.9)	4 (19.0)	5 (45.5)	3 (42.9)
Number Cl-[3]	14 (17.5)	3 (42.9)	9 (42.9)	2 (18.2)	0 (0.0)

[a] The highest *S. aureus* count in one sample was 22.000 cfu/ml

[1] In 12 (26%!) of 46 *S. aureus* positive bulk milk samples 2 different *S. aureus* phenotypes were detected, in one samples three different phenotypes and in three samples CNS were not distinguishable from *S. aureus* colonies on BP.

[2] α and ß-haemolysis were not confidently distinguished

[3] The prevalence of clumping factor negative strains

Table 9: *S. aureus*-values (cfu/g) of different raw milk products during 1996-2002 (MLF Wangen; unpublished results)

S. aureus **content in:**	**Yoghurt** (n=81)		**Fresh cheese** (n=165)		**Soft cheese** (n=405)		**Semi-hard cheese**		**Hard cheese**[1] (n=167)		**All Products** (n=1484)	
cfu/g	**Number**	**(%)**	**Number**	**(%)**	**Number**	**(%)**	**Number**	**(%)**	**Number**	**(%)**	**Number**	**(%)**
$<10^2$	56	69,1	99	60,0	202	49,9	314	47,1	113	67,7	784	52,8
10^2-10^3	7	8,6	13	7,9	57	14,1	74	11,1	22	13,2	173	11,7
10^3-10^4	7	8,6	17	10,3	58	14,3	93	14,0	11	6,6	186	12,5
10^4-10^5	6	7,4	27	16,4	38	9,4	112	16,8	12	7,2	195	13,1
10^5-10^6	4	4,9	9	5,5	39	9,6	56	8,4	8	4,8	116	7,8
10^6-10^7	1	1,2	0	0,0	7	1,7	17	2,6	1	0,6	26	1,8
$>10^7$	0	0,0	0	0,0	4	1,0	0	0,0	0	0,0	4	0,3
$>10^{4*}$	12	14,8	36	21,8	88	21,7	185	27,8	21	12,6	341	23,0
$>10^{5**}$	5	6,2	9	5,5	50	12,3	73	11,0	9	5,4	146	9,8

[1] These hard cheeses did not have reach a maximum temperature of 50° C during cheese making

Table 10: Relation between *S. aureus*- values and *E. coli*- values (cfu/g) of different raw milk products during 1996-2002 (see Table 9 above; MLF Wangen; unpublished results)

E.Coli (CFU/g)	**Distribution *S. aureus*** (CFU/g)[1]									
	$<10^3$		**10^3-10^4**		**10^4-10^5**		**$>10^5$**		**$>10^6$**	
	Samples (n)	**%**	**Samples** (n)	**%**	**Samples** (n)	**%**	**Samples** (n)	**%**	**Samples** (n)	**%**
$\leq 10^2$	446	**75.3**	86	**51.8**	65	**34.6**	22	**18.5**[a]	5	**17.2**[a]
10^2-10^3	63	**10.6**	26	**15.7**	40	**21.3**	28	**23.5**	9	**31.0**
10^3-10^4	45	**7.6**	26	**15.7**	34	**18.1**	32	**26.9**	4	**13.8**
10^4-10^5	19	**3.2**	16	**9.6**	30	**16.0**	21	**17.6**	4	**13.8**
10^5-10^6	11	**1.9**	6	**3.6**	15	**8.0**	6	**5.0**	2	**6.9**
$>10^6$	8	**1.4**	6	**3.6**	4	**2.1**	10	**8.4**[a]	5	**17.2**[a]
Sum	**592**	100.0	**166**	100.0	**188**	100.0	**119**	100.0	**29**	100.0

[a] These results show the close correlation between high *S. aureus* and high *E. coli* counts

Figure 14: Effect of sample handling (fresh or frozen) and sampling time on the distribution of S. *aureus* positive quarters (in cfu/ml) according to Godden et al. (2002)

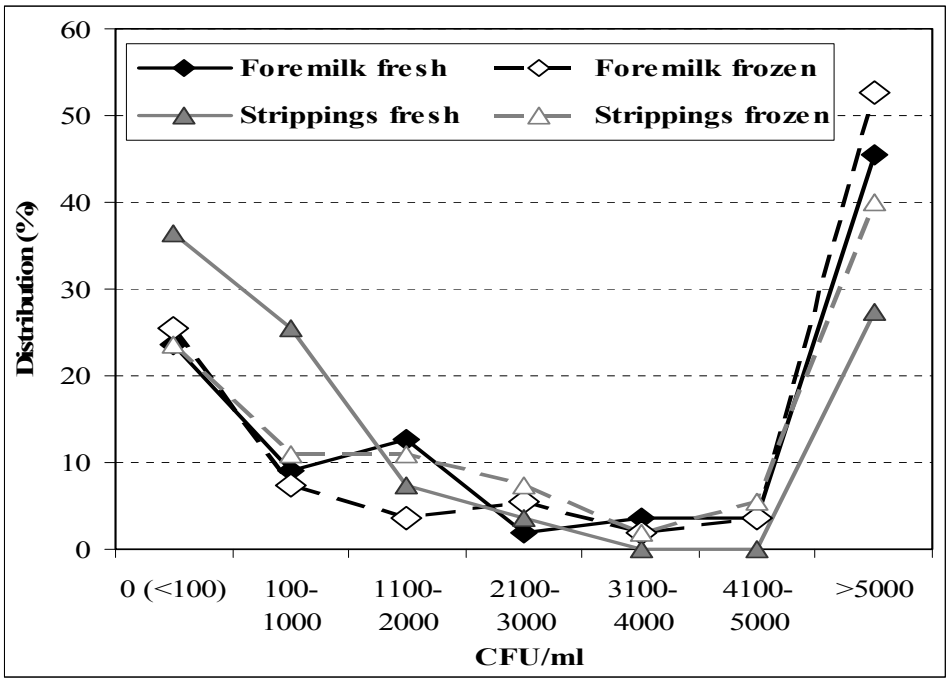


Figure 15: The shedding course of the somatic and bacteria cells from the first milk before cleaning (f_1), after cleaning (f_2) and during milking (0 = start of machine milking) from quarters with different kind of infections (Fig. A-C) according to Vries (1976):

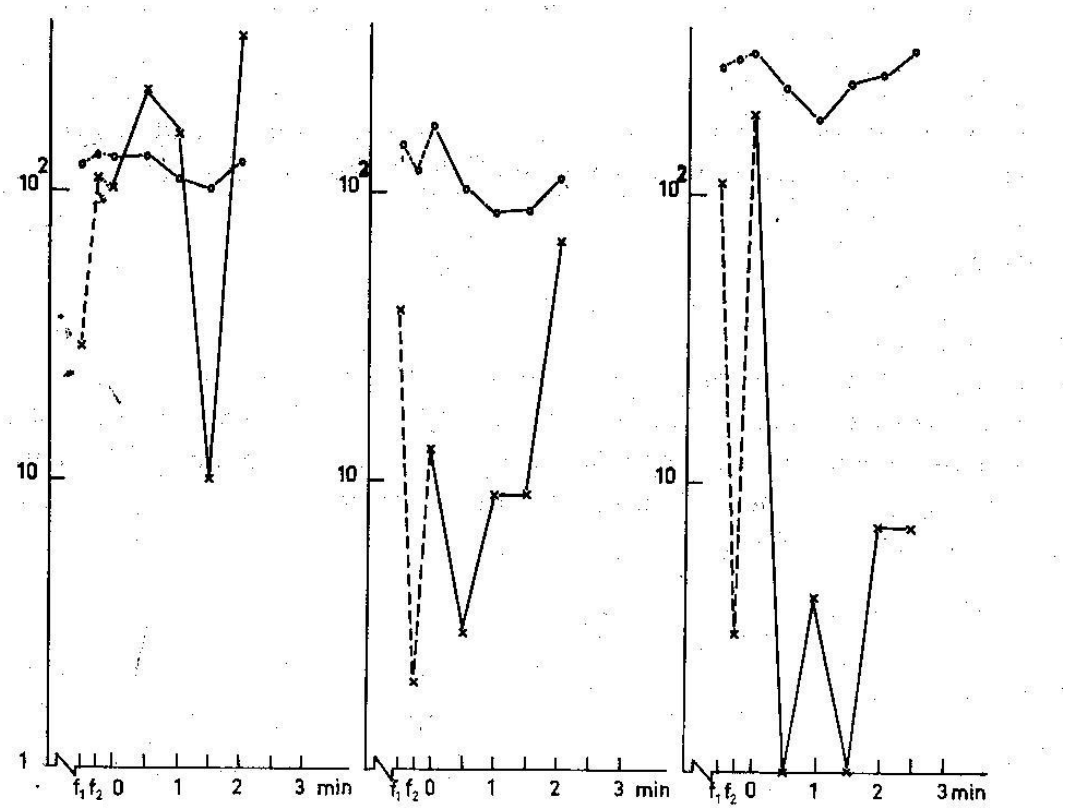
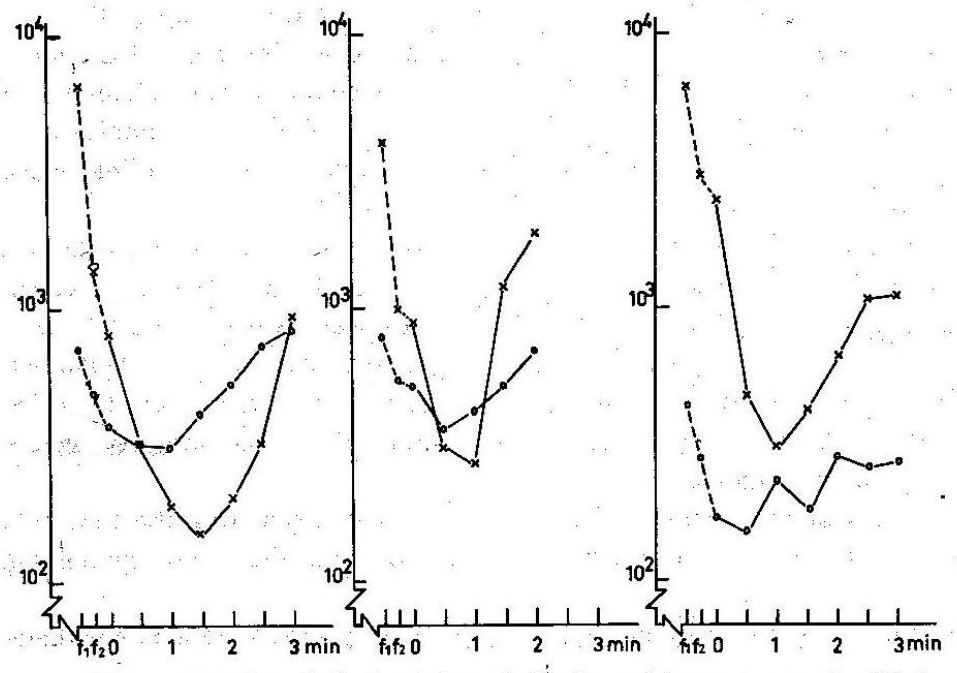
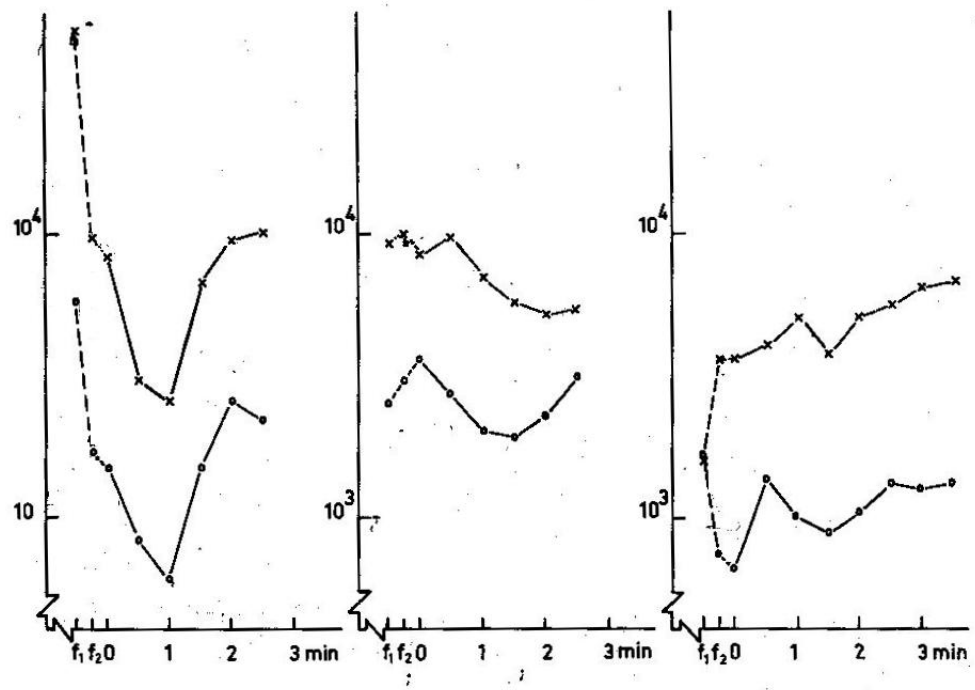


Fig. 2. Three examples of the variation of the bacterial count per ml milk (x——x) and the cell count per μl milk (0——0) during milking of slightly infected quarters.

Continued on Figure 15: Fig B and C (Vries, 1976)



Three examples of the variation of the bacterial count per ml milk (x——x) and the cell count per μl milk (0——0) during milking of quarters infected with staphylococci.



Three examples of the course of the bacterial count per ml milk (x——x) and the cell count per μl milk (0——0) during milking of quarters infected with streptococci.

Table 11: Average of the BMSCC, the herd size, prevalence of cows with *S. aureus* IMI in the herds and from heifers of HCPS and LCPS herds according to Roberson et al. (1994)

LCPS/HCPS Herds:	Average BMSCC (SCC´000/ml)	Initial herd prevalence		Heifers (1th lactation) at parturition	
		Herd size Number of cows	S.aureus IMI (%)	Cows Sampled (n)	S.aureus IMI (%)
LCPS herds[5] (n=9)					
A	341	137	1.5	38 (28)†	5.3
C	176	88	3.4	11 (13)	0
D	142	119	2.5	30 (25)	6.7
E	164	76	1.3	21 (28)	9.5
F	164	166	0	67 (40)	13
H	217	299	0.3	111 (37)	5.4
L	157	241	1.2	28 (12)	7.1
P	270	329	5	50 (15)	4
R	173	118	1.7	36 (31)	5.6
HCPS herds[5] (n=9)					
B	270	131	27	24 (18)	13
G	360	104	13	56 (54)	3.6
I	562	133	16	77 (58)	9.1
J	**301**	**39**	**56**	11 (28)	**27**
K	214	200	27	31 (16)	6.5
M	584	135	27	51 (38)	16
N	370	323	27	97 (30)	8.2
O	704	152	65	58 (38)	10
Q	426	88	28	31 (35)	3.2

†Prevalence of sampled heifers in relation to all sampled cows in brackets

Table 12: Prevalence of IMI by pathogen as determined in composite milk samples from primiparous and multiparous cows at two different time periods over 1 year in 18 northwestern US dairies according to Roberson et al. (1994)

LCPS/HCPS herds: Cows and sampling periods	Sampled (n)	Number of lactations and percentage of cows with IMI (Distribution %)									
		Total cows with IMI		*S. aureus*		CNS		Streptococci		Coliforms	
		1th	>1	1th	>1	1th	>1	1th	>1	1th	>1
LCPS herds (n=9)											
After calving	1325	17.3	27.2	2.0	2.4	12.9	15.2	2.3	4.5	1.5	4.2
Dry off	1025	10.8	22.0	1.2	2.1	8.8	13.0	0.5	3.6	0.8	0.8
Total	2350	12.3	21.6	1.2	1.8	9.5	13.0	1.0	3.5	0.7	1.9
HCPS herds (n=9)											
After calving	1131	19.7	30.8	3.5	16.5	13.1	9.6	2.7	2.4	2.4	2.6
Dry off	600	19.0	33.3	7.5	22.5	10.3	6.8	0.3	1.0	0.2	0.3
Total	1731	16.3	32.0	6.1	20.4	11.5	8.3	1.1	1.4	0.9	1.3

Figure 16: Incidence of new IMIs caused by *S. aureus* in previously uninfected dairy cattle in 9 herds during an 18-month control program according to Zecconi et al. (2003) (herds A, B,C, E, and F with initially < 20% positive *S. aureus* positive quarter samples were defined as the low prevalence group)

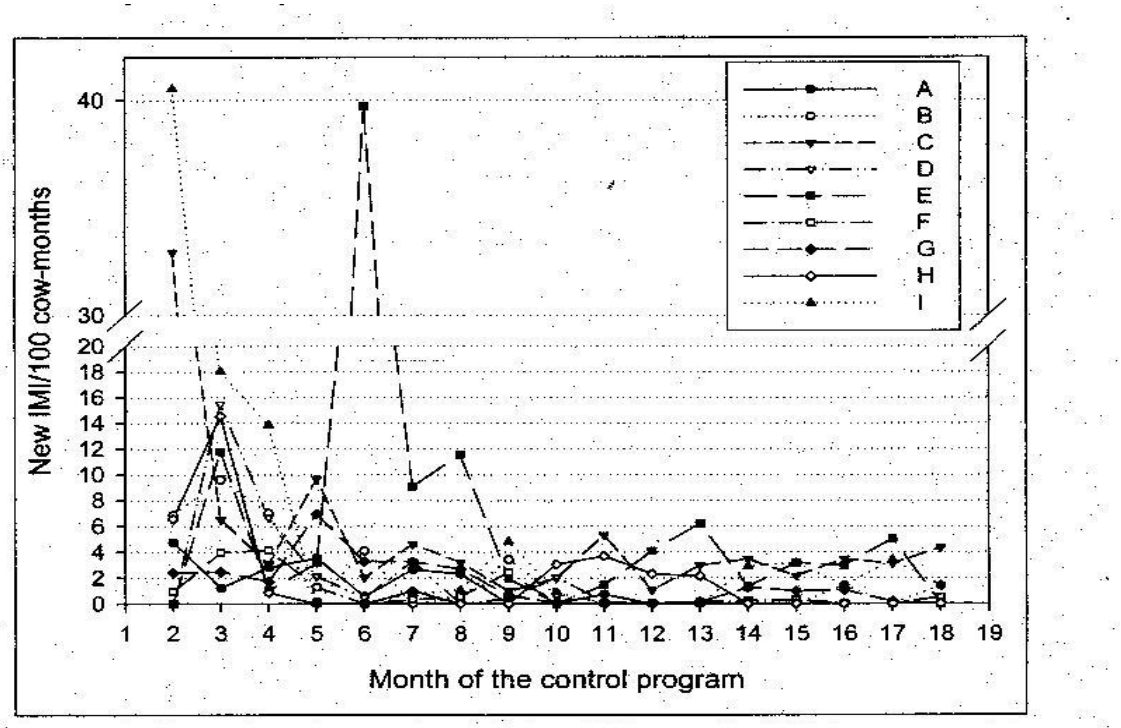


Figure 17: *S. aureus* IMI in 5 herds of the low prevalence group (LP) and 4 herds of the high prevalence group (HP) of infected cattle during various intervals for the duration of lactation according to Zecconi et al. (2003) * Signficant (P< 0.05) difference between groups

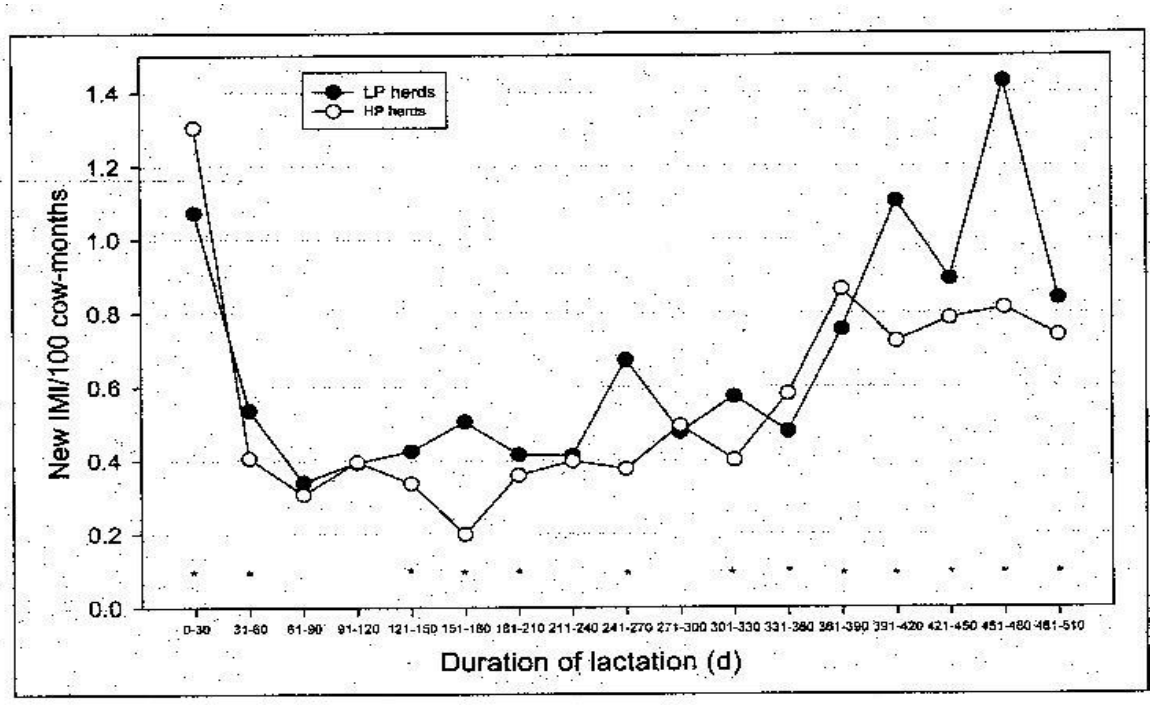


Figure 18: Mean incidence of new IMI`s with *S. aureus* in previously uninfected dairy cattle of various parities (1 to < 4) in 9 herds according to Zecconi et al. (2003)

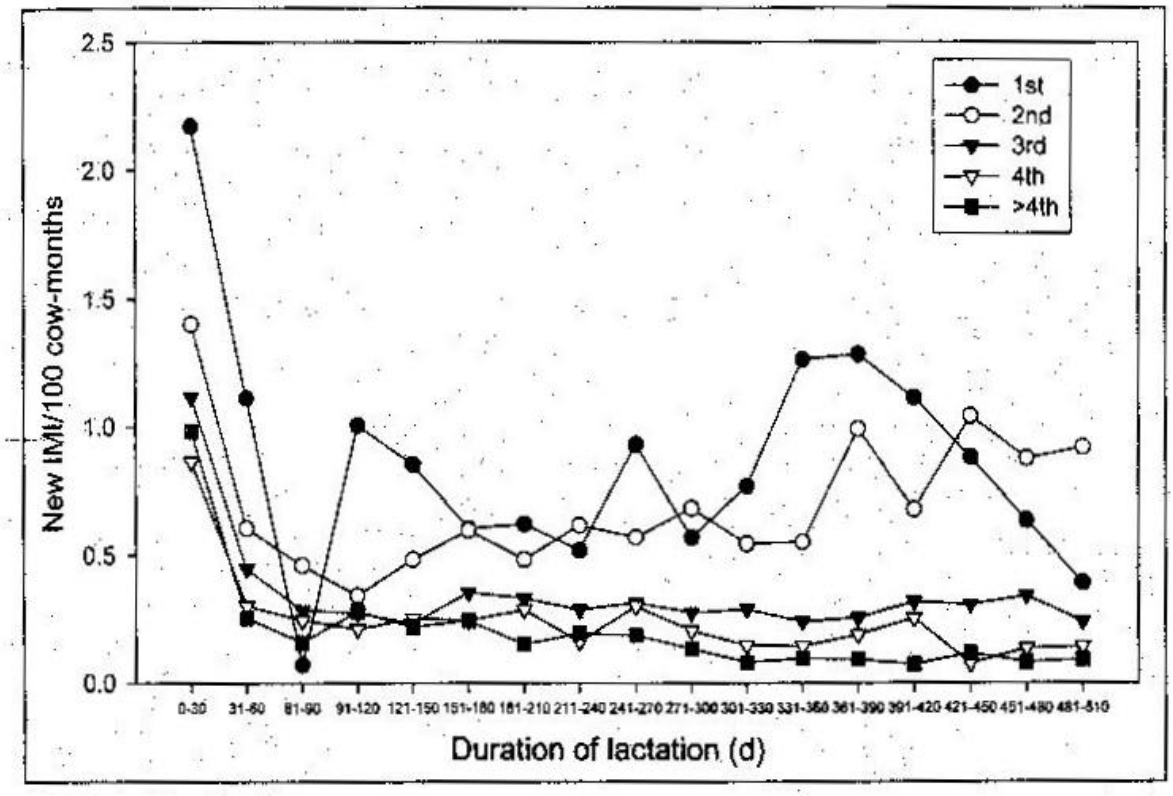
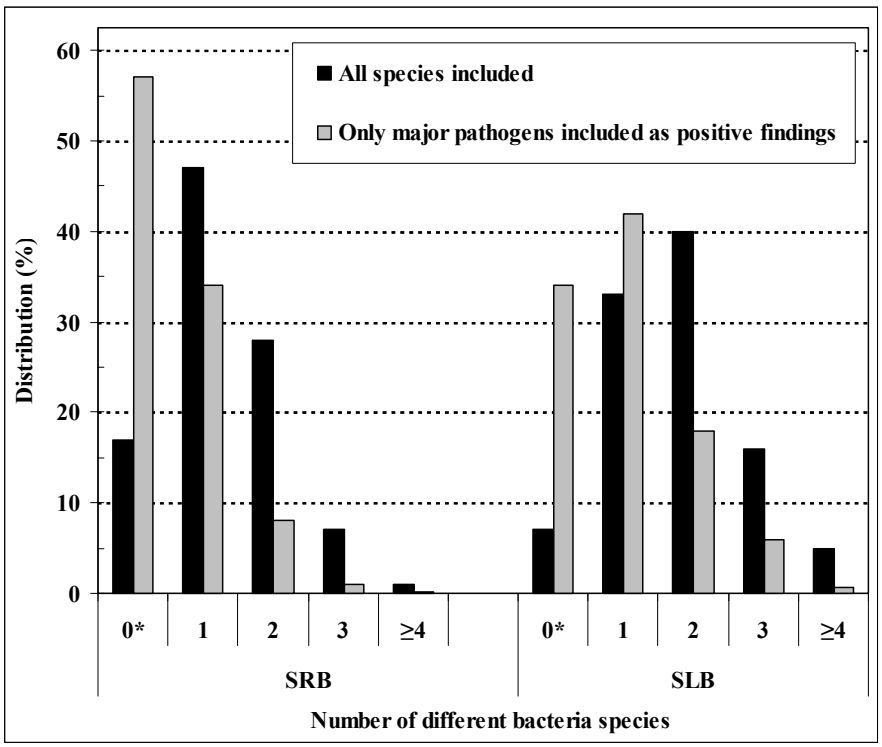


Table 13: Findings at the subsequent monthly sampling (%) of single quarters in relation to previous sampling intervals for the Swedish red white breed (SRB) and Swedish Friesian breed (SLB) according to Brolund (1985)

	Findings at the subsequent monthly sampling (%)				
	Negative	Staphylococci (CNS)	*S. aureus*	Streptococci	Gram negative
●SRB					
Negative	**81**	14	2	2	< 1
Staphylococci (CNS)	45	**45**	4	3	< 1
S. aureus	27	16	**54**	1	< 1
Streptococci	49	19	2	**30**	<1
Gram negative	39	16	5	5	**31**
Number of samples (n=25,568)	68.9	21.0	4.4	5.2	0.5
●SLB					
Negative	**70**	20	4	3	< 1
Staphylococci (CNS)	36	**47**	8	4	< 1
S. aureus	20	24	**49**	4	< 1
Streptococci	27	19	5	**46**	2
Gram negative	28	20	6	8	**33**
Number of samples (n=14,959)	50.6	28.4	9.4	10.5	1.1

Figure 19: Distribution (%) of the number of different bacteriological species[1] isolated within quarter and lactation for the Swedish red white breed (SRB) and Swedish Friesian breed (SLB) according to Brolund (1985)



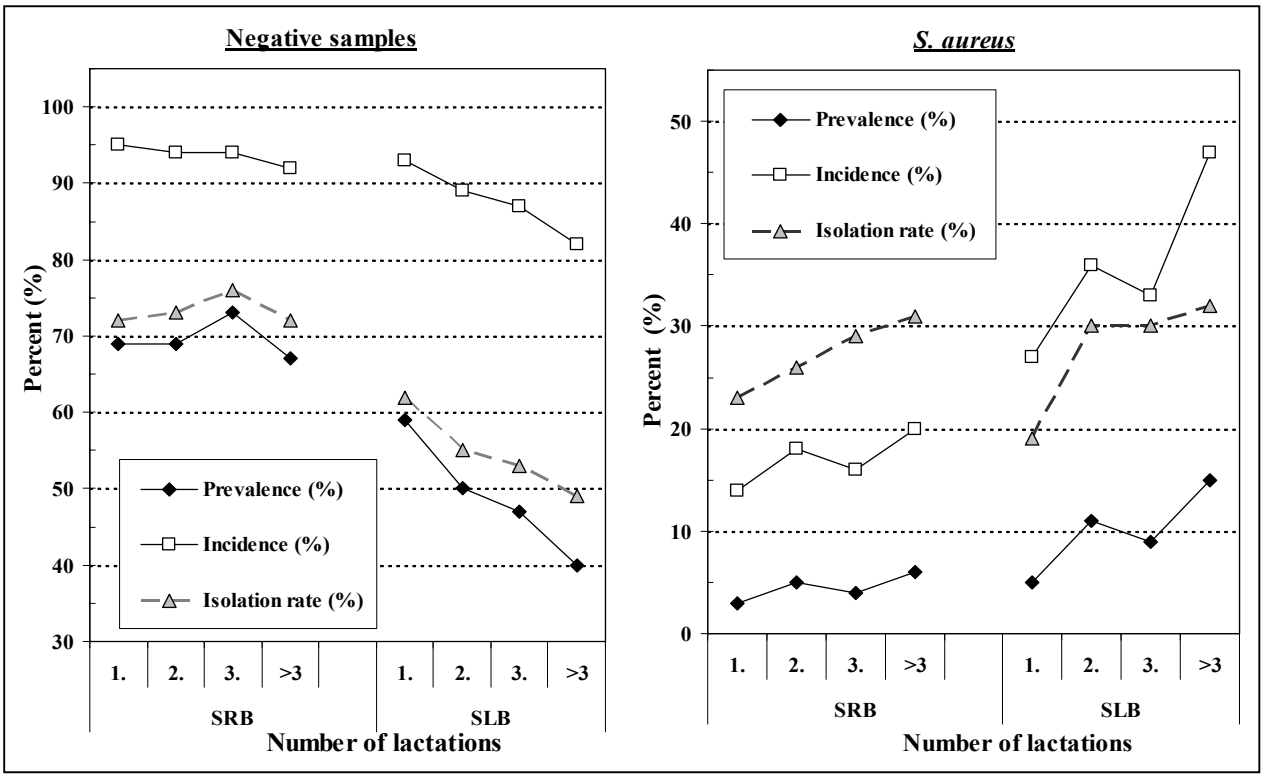
[1] Streptococci were classified as one species
[*] Only one bacteria species was isolated

Table 14: Prevalence of bacteriological/inflammatory status of quarters at 1th sample (one or six weeks before drying off) in comparison to the second samples at drying off according to Oesteras et al. (1991)

	1th sample		Distribution (%) of findings at drying off (2nd Sample)				
	Number of samples	(%)	Healthy[1]	Negative (high SCC)[2]	*S. aureus*	Staphylococci (CNS)	Streptococci
Healthy[1]	1,526	55.3	**66.4**	12.6	14.1	3.2	3.7
Negative (high SCC)[2]	207	7.5	**42.5**	**33.8**	13.5	4.8	5.3
S. aureus	615	22.3	22.9	5.9	**63.3**	3.6	4.4
Staphylococci (CNS)	231	8.4	31.2	12.6	16.9	**35.5**	3.9
Streptococci	181	6.6	27.6	9,9	12.7	4.4	**45.3**
Total	2,760	100.0	49.5	12.5	25.1	6.2	6.7

[1] No bacteriological diagnosis and no positive CMT
[2] Positive CMT but no bacteriological diagnosis

Figure 20: Prevalence[1] (%), incidences (%) and isolation rates (%) on a lactation basis for bacteriological negative and *S. aureus* positive findings in quarter foremilk (QFM) samples at monthly sampling during the whole lactation period for the Swedish red white breed (SRB) and Swedish Friesian breed (SLB) according to Brolund (1985)



[1]In the case of repeated examinations of quarters the prevalence (percentage of samples with a given bacteriological finding is a function of Dodd and Neave, 1970 quoted by Brolund (1985):

A. The incidence (percentage of quarters having the actual finding at least once during the lactation)

B. The isolation rate (%) of the actual finding within quarter and lactation given isolated at least once

C. A factor correcting for unequal number of observations per lactation.

Consequently the prevalence = A×B×C

Figure 21: Distribution of the number of *S. aureus* positive findings period for the Swedish red white breed (SRB) and Swedish Friesian breed (SLB) within quarter and lactation according to Brolund (1985)

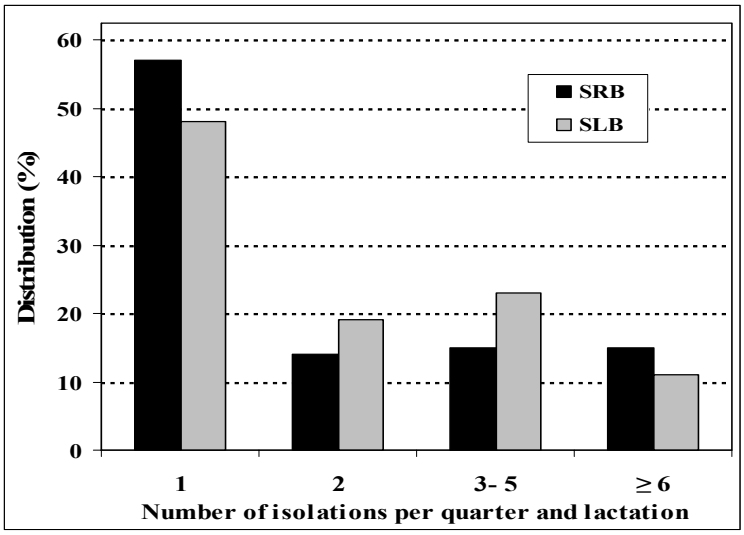


Figure 22: Relation between proportion of cows testing positive for major mastitis pathogens of the Streptococcus species and streptococcal counts in raw bulk tank milk. R2= 0.02 for log-transformed counts according to Zadoks et al. (2004)

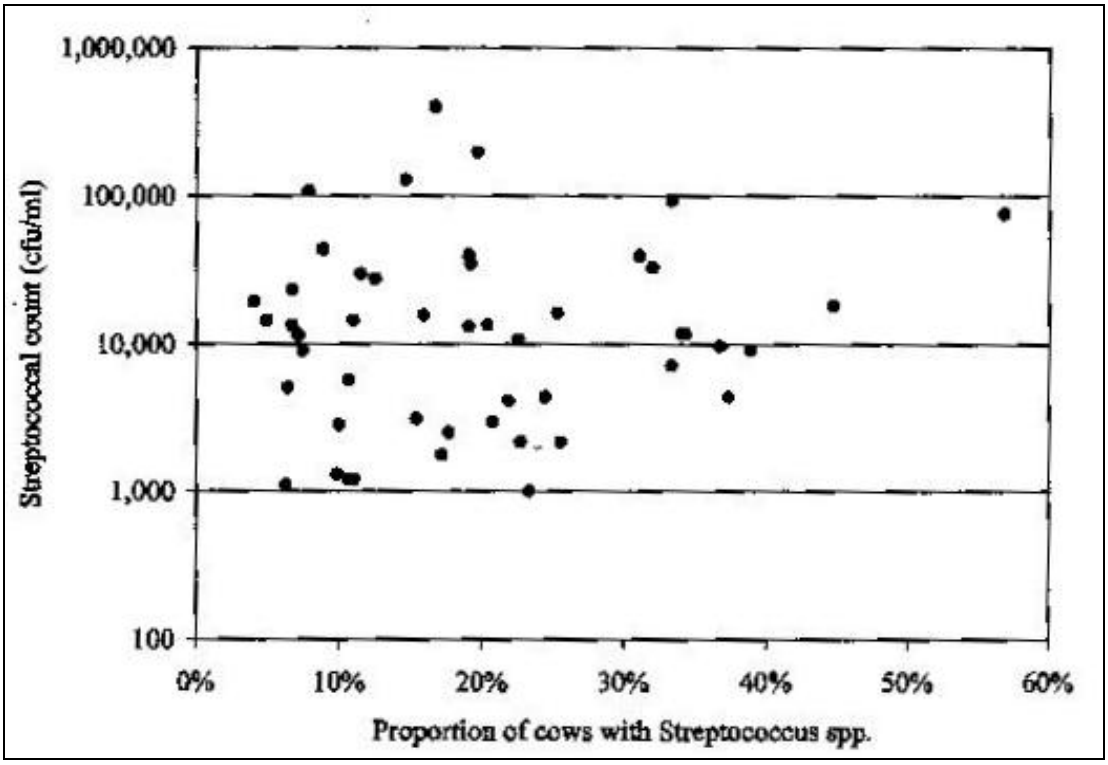


Table 15: Herd specific parameter of three commercial herds participating in a longitudinal study on risk factors for new IMI on the quarter level with *Str. uberis* and *S. aureus* in the Netherlands according to the results of Zadoks et al. (2001)

Characteristic	**Herd A**	**Herd B**	**Herd C**
Herd size[1]	67± 3	95± 5	41± 2
BMSCC[2]	266± 76	235± 75	205± 69
Milking parlour	2×5 herring bone	2×4 open tandem	2×5 herring bone
Udder health management:			
Teat dipping	no	yes/no[3]	yes
Antibiotic treatment clinical cases of mastitis	yes	yes	yes
Blanket dry cow treatment	yes	yes	yes
Routine culling of chronically infected cows	no	yes	no
Milking clusters were flushed with hot water (90°C) after milking of *S.aureus* infected cows	no	no	yes
Prevalence new *S. aureus* IMI	41.0 %	19.0 %	40.0 %
Prevalence new *S. uberis* IMI	24.7 %	49.5 %	25.8 %

[1] Mean number of cows at each routine sampling during the study ± standard deviation

[2] Arithmetic mean of bulk milk SCC (in 1000 cells/ml) in year preceding study

[3] Teat dipping was practiced in Herd B from sampling 1 to 7, and from sampling 16 to 20

Figure 23: Number of new infections with *S. uberis* and *S. aureus* per 3 week sampling interval in Herd A, B and C (Appendix: Table 15 before) according to Zadoks et al. (2001)

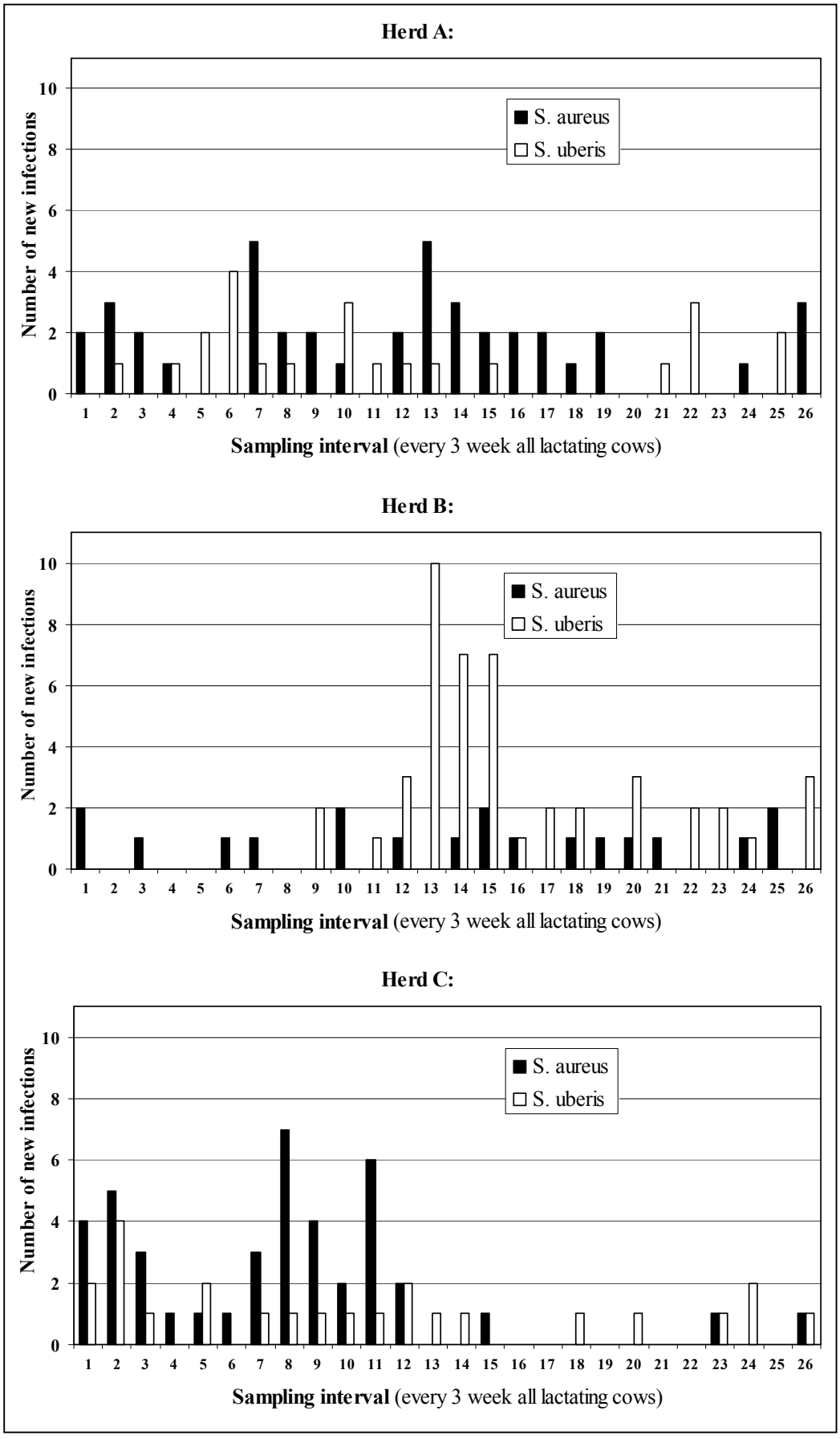


Table 16: Risks of cases treated by veterinarians for 41989 multiparous Finnish Ayrshire cows during the lactation according to Gröhn et al. (1990)

	Lactational incidence risk		Median week post- partum of diagnosis
	Number of cows	%	
Ketosis	2755	**6,6**	**4,0**
Rumen acidosis	131	0,3	8,0
Udder edema	141	0,3	2,0
Acute mastitis	2946	**7,0**	**6,3**
Chronic mastitis	860	2,0	**14,7**

Figure 24: Mean values for calculated energy balances (EB) and calculated protein balances (PB) of cows during the first 14 weeks of lactation according to Beerda et al. (2007). Changes in time were significant for both EB and PB.

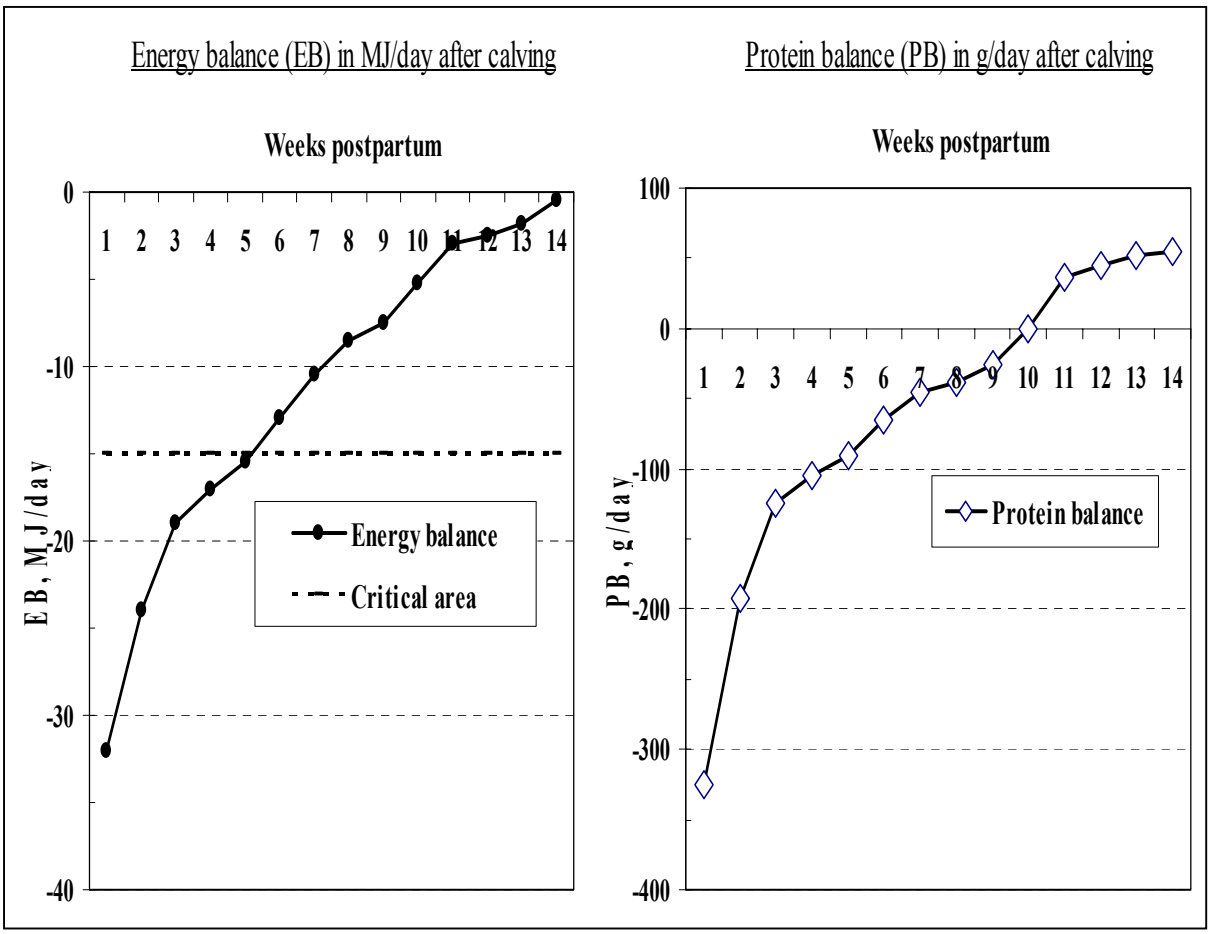


Table 17: Influence of different deficiencies in feeding (ration with to low energy and high protein content) on SCC, bovine mastitis and the healthy status of cows (Wendt et al., 1998)

Factor	Effect	Consequences
• **Negative energy balance after calving** Ketosis: - subclinical - clinical	• Liver disease • reduced phagocytosis • supressed immune defense	• mastitis • elevated SCC • elevated fat content
• **Too high protein content**	• NH_3 / Rumen • high urea content • stress of the liever	• subclincial mastitis • clinical mastitis • elevated SCC
• **Too high content of carbohydrates lack of crude fibre**	• Rumen acidosis • deficiency of calcium	• elevated SCC • clinical mastitis
• **Toxic fooder** -Phytoestrogen -Mycotoxins	• Estrogen effect • supressed immune defense • reduced phagocytosis	• Edema • elevated SCC • acute mastitis
• **Nitrate/Nitrite** Liquid manure- N Fedding of cabbage	• supressed immune defense • reduced oxygen content in the blood • Irritation of the mucous membrane	• elevated SCC • rising need of vitamins • mucous membrane diseases
• **Deficiency of carotin, Vitamin E or Selen**	• supressed immune defense • Deficiency of antioxidants	• subclinical mastitis • latent infections

Table 18: Influence of feeding (ration with to low energy and high protein content) on the number of *S. aureus* shedding cows in herd F, SCC and urea content (> 30mg/100ml) when consecutive sampling of 32 cows (3rd sampling interval see Table 14)

SCC in thousand / ml	Number of cows with the corresponding SCC- and *S. aureus* - values							
	S. aureus - values of shedding cows in cfu/ ml						Identical cows 13.10./12.11	Elevated SCC-values 13.10./12.11
	Sampling at 13.10. 03			Sampling at 12.11. 03				
	10^1-10^2	10^2-10^3	10^3-10^4	10^1-10^2	10^2-10^3	10^3-10^4		
< 50	1		2		4		3	2
50-99				1	1	1		
100- 199	1		1	1	11	1		10
200- 499		1		1	2		1	1
500-1000	2							1
> 1.000				1				
Sum	**8**			**24**[a]			**4**	**14**
Urea content >30 mg	**2**			**17**[b]			**2**	**13**

[a] One *S. aureus* shedding cow came in the dry off period and three shedding cows had calved since the 13.10. 2003

[b] 12 of 17 cows had at the 13.10. 2003 a urea content of less than < 21 mg/ml

Table 19: Frequency of lytic reactions at RTD or 100×RTD of poultry, human and bovine strains with a set of poultry, human or bovine phages according to Hajek and Howak (1978)

Phag set / origin of strains (Biotype)	Number of strains	Phage group									
		I	II	III	IV	96	80/81	I/96	M	Mixed groups	Not typeable
		Distribution (%)									
Poultry phage set[1]						xxx[4]	xxx	xxx	xxx		
Human clinical material (A)	50	- / 2*	-	2 / 2	-					2 / -	96 / 92
Chicken (B)	100	74 / 1	18 / 1	2 / 0	-					- / 1	6 / 3
Cow (B)	90	- / 1	- / 12	4 / 8	-					2 / 33	93 / 40
Human phage set[2]					xxx						
Human clinical material (A)	50	8 / 4	2 / -	42 / 20		-	4 / 0	-	-	-	44 / 20
Chicken (B)	100	-	-	14 / 20		-	-	-	-	-	86 / 66
Cow (B)	90	11 / -	- / 1	33 / 3		7 / -	-	17 / 5	-	- / 1	32 / 22
Bovine phage set[3]				xxx		xxx	xxx	xxx			
Human clinical material (A)	50	-	-/1		-				-	-	100 / 98
Chicken (B)	100	- / 2	-		1 / 1				-	-	99 / 96
Cow (B)	90	42 / -	-		56 / -				-	1 / -	1 / 1

* RTD / 100×RTD

[1] Phage set with 22 poultry phages of Shimizu (1976)

[2] The new international human phage set since 1975 was used. It does not contain phage 42D of phage group IV anymore.

[3] Following phages were used: Group I: bovine phage AC1; Group II: bovine phage 116; Group IV: bovine phages 102, 107, 108, 111, 117 and the human phages 42D, 42F; Miscellaneous (M): the human phage 78 and the bovine phage 118 and 119

[4] This phage group do not exist in this phage set.

Table 20: Classification of *S. aureus* from different hosts in certain biotypes according to Hajek and Marsalek et al. (1971)

Biotype	Classificatory							Typical occurence
	Fibrinolysin	Pigment	Coagulation of human plasma	Coagulation of bovine plasma	α- haemolysin	β- haemolysin	Sensitivity to phages	
A	+[1]	+	+	-	+[1]	V-	H	Men
B	-	+	+	-	V+	V+	H/B	Swine, poultry
C	-	+	+	+	V	+	H/B	Cattle, sheep
D	-	V-	+	-	-	+	H	Hares
E[2]	-	-	-	+	-	+	C	Dogs, horses, mink
F[2]	-	-	+	+	V-	+	-	Pigeons

+ = more than 80% of organisms positive; - = more than 80% of organisms negative;

V = variable (V+ and V-: predominace of positive or negative organisms)

H= international basic set for human strains, still with phage 42D; B= Davidson`s set of phages (with huamen and bovine phages); C= phages from staphylococci adapted to dogs (canine)

[1] This characteristics are according to Pulverer (1964), Meyer (1966) and Gedek, (1972) high correlated to a positive egg yolk reaction.

[2] This strains were later according to Hajek and Marsalek (1976) the type strains for the new species *Staphylococcus intermedius*.

Table 21: Classification of *S. aureus* from different host to the corresponding biotypes according to Appendix Table 20 (see above) and their production of enterotoxins (Hajek and Marsalek et al., 1973)

Origin of strains (n)	Biotype (%)	Enterotoxin produced (%)			
		A	B	C	AB
		(n= number of strains per host respectively side)			
Human pneumonia (n=91)	**A** (99)	**7**	**40**	**8**	**7**
	C (1)	-	-	-	-
Human carriers (n=53)	**A** (100)	**8**	**15**	**15**	-
Pigs (n=65)	**A** (23)	-	**4**	-	-
	B (55)	-	-	-	-
	C (20)	-	-	-	-
	NT[1] (2)	-	-	-	-
Cows-mastitis (n=79)	**A** (2)	-	-	**1**	-
	C (94)	-	-	-	-
	NT (4)	-	-	-	-
Cows-carriers (n=74)	**A** (9)	-	-	-	-
	C (86)	-	-	-	-
	NT (4)	-	-	-	-
Sheep mastitis (n=25)	**A** (12)	-	-	-	-
	C (88)	-	-	**16**	-
Horses (n=65)	**A** (23)	-	**3**	-	-
	B (55)	-	-	-	-
	C (20)	-	**3**	-	-
	NT (2)	-	-	-	-
Chicken (n=20)	**A** (20)	-	**5**	-	-
	B (70)	-	-	-	-
	NT (10)	-	**10**	-	-
Dogs (n=58)	**A** (17)	**9**	**3**	**2**	-
	E (83)	-	-	-	-

[1] Nontypable to certain biotypes

Table 22: Characterisation and host-specific allocation of 662 *S. aureus* strains from cattle according to Hummel et al. (1992)

Host- specific allocation of strains and in 11 herds (number of isolates in brackets)	**Phage group**				
	I	**I/119[b]**	**II**	**III**	**IV (Variety bovis)**
	Distribution (%)				
Percent of strains (662)	3	52	21	2	22
Number of strains from mastitis cases (54)	0	48	26	2	24
Clumping factor negative	0	**93**	26	0	8
Biochemical reactions					
Coagulation of rabbit plasma	+	+	+	+	+
Coagulation of human plasma	+	+	+	+	+
Coagulation of bovine plasma	-	-	-	-	+ / (+)[1]
Crytal violet type	C	C	A	C	A
Haemolysin type	β	β	β or α	β	β
Fibrinolysin type	-	-	- or +	-	-
Herd 1 (53)	2	**92**	3	0	0
Herd 2 (58)	0	**86**	9	2	2
Herd 3 (29)	0	**89**	2	0	3
Herd 4 (71)	0	**79**	13	1	7
Herd 5 (33)	12	**76**	10	0	3
Herd 6 (31)	10	**58**	16	0	16
Herd 7 (26)	12	50	19	0	19
Herd 8 (72)	0	33	46	0	21
Herd 9 (25)	0	36	0	0	**64**
Herd 10 (32)	0	3	28	0	**69**
Herd 11 (28)	4	0	7	18	**72**

[1](+) delayed positive reaction
[b](+) Phage group I with the HPS and phage type 119 with the BPS

Table 23: Phenotypic characteristics and drug resistance from *S. aureus* of different collections in the dairy chain or human isolates (Following important antibiograms: Peniclin (Pen), Penicillin and Tetracyclin (Pen+TE), Penicillin and Streptomycin (Pen+S))

Source of isolates	Number of strains	Phenotypic characteristics (%)						Antibiotic resistence (%)			
		Haemolysis				CF-	Egg-yolk positive	Pen	Pen+TE	Pen+ S	Other
		no	weak	α	β/αβ						
Strains from the Dairy chain:											
1. Collection A: bovine mastitis	213	4	6	10	80	31	53	24	1	0	4
2. Collection B: bovine mastitis	72	10	17	21	52	35	36	37	0	0	1
4. Germany: Bulk milk[1]	82	18	8	8	64	35	55	30	1	0	0
6. Germany: Cheese making farms[2]	93	15	8	24	53	8	41	36	1	0	3
3. Austria: Bulk milk[3]	115	7	13	9	71	6	36	42[a]	4[a]	7[a]	6
5. Austria: Cheese making farms[4]	120	8	13	27	53	5	53	30	1	1	2
Human strains:											
7. Switzerland: Nasal[5]	147	49	18	21	12	1	90	45	1	0	1
8. Austria: Human milk[5]	188	54	18	5	23	4	77	42	7	1	5

[1] These isolates come from small dairies in the south-west of Germany producing raw milk hard cheese (the dominating *S. aureus* subpopulation were the clumping factor negative biotypes)

[2] These strains come from raw milk cheese making farms in the southern Germany (no dominating subpopulation many intermediary biotypes in mixed groups with human and bovine phages.

[3] These *S. aureus* isolates are from the collection of Zangerl (1999) considering only bulk milk samples from Tirol and Salzburg (The dominating suppopulation belongs to phage group IV)

[4] These strains are from the collection of Zangerl (1999) considering only *S. aureus* from raw milk cheese making farms (no dominating subpopulation many intermediary biotypes in mixed groups with human and bovine phages).

[5] The phage typing results from these strains are shown in detail in Appendix Table 38

[a] Most of these antibiotic resistant strains belong to phage group IV

Table 24: 49 bovine S. *aureus* dairy isolates, two strains from bovine mastitis inclusive one from goat´s and sheep´s cheese with enterotoxin genes (*se*) listed in order to the enterotoxin-profile[1] with corresponding characteristics and phage typing results[2] at the Robert Koch Institute using all 37 phages for routine typing. The strains with exception of six isolates are part of the collection from Austria according to Gonano et al. (2009).

Strain number	Elective criteria and other characteristics					Phage typing with all Phages of the human and bovine phage set inclusive 7 additional Phages					
	Phenotypic characteristics			Enterotoxin genes	Antibio-gram	Human set and additional Phages				Bovine set and additional Phages	
	CF	Egg yolk	Haemolysis			Phage group I	Phage group II	Phage group III	M / Group V	Lyogruppe IV	M
Mastitis 1[a]	+	+	αβ	sea	**P**	-	-	-	-	**117**	-
Mastitis 2[a]	+	+	αβ	sea	**P**	-	-	-	-	**117**	-
111	+	+	β	sea	**P**	-	-	85	-	117	-
327[b]	+	+	α	sea/sed	**P**	-	-	**6**/42E/**47**/**53**/**54**/**75**	81	42F/117/111	-
326[c]	+	+	α	sea/sed	**P**	29/79	-	6/42E/47/**53**/54/75/84/85	81/95	42F/**102**/**117**/**111**	78/118
2	+	+	α	sea/sed/seg/sei/sej	**P**	-	-	6/42E/47/**53**/75	81/825	42F/102/117/**111**	78
34[d]	+	+	α	sea/sed/seg/sei/sej	**P**	79	-	53/77/84	-	102/111	-
159	+	+	α	sea/sed/seg/sei/sej	**P**	29/52/52A/79/80	-	6/47**53**/54/75	81/812/825	102/**111**	-
84	+	+	α	sea/sed/sej	S	-	-	53	95	-	-
21	+	+	w	sea/seg/sei	S	**79**	-	-	95	-	-
115	+	+	w	sea/seg	**P**	-	-	75	-	-	-
242	+	+	no	sea/seg	**P**	**29**/**52**/52A/**79**/**80**	-	75	-	-	118
42	+	+	w	sea/seh/sej	S	**79**	-	42E/47/54/85	-	42F/107/117	-
59	+	+	α	SEA[3]; /seb/sec	S	-	-	-	- 187	-	-
237	+	+	β	seb	**P**	52/79/80	-	-	-	102/**108**	118
238	+	-	β	seb	**P**	**52**/52A/79/**80**	-	-	-	42D/102	118
200	+	+	w	seb/sec	S	**Not typeable**					
205	+	+	αβ	seb/seg/sei	S	-	-	-	**94**/**96**	42D	-
204	+	+	w	seb/seg	**P**	**Not typeable**					
209	+	+	w	seb/seg	**P**	**Not typeable**					
174	+	+	α	sec	S	79	-	-	-	-	118
253	+	+	α	sec	S	-	-	-	-	-	118
290	+	-	β	sec	S	-	-	-	-	-	**78**
207	+	+	w	sec/seg/sei	S	52/79	-	-	95	-	-
178	+	+	β	sec/seg/sei	S	-	-	-	95	-	-
100	-	-	αβ	sec/seg/sei	S	29/**52**/**52A**/79/**80**	**55**	-	**94**/**96**	102/108	**119**
281	-	-	αβ	sec/seg/sei	S	52A/**80**	55	-	**96**	-	**119**
158	-	-	αβ	sec/seg/sei	S	79/80	-	-	**96**	-	**119**
29[e]	+	-	β	sec/seg/sei/sej	S	-	116	-	-	107	737/**740**
216	+	-	β	sec/seg/sei/sej	S	52A/80	55	-	**96**	-	119
212	+	+	αβ	sed/sej	**P**	-	-	6/**47**/53/54	81	**108**	-

Continued on Table 24: Elective criteria and phage typing results of strains with no enterotoxin production but enterotoxin genes *sei, seg*, or *sej*

Strain number	Elective criteria and other characteristics					Phage typing with all Phages of the human and bovine phage set inclusive 7 additional Phages					
	Phenotypic characteristics			Enterotoxins	Antibio-gram	Human set and additional Phages				Bovine set and additional Phages	
	CF	Egg yolk	Haemolysis			Phage group I	Phage group II	Phage group III	M / Group V	Lyogruppe IV	M
330	-	-	αβ	seg/sei	S	52/52A/79/80	-	-	-	-	**119**
157	-	-	αβ	seg/sei	S	29/**52**/52A/80	-	-	**94/96**	-	**119**
106	+	-	αβ	seg/sei	S	**52/52A**/79/**80**	55	-	**94/96**	-	**119**
63	+	-	αβ	seg/sei	S	29/**52**/52A/79/**80**	55/116	-	**94/96**	107/111	**119**
145	+	+	αβ	seg/sei	S	-	-	-	**94/96**	-	-
63	+	-	αβ	seg/sei	S	-	-	-	**94/96**	-	-
164	+	+	w	seg/sei	S	-	-	-	**94/96**	-	-
210	+	+	w	seg/sei	**P**	-	55/**116**	-	-	-	-
114	+	+	w	seg/sei	**P**	-	**3A/116**	-	-	-	-
120	+	+	β	seg/sei	**P**	-	**3A/116**	-	-	-	-
160	+	+	β	seg/sei	S	-	**3A/55**/116	-	-	-	-
44[e]	+	+	β	seg/sei	S	79	**3A/116**	6/42E/47/**53**/54/75/84	81/812/825	42D/102/117/108/111	78/737
66	+	+	β	seg/sei	S	-	**3A/116**	-	81/812/825	102	-
230	+	-	β	seg/sei	**P**	-	-	-	-	42F/102/107/108/111	-
109	+	+	β	seg/sei	S	**Not typeable**					
223	+	+	α	seg/sei	**P**	**Not typeable**					
224	+	+	w	seg/sei	**P**	**Not typeable**					
258	+	+	β	sej	**P**	-	-	-	-	42F/**102**/**107**/117/ **108/111**	-
3	+	+	β	sei	S	**Not typeable**					

[1]All enterotoxin A and D producing isolates were tested bovine plasma positive on the Robert Koch institute (all were bovine plasma positive; with exception of one strains all showed strong reactions still after 3 hours). **CF**, clumpingfactor. Antibiogram: **P**, Penicillin resistance; S, susceptible.

[2] Phage reactions >20 plaques were considered as positive result. Bold marked phages had semiconfluet or confluent lysis at 100×RTD.

[3] This strain was the single isolate with a enterotoxin production but no corresponding *se* gene (Only SEC and SEB but no SEA was confirmed by PCR).

[a] These are two enterotoxin producing strains (tested by VIDAS) from 20 penicillin resistant isolates from collection A (the national monitoring program, Germany, 2001).

[b] This strain comes from a food poisoning outbreak in France (probably outbreak 3 according to Kerouanton et al., 2007, Appendix Table 49).

[c] This strain comes from a food poisoning outbreak in Austria with fresh cheese.

[d] This strain form fresh cow´s milk cheese comes from the same farm how strains number 2.

[e] These are the only two isolates, which come from sheep´s (29) or goat´s raw milk fresh cheese (44). (phage 740 and 737was isolated from ovine and caprine milk!)

Table 25: A comparison of the enterotoxin profile from *S. aureus* strains of different investigators from bovine milk and mastitis samples

Source of bovine milk sampels	No. of isolates	Prevalence of Enterotoxins (%)					
		A	AD	B	D	C	C / TSST-1[e]
Raw milk and raw milk Products (Morandi et al., 2007)[a,b]	71	19.7	18.3	--	15.5	1.5	ND[1]
Bulk tank milk (Stephan et al., 2002)[a]	200	8.5	3.5	1.0	--	17.0[d]	2.5
Mastitis (Stephan et al., 2001)	97	--	8.2	--	3.1	1.0	22.7
Mastitis (Zschöck et al., 2005)[c]	105	--	--	--	23.8	1.0	28.9
Bulk tank milk (Mayer, 1975)	297	3.7	1.0	3.4	15.1	1.3	ND
Mastitis (Untermann,1973)	120	--	--	--	ND	4.2	ND
Mastitis (Gonano et al., 2009)	91	--	--	2.2	1.1	6.6	ND
Raw milk and raw milk Products (Gonano et al., 2009)	247	2.4	1.6	2.4	0.4	5.3	ND

[a] These *S. aureus* were cultured on BP-RPF.,

[b] 11 (15,%) *S. aureus* strains with *sed* produced no enterotoxin and three strains with no detectable *se* produced enterotoxins.

[c] This collection with *S. aureus* of German dairy herds included only strains of subclinical mastitis. All strains showed αβ-haemolysis.

[d] This is the type C[1] (Appendix: Table 26) or probably the type in phage profile 22 in Table 19 and 20.

[e] These should belong to the same clonal complex such as the strains with a strong phage reaction for phage 119 in Table 8 and 16.

[1] TST-1 was not investigated. ND, Not determined.

Table 26: Pheno- and genotypic characteristics of 34 enterotoxin producing *S. aureus* strains from bovine mastitis according to Stephan et al. (2001) (shown in Appendix Table 24 above)

n[a]	Toxins		Phenotype				Protein A (spa) gene (bp)			Size of amplicons of coa gene (bp)	PFGE pattern
	Enterotoxin[b]	TSST-1	Haemo-lysis	Egg yolk	Staph-aurex[c]	Resi-stances[d]	IG-binding region	X region	Repeats X region		
1	AD	−	α	−	+	−	920	300	11	580	Ia
2	AD	−	δ	−	−	−	920	300	11	580	Ia
1	AD	−	δ	+	+	−	920	200	6	580	Ia
2	AD	−	α	+	+	−	920	300	11	580	Ib
1	AD	−	δ	+	+	−	750	300	11	580	If
1	AD	−	δ	+	+	−	750	300	11	580	Ig
16	C	+	αβ	−	−	−	920	100	2	580	IIa
1	C	+	αβ	−	−	Poly	920	100	2	580	IIa
1	C	+	αβ	−	+	−	920	100	2	580	IIa
1	C	+	β	−	−	−	920	100	2	580	IIa
1	C	+	β	−	−	−	920	100	2	580	IIb
2	C	+	αβ	−	−	−	920	100	2	580	IIc
1	**C[1]**	**−**	**δ**	**+**	**+**	**Pen G/Amp**	**750**	**300**	**11**	**660**	**III**
1	D	−	αβ	+	+	−	920	300	11	580	Ic
1	D	−	αβ	+	+	−	920	280	10	580	Id
1	D	−	αβ	+	+	Pen G/Amp	920	300	11	580	Ie

[a] Number of strains,

[b] Results obtained with SET RPLA and PCR.

[c] Combined agglutination test for protein A and clumping factor reaction

[d] Amp: ampicillin, Pen G: penicillin G; Poly: polymyxin B.

[1] This is probably the *S. aureus* type of Table 20 and 21

Table 27: Percentage of *S. aureus* producing various kinds of haemolysis or other elective criteria by various Authors (quoted according to Munch-Peterson, 1965 inclusive other results from the literature after 1970)

Source	No. of strains	% of strains producing haemotoxin								Egg yolk [2] (%)	CF-[3] (%)
		No	α	β	δ[1]	αβ	αβ(δ)	β(δ)	α(δ)		
Edwards and Rippon (1957)		-	2.5	-	-	-	97.5	-	-	-	
Nakagawa (1958)	189	12.7	6.7	3.7	3.2	30.7	13.2	21.2	8.5	-	
Reid and Wilson (1959)	95	-	1.6	50.5	46.3					21.4	
Czenicki (1961)	341	-	10.7	24.1		65.2				-	
White and McDonland (1961)		1.3	1.5	2.5		94.4				-	
Pillet et al. (1962)	165	-	10.9	6.7	-	82.4	-	-		-	
St. George et al. (1962)	688	-	24.3	18.9	8.7	37.8	-	10.2		-	
Loken et al. (1962)	479	-	0.4	10.6	1.0	1.9	22.1	62.4	1.5	-	
Betseth (1963)	209	-	6.7			93.3				-	
Munch- Peterson (1965)[a]	227	24.7	18.9	23.8		7.5			1.5	-	9.1
Gedek and Kopp (1967)	225	-	-	1.8	-	-	86.7	5.3	2.2	16.9	
Grigorova and Bajljosov (1972)	97 [d]	1.2	-	6.0	-	15.7	7.1	14.3	66.6	31.0	
	127 [e]	-	-	17.6	-	4.8	52.8	13.9	-	25.0	
Untermann et al. (1973)	120	-	5.0	3.3		91.7				8.3	
Mayer (1975)	297	-	-	6.7	-	2.4	83.7	2.1	6.1	30.6	
Becker et al. (1987)	267	11.6	13.1	20.6		54.7				34.5	15.7
Zangerl (1999)[b]	254	2.0	18.5	53.1	12.6	13.8				45.7	7.9
Bulk milk[c]: (MLF Wagen 1996-2002)	642	15.5								57.7	13.0
Products[c]: (MLF Wagen 1996-2002)	1484	14.4								54.1	13.3

[1] This indicates only weak- haemolysis and is frequently not detected.

[2] Prevalence (in %) of egg-yolk positive isolates

[3] Prevalence in % of clumping factor negative isolates

[a] This is the single study investigating all cows of a herd.

[b] These *S. aureus* were in contrast to the other samples only isolated from raw milk or raw milk products.

[c] α and ß-haemolysis were not confidently distinguished.

[d] These isolates are bovine strains from Cuba.

[e] These isolates are bovine strains from Bulgaria.

Table 28: Sensitivity to human and bovine phages at RTD and 1000×RTD of human and bovine strains according to Meyer (1967) (The bold market numbers indicate the most frequent different phage patterns when using additional bovine phages of phage group IV)

Phage groups	Bovine strains[1] (n= 307)						Human strain[2] (n= 614)					
	With basic set (%)			With combined set[3] (%)			With basic set (%)			With combined set (%)		
	RTD	RTD × 1000	Total	RTD	RTD × 1000	Total	RTD	RTD × 1000	Total	RTD	RTD × 1000	Total
I	4.2	2.3	6.5	2.9	0.3	3.3	31.3	12.2	43.5	30.5	9.4	39.9
II	2.3	1.0	3.2	1.6	1.0	2.6	4.4	1.8	6.2	4.4	1.8	6.2
III	9.1	21.2	**30.3**[a]	2.6	1.0	3.6[b]	12.2	4.9	17.1	10.9	3.3	14.2
IV	9.5	2.6	12.0	**55.0**	0.7	55.7[c]	-	-	-	1.0	0.2	1.1
187	0.7	0.3	1.0	0.7	0.3	1.0	1.5	7.2	8.6	1.5	7.2	8.6
I/II/III	0	3.3	3.6	0	0	0	0.2	0.2	0.3	-	0.2	0.2
I/III	4.6	21.8	**26.4**[a]	0	0.3	0.3[b]	4.7	7.5	12.2	3.3	5.9	9.1
II/III	0.7	1.6	2.3	0	0	0	-	-	-	-	-	-
III/IV	0.7	4.6	5.2	5.9	0.7	6.5	-	0.3	0.3	1.0	1.5	2.4
Other[4]	1.0	5.5	6.5	19.9	4.2	24.1	0.3	1.8	2.1	4.4	7.2	11.6
Not-typeable	**67.4**		6.0	11.4		0.6	45.4		9.8	43.2		8.8

[1] 18.6 % of bovine strains are only human plasma positive; the rest of bovine strains are human and bovine plasma positive.

[2] All human isolates are only human plasma positive.

[3] Additional to the baisc set with the only human phage 42D IV following bovine phages of phage group IV from Davidson (1961) were used: 102, 105, 107, 108 and 111.

[4] These strains show all positive phage reactions in mixed phage groups with phages of phage group IV and other phages.

[a] More than 80 % are bovine plasma positive and belong at RTD wiht bovine phages almost all to phage group IV.

[b] About 90% are only human plasma positive.

[c] Except of one strain are all bovine plasma positive.

Table 29: The association between phage types of the IPS and the BPS with additional phages of phage group IV to penicillin of 211 strains from South Africa according to Giesecke et al. (1972)-(Following additional phages were used: Phage 105 (Davidson, 1961) and phage 129-16/ P42D-E193/ and 88A (Smith (1948).)

Serial No.	Lysogenic phage pattern	Phage group	Number of isolates lyzed		Penicillin resistant (%)	
			RTD	1000×RTD	RTD	1000×RTD
1	80/81	I	1	-	0	-
2	**52A**/80/ 81	I/III	1	-	0	-
3	**29**/77/**42D**	I/III/IV	-	2	-	50
4	**52A**/**107**/**117**/105/ 129-16/ P42D-E193/ 88A	I/IV	1	-	0	-
5	52/42D/**102**/ 129-16/ P42D-E193/ 88A	I/IV	-	1	-	100
6	77	III	-	1	-	100
7	77/**42D**	III/IV	1	-	0	-
8	77/**42D**/**117**	III/IV	1	-	0	-
9	**42E**/**53**/**42D**	III/IV	-	1	-	100
10	83A/**42D**	III/IV	-	1	-	100
11	**53/ 102**	III/IV	-	1	-	100
12	77/**42D**/P42D-E193	III/IV	-	2	-	50
13	**102**/105/ 129-16/ P42D-E193/ 88A	IV	38	8	34	50
14	**102**/ 129-16/ P42D-E193/ 88A	IV	25	-	8	-
15	**102**/105/ 129-16/ P42D-E193	IV	3	-	67	-
16	**102/42D/107/ 117**	IV	1	-	0	-
17	105/ 129-16/ P42D-E193/ 88A	IV	3	3	0	67
18	**102**/ 129-16/ P42D-E193	IV	5	-	20	-
19	**102**/ 105/ 129-16	IV	6	-	0	-
20	**102/107**	IV	4	-	0	-
21	**102/117**	IV	1	-	0	-
22	**102**/ 129-16	IV	12	2	0	50
23	**102**/ P42D-E193	IV	3	-	0	-
24	**102**/ 105	IV	7	4	0	25
25	**42D**/P42D-E193	IV	-	4	-	75
26	105/ 129-16	IV	-	2	-	100
27	**42D**	IV	-	13	-	100
28	**102**	IV	12	5	0	100
29	**117**	IV	-	1	-	100
30	129-16	IV	1	1	0	100
31	P42D-E193	IV	-	2	-	100
32	105/ 129-16/ P42D-E193/ **119**	IV/M	1	-	0	-
33	**42D**/**102**/**117**/ 129-16/ P42D-E193/88A/**119**	IV/M	-	1	-	100
34	**102**/**107**/105/ 129-16//**119**	IV/M	-	1	-	100
35	**119**	M	1	-	0	-
36	**NT**[1]			17		6

[1] Non-typable isolates: 17 strains (8%) showed no phage reactions at 1000×RTD.

Table 30: The strength of phage reactions with different phage dilutions according to Davidson (1972)

Propagating strain	Strength of phage	Phage																	
		29	52A	3A	883	6	42E	53	75	77	84	42D	102	107	117	78	118	119	AC1
102	RTD	-	-	-	-	-	-	-	-	-	-	(+)	SCL	++	-	-	-	-	-
	1000×RTD	(+)	-	-	-	-	(±)	-	-	(+)	-	SCL	CL	CL	-	-	-	-	(±)
107	RTD	-	-	-	-	-	-	-	-	-	-	(±)	SCL	SCL	SCL	-	-	-	-
	1000×RTD	-	-	-	-	-	-	-	-	(+)	-	CL	CL	CL	CL	-	-	-	-
M 8	RTD	-	-	-	-	-	-	-	-	-	-	++	SCL	SCL	SCL	-	-	-	-
	1000×RTD	-	-	-	-	-	-	-	-	(±)	-	CL	CL	CL	CL	-	-	-	-
118	RTD	-	-	-	-	-	(±)	(±)	-	(±)	(±)	-	(±)	(±)	-	-	SCL	-	-
	1000×RTD	(0)	-	-	-	++	CL	CL	-	++	SCL	-	++	++	-	-	CL	-	-
119	RTD	-	-	-	-	-	-	-	-	-	(±)	(±)	++	(±)	-	-	-	SCL	-
	1000×RTD	0/CL	0/++	(0)	(0)	(0)	-	+	(0)	+	0/CL	SCL	CL	CL	CL	0/++	-	CL	CL
HAC 1/2	RTD	SCl	-	-	-	-	-	(±)	-	++	(+)	-	-	++	-	-	-	-	SCL
	1000×RTD	CL	0/+	-	-	-	(±)	CL	+	CL	CL	(0)	+	CL	-	(0/+)	(0)	-	CL
94	RTD	-	-	-	SCL	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1000×RTD	-	-	-	CL	-	-	-	-	-	-	-	-	-	-	-	-	-	-

CL= confluent lysis ++ = > 50 plaques ± = 20- 50 plaques ()= reaction not always present
SCL= semiconfluent lysis + = 20- 50 plaques 0 = inhibition reaction

Table 31: Phage types (Strain 1-6) observed among 25 typings of each culture typed at conventional concentrations (RTD; 1000×RTD) and at 100×RTD according to Degree (1967)

	Types observed at various concentrations used for typing					
Strain	RTD	%	RTD×100	%	RTD×1000	%
1	**Not typeable**	**8**	7/47/53/54/83A	48	-	
	54	48	**7**[a]/42E/53/**54/83A**	28		
	7/54	16	**7**/53/**54/83A**	16		
	54/83A	12	**7**/47/42E/53/**54/83A**	8		
	7	8				
	83A	4				
	7/54/83A	4				
2	**Not typeable**	**100**	47/53/54/75/77	32	79/**47/53/54/75/77**/83B	36
			47/77	20	**47/53/54/75/77**/83B	16
			47/75/77	16	**47/53/75/77**/83B	16
			47/54/75/77	12	79/7/**47/53/54/75/77**/83B	12
			47/53/75/77	8	79/**47/53/54/75/77**	4
			47	8	79/**47/53/75/77**/83B	4
			77	4	7/**47/53/54/75/77**/83B	4
					47/53/75/77	4
					47/53/54/75/77	4
3	**Not typeable**	**4**	3A/3B/3C	84		
	3A	96	**3A**/3B	16		
4	**Not typeable**	**100**	3A	84	**3A**/3B	68
			3A/3B	16	**3A/3B**/3C	24
					3A	8
5	52/52A/80/81/KS6	88	**52/52A/80/81/KS6**	96		
	52/52A/81/KS6	8	**52/52A**/79/**80/81/KS6**	4		
	52A/80/81/KS6	4				
6	**Not typeable**	**100**	81	96	**81**/KS6	80
			29/81	4	**81**	12
					29/81/KS6	4
					29/81/42E	4

[a] Bold market phage numbers indicate the same phages, which showed still > 50 plaques at the lower phage dilution.

Table 32: The sample collections and phage sets of corresponding collaborating laboratories according to Davidson (1972)

Collaborating laboratory	Number of cultures	Sources of cultures	Phages used[a]
1. Australia	873	>288 herds in 7 districts: normal and mastitis milk	Set A except 85 and 187; Additional phages: 3B; 7, 42DA; 101,367,425, 600, 10, 186, 373
2. Bulgaria	113	milk of individual cows	Set A
3. Denmark	812	494 cultures from bulk milk samples of 223 herds; 318 cultures from milk of individual cows from 9 herds	Set A except 85; Additional pahges: 812
4. Finland	1344	650 herds: normal and mastitis milk	Set A except 52, 79, 85, and 186
5.Germany	1034	more than 40 herds of individual cows from whole Germany	Set A except 80, 83A and 85; Additional pahges: 7, 812
6.Germany	200	200 herds: milk of individual cows	Set B except 78
7. Ireland	128	91 cultures from bulk milk of different herds; 37 cultures of individual cows	Set B
8. Israel	97	31 herds: normal and mastitis milk	Set B
9. Japan	300	northern and central Japan: milk from individual cows	Set A except 85. Additional phages: 3B and 7
10. New Zealand	190	Several districts, 10 cultures of cheese, 2 from calves, remainder from normal and mastitis milk	Set A except 85, Additional phages: 3B, 7, 101, 105, 108, 110, 111, A13
11. Sweden	226	61 herds: mastitis milk	Set A
12. England	454	>300 herds from all districts: normal and mastitis milk	Set A, Additional phages: 3B, 7, 129/16, P42D/E193; 88A, 11, H98, 365, T90, 257
13. USSR A	120	3 herds: 10 cultures from milkers hands, 22 from milking apparatus, the remainder from normal and mastitis milk	Set A except 85. Additional phages: 3B and 7
14. USSR B	180	98 cultures of mastitis milk, 82 from dairy products	Set A except 85. Additional phages: 3B and 7
15. USA A	442	1 herd sampled over 3 years: milk from individual cows	Set A except 84 and 85; Additional phages: 3B
16. USA B	237	> 50 herds in 12 States, 9 cultures of canada: normal and mastitis milk	Set A except 84, 85 and 78; Additional phages: S2

Table 33: Phages in use of corresponding laboratories according to Davidson (1972)

Set A (30 Phages):

Current international set and bovine phages from provisional bovine set: 29, 52, 52A, 79, 80, 3A, 3C, 55, 71,
6, 42E, 47, 53, 54, 75, 77, 83A, 84, 85, 42D, 81, 187, 78, 102, 107, 117, 118, 119, 116, AC1

Set B (17 phages):

Provisional bovine set : 29, 52A, 3A, 6, 53, 75, 77, 84, 42D, 78, 102, 107, 117, 118, 110, 116, AC1

Additional phages:

3B, 7, 42F- phages of international series not in the international basic set
129/16, P42D/E193, 88A- Smith (1948)
S2- Seto and Wilson, J. B. (1958)
A13- Coles and Eisenstark, A. (1959)
H98, 365, T90, 257- Nakagawa (1960)
101, 105, 108, 110, 111- Davidson (1961)
812, 825- Meyer (1966)
367, 425, 600, 10, 186, 373- Frost (1967)
42DA- a variant of phage 42D obtained in Australia

Table 34: Results of phage typing at RTD and > 50 plaques in the corresponding collaborating laboratories according to Davidson (1972)

Phage group	Collaborating laboratory[1]															
	1 Australia	2 Bulgaria	3 Denmark	4 Finland	5 Germany	6 Germany	7 Ireland	8 Israel	9 Japan	10 New Zeland	11 Sweden	12 England	13 USSR A	14 USSR B	15* USA A	16 USA B
	Distribution (%)															
I	2	1	0	2	3	6	9	2	3	2	2	3	0	1	0.5	3
I/M	1	1	2	6	1	3	1	0	1	1	0	2	0	1	1.4	12
II	2	0	46	0	1	1	0	0	12	2	23	0	0	1	0	1
II/M	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0.5	0
III	14	1	2	4	3	5	5	9	6	8	12	6	0	2	0.5	3
III/M	1	0	0	2	0	0	0	1	1	3	0	2	0	0	0	6
IV	29	24	7	64	41	75	20	35	26	23	11	30	9	58	8.1	10
IV/M	0	4	0	0	0	0	0	0	0	3	0	1	7	2	1.6	4
I/III	3	0	1	1	6	0	23	1	1	5	2	7	0	1	0	1
I/III/M	0	0	0	1	0	0	1	0	1	1	0	6	0	0	0	2
I/IV	0	0	0	0	0	1	13	0	0	0	0	0	1	0	0	2
I/IV/M	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	4
II/IV	0	0	0	0	0	0	1	0	0	0	0	0	4	0	0	0
III/IV	15	1	0	4	2	1	2	0	0	12	0	3	35	13	4.5	1
III/IV/M	1	0	0	1	0	0	1	1	0	2	0	1	0	1	2	4
I/III/IV	2	1	0	0	7	0	7	0	0	11	0	13	0	1	0	1
I/III/IV/M	0	0	0	0	0	0	6	0	1	5	0	10	0	0	0.9	3
II/III/IV	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
AC1	0	4	0	0	0		0	0	0	0	0	0	0	0	0.5	1
78	0	0	2	0	0		0	0	1	3	0	2	0	0	0	0
118	0	0	0	0	1	1	0	2	3	0	0	0	0	0	6.6	1
119	1	0	13	3	0	0	2	0	0	0	1	1	0	0	50.2	20
Others	2	3	2	1	0	1	5	0	1	2	1	3	1	0	0.7	6
NT[2] at RTD:	26	60	25	9	32	8	3	48	42	19	48	11	28	22	22	15
NT[2] at 1000×RTD:		15	2		15	2				12	9		0		15	
total (n)	873	113	812	1344	1034	200	128	97	300	190	226	454	120	180	442	237

[1]Collaborating laboratory see Apendix: Table 32 page before

[2] Nontypeable strains at the corresponding phage dilution

*All strains in this collection come from a monitoring program in a single herd during three years.

Marked fields:

The dominating phage types in the corresponding laboratory

The phage types with the second highest prevalence in the corresponding laboratory

The phage types with the third highest prevalence in the corresponding laboratory

Table 35: The sensitivity of single phages at RTD in the corresponding collaborating laboratories according to Davidson (1972)

	Collaborating laboratory[1]																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
	Australia	Bulgaria	Denmark	Finland	Germany	Germany	Ireland	Israel	Japan	New Zeland	Sweden	England	USSR A	USSR B	USA A	USA B	Average reactions[2]
Phage	Distribution (%)																
29	5	2	2	9	17	10	61	1	6	21	1	38	0	1	2	13	11 (5)[3]
52	7	4	2		13				1	23	4	34	1	2	1	11	9 (3)
52A	0	3	2	4	11	2	43	4	2	10	3	25	1	1	0	8	6 (1)
79	0	3	0		8				1	2	2	2	0	1	0	17	3 (1)
80	1	3	3	6					6	8	2	33		1	0	11	7 (2)
AC1	0	9	1	3	0	1	1	1	3	1	0	9	4	1	2	5	2 (10)
3A	1	1	43	0	0	1	2	0	0	2	15	2	0	1	0	4	6 (47)
3C	0	1	1	1	1				1	3	0	0		0	0	2	1 (6)
55	1	0	0	0	1				0	0	0	0	1	0	0	2	1 (3)
71	1	1	0	0	2				0	0	0	0	1	0	0	2	1 (13)
116	2	2	22	0	0	1	3	0	11	0	23	2	5	0	0	2	7 (21)
6	7	1	1	2	11	4	37	6	1	25	8	31	3	2	1	2	6 (2)
42E	30	2	0	6	14				1	34	0	37	52	9	0	3	14 (6)
47	5	1	1	1	10				3	21	7	30		2	7	11	7 (1)
53	3	3	1	1	4	4	21	6	4	14	7	7	0	1	1	4	4 (5)
54	13	2	1	2	11				1	20	8	30		2	1	3	8 (3)
75	11	2	1	1	9	3	43	2	2	22	4	22	6	1	0	3	7 (4)
77	3	1	0	6	0	2	1	2	3	7	4	3	11	1	1	5	3 (9)
83A	7	0	1	1					3	2	0	28	3	1	1	5	5 (2)
84	2	3	1	3	4	1	9	5	1	10	4	13		0			4 (7)
85		3									3	5					3 (0)
81	2	0	2	1	12				5	19	3	30	0	7	1	5	6 (5)
187		0	0		0				0	0	0	0	0	0	0	3	0 (0)
42D	23	13	4	20	5	40	12	2	20	22	0	20	68	34	1	7	17 (9)
102	9	25	5	26	30	67	27	26	25	29	0	18	32	61	13	24	24 (13)
107	22	19	3	33	25	73	9	9	24	18	5	14	24	50	15	22	25 (6)
117	28	18	5	60	30	59	32	32	25	41	11	49	67	62	11	16	36 (16)
78	1	0	2	3	0		0	0	0	15	1	9	0	0	0		2 (29)
118	1	4	0	3	3	2	9	2	6	3	0	6	2	2	12	9	3 (26)
119	4	4	15	9	2	2	5	1	0	0	1	9	23	2	52	53	6 (59)
Total (n)	873	113	812	1344	1034	200	128	97	300	190	226	454	120	180	442	237	

[1]Collaborating laboratory see Appendix: Table 32 two pages before

[2]The mean value between all collaborating laboratories of all phage reactions for the corresponding phage

[3] The number in brackets is the percentage of positive reactions for each phage, where no or less than 50 plaques for any other phage was detectable.

Marked fields:

The phage with the highest prevalence in the corresponding laboratory

The phage with the second highest prevalence in the corresponding laboratory

The phage with the third highest prevalence in the corresponding laboratory

Table 36: Occurrence of phage types among Danish *S. aureus* isolates from bovine mastitis from the 1950´s, 1992 and 2000 with the HPS according to Vintov et al. (2003a)

Phage group	Phage type	1952- 1956 (n=86)	1992 (n=107)	2000 (n=99)
		Distribution (%)		
Phage group I	29/52		1.9[a]	
	29/52/52A/80		1.0	6.1[a]
80/81-complex	80	20.9	13.1	21.2
	52	1.2	4.7[a]	1.0[a]
	52/80	2.3		
	52/52A/80	1.2	1.9[a]	
Total		**25.6**	**21.5**	**28.3**
Phage group II	3A	9.3	2.8	1.0
	3C		1.0	
	3A/3C	1.2	1.0	1.0
	3A/3C/55		1.0[a]	
	3C/55	1.2		
	3C/55/71	1.2		
Total		**12.9**	**5.8**	**2.0**
Phage group III	42E	3.5	1.0	2.0
	47		10.3[a]	2.0
	42E/47	1.2		1.0
	47/75		5.6	1.0[a]
	75		2.8	2.0
	77	9.3[a]	1.9[a]	6.1
	77/83A		1.0	
	54/83A	1.2		
	Other group III	10.3[a]	6.6[a]	4.1[a]
Total		**25.5**	**29.1**	**18.3**
83A-complex	83A	2.3[a]	1.0[a]	
	83A/93	1.2		
	84	1.2		1.0
	85		1.0[a]	
	84/89			1.0
Total		**4.7**	**2.0**	**2.0**
Type 95	95		4.7	3.0
94/96-complex	94/96		3.7[b]	
Mixed group (NI)	I + 95	1.2		
	I+ 94/96			2.0[b]
	I+ II+ III + 95			2.0[a]
	I+ II+ III		1.9	1.9
	I + III	1.2[a]	9.3[a]	4.1[a]
	I+ 83A	2.3	2.8	
	I+ III + 95		4.7[a]	5.1[a]
	III + 95		2.8	3.0[a]
Total		**5.7**	**21.6**	**16.2**
Nontypable		**27.9[a]**	**11.2[a]**	**32.3[a]**
	Penicillin resistance:	**11.6**	**18.7**	**11.1**

[a] This phage type included one or more isolates with penicillin resistance.

[b] This results agree with Hajek and Howak (Table A: 19) when using human phages of the HPS instead of bovine phage AC1. It shows furthermore the necessity of typing instantly at 100×RTD and justifies the discrimination between confluent and weaker according to our results.

Table 37: A comparison of the results according to Vintov et al. (2003) and Davidson (1972) inclusive the mean values of important parameters according to the IDF (367/2001)

		Phage groups								Penicillin resistant	The mean value of important paramters in the correponding countries			
		I[a]	II	III[b]	81	95[c]	96[d]	Others	Not typable[1]		Number of lactations	Milk production per cow (kg milk/ lactation)	Average BMSCC (SCC/ml)	Average Herd size (number of cows)
	Year	Distribution (%)												
Denmark	**1972**	**15**	**43**	**3**	**0[e]**		**0[e]**	**4**	**35**					
	2003	23 (7)[f]	6 (1)	31 (5)	0	5 (0)	4 (0)	22 (5)	12 (2)	19	2,3	7,532	247,000	65,0
Germany	**1972**	**7**	**2**	**6**	**0**		**0[d]**	**7**	**80**					
	2003	44 (3)	4 (2)	25 (14)	0	1 (1)	13 (3)	3 (1)	8 (1)	25	2,3	5,990	171,000	31,6
Finland	**1972**	**8**	**1**	**10**	**0**		**0[d]**	**3**	**78**					
	2003	38 (1)	8 (6)	32 (8)	3 (3)	2 (1)	1 (1)	2 (1)	13 (7)	29	2,5	6,230	166,000	14,5[g]
Sweden	**1972**	**2**	**23**	**12**	**0[e]**		**0**	**3**	**59**					
	2003	33 (1)	11 (2)	14 (14)	0	11 (8)	0	1 (0)	31 (2)	29	2,5	8,300	200,000	31,5
USA	**1972**	**41**	**2**	**14**	**0**		**0[d]**	**12**	**31**					
	2003	26 (1)	11 (4)	19 (6)	3 (3)	2 (2)	0	9 (2)	39 (33)	50	2,5	7,689	363,000[h]	78,8
England	**1972**	**5**	**0[d]**	**11**	**1**		**0**	**39**	**46**					
	2003	16 (7)	0	45 (38)	2 (2)	0	0	8 (3)	27 (13)	67	3,4	6,700	170,000	67,9
Ireland	**1972**	**24**	**1**	**9**	**0**		**0**	**42**	**25**					
	2003	0	0	67 (67)	0	0	0	34 (5)	0	71	5	4,500	300,000	31,0
Norway	2003	25 (0)	1 (0.5)	7 (2)	0	0	8 (0)	0	62 (1)	**2**	2,3	6,160	142,000	14,4[g]
Switzerland	2003	43 (0)	1 (1)	3 (2)	0	0	8 (0)	16 (15)	19 (13)	32	3- 4	4,475	101,000	15,0[g]

[1] All strains being only sensitive to phages of the BPS except of phage 119 according to Davidson (1972) were assigned as non-typeable isolates

[a] All strains in phage group I/M, I/119 and I/IV or I/IV/M according to Davidson in Table A 33 were grouped in phage group I, because 118,119 and the phage of phage group IV are not part of the HPS.

[b] All strains with only reactions for phages of the 83A- complex were added to the strains being only sensitive to the other phages of phage group III

[c] This phage was not used 1972 according to Davidson (1972).

[d] Strains of phage type AC1 according to Davidson (1972) were added according to the results of Hajek and Howak (1978) to the phage type 96 respectively 94/96- complex.

[e] Single isolates were detectable.

[f] The prevalence of penicillin resistant strains is shown in brackets.

[g] >85 % of the herds has only tie stalls.

[h] This result is borrowed from Schaik et al. (2002).

Table 38: Phage typing of isolates from human milk (Collection of Austria) and human nasal strains (Collection from Switzerland) with the phage set according to Table 3 considering only human phages and all 30 phages with weak and only strong phage reactions

Phage groups	Phage reactions: Mixed phage set (%)		Phage reactions: Only human phages (%)	
	Nasal (n=147)	Human Milk (n=188)	Nasal (n=147)	Human Milk (n=188)
	≥ 20 / >1000	≥ 20 / >1000	≥ 20 / >1000	≥ 20 / >1000
Phage group I	**5 / 4**	**1 / 0**	**5 / 4**	**1 / 0**
Phage group II	**16/ 19**	**6 / 5**	**16/ 19**	**6 / 5**
116[a]	0 / 0	**5 / 6**		
Phage group II all	**16 / 19**	**10 / 11**		
Phage group III	**5 / 5**	**5 / 5**	**5 / 7**	**12/12**
Miscellaneus humane:				
81[b]	**1 / 1**	**1 / 9**	**1 / 1**	**3 / 9**
95	**3 / 8**	**6 / 17**	**3 / 8**	**6 / 18**
96[c]	**5 / 5**	0 / 0	**5 / 5**	**1 / 0**
187	0 / **1**	0 / **2**	**1 / 1**	0 / **2**
812	0 / 0	0 / 0	0 / 0	**2** / 0
Human phage groups[d]	**35 / 43**	**24 / 44**	**36 / 45**	**31 / 45**
Bovine phage group IV	**1 / 1**	**2 / 9**		
42D[e]	0 / 0	**1 / 1**	0 / 0	**1 / 1**
Miscellaneus bovine:				
78	0 / 0	0 / 0		
118	0 / 0	0 / 0		
119	0 / 0	**1 / 1**		
Bovine phage groups	**1 / 1**	**4 / 11**		
Mixed groups (NI)	**41 / 17**	**58 / 25**	**39 / 13**	**41 / 7**
Not typable (NT)	**24 / 40**	**18 / 31**	**25 / 42**	**27/ 49**
Phage types:				
Numbers	**67/ 34**	**64 / 48**	**49 / 29**	**45 / 25**
Phage types per isolate	**2.2 / 4.3**	**2.9 / 3.9**	**2.7 / 5.1**	**4.2 / 7.5**

Table 39: The most important phage types of the collections according to Appendix: Table 37 above

Specimen source	Phage patterns (weak reactions < 1000 plaques)	Prevalence of isolates Number of strains	(%)
Human milk: (n=188)	**95** (79)	32	17.0
	81 (95, 812, 42D, 80+)	18	9.6
	116	10	5.3
Human nasal: (n=147)	**55** (3A)	23	15.6
	95 (54)	8	5.4
	96	8	5.4
	29/81 (52, 52A,79, 80, 42E, 95, 812)	8	5.4

Table 40: The sensitivity to phage of BPS and additional bovine phages of human nasal and milk isolates according to Appendix Table 38 above

Phage groups	Phages	Human Nasal (%) (Switzerland) ≥ 20 / ≥ 1000 (n= 147)	Human milk (%) (Austria) ≥ 20 / ≥ 1000 (n= 188)
II	116	3 / 1	9 / 8
IV	42D	4 / 0	15 / 4
	102	10 / 2	15 / 10
	107	5 / 2	16 / 10
	117	14 / 5	18 / 11
	108	2 / 0	7 / 6
	111	12 / 1	26 / 16
M (BPS)	78	5 / 0	2 / 0
	118	12 / 5	16 / 12
	119	1 / 0	3 / 2

Table 41: Characterization of 71 *S. aureus* strains according to Hennekinne et al. (2003)
*Phages of the HPS; †NT, not typable; ‡ND, not done

PFGE[1]		Phage	Source	Devriese´s biotyping scheme modified by Isigidi et al. (1990)					
Cluster	Pattern	group*		Biotype	Staphylo-kinase	β haemolysin	Bovine plasma coagulase	Crystal-violet Type	Protein A
	A1	NT†	Goat`s milk	Ovine	-	+	+	C	
A	**A2**	NT	Goat`s milk	Ovine	-	+	+	C	
	A3	NT	Goat`s milk	Ovine	-	+	+	C	
B	B1	ND‡	Pig nare	**Poultry**	-	-	-	A	-
	C1	NT	**Live chicken**	**Poultry**	-	-	-	A	-
C	C2	NT	**Live chicken**	**Poultry**	-	-	-	A	-
	C3	NT	**Live chicken**	**Poultry**	-	-	-	A	-
	D1	ND	Pig nare	**Human**	+	+ or -	-	A or C	
	D2	I+III+95	Pork meat	**Human**	+	+ or -	-	A or C	
	D3	II	Minced meat	**Poultry**	-	-	-	A	-
	D4	III	**Chocolate milk**[2]	**Human**	+	+ or -	-	A or C	
D	D5	NT	Venison meat	**Human**	+	+ or -	-	A or C	
	D6	**III+95**	**Ewe´s milk cheese**[2]	**Human**	+	+ or -	-	A or C	
	D6	**III**	**Ewe´s and cow´s milk cheese**[2]	**Human**	+	+ or -	-	A or C	
	D7	NT	Guinea fowl meat	**Poultry**	-	-	-	A	-
	D8	I+III	Pork meat	**Human**	+	+ or -	-	A or C	
	D9	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	E1	III	**Live chicken**	**Poultry**	-	-	-	A	-
	E1	III	**Live chicken**	**Poultry**	-	-	-	A	-
	E2	III	**Live chicken**	**Poultry**	-	-	-	A	-
	E3	III	**Live chicken**	**Poultry**	-	-	-	A	-
E	**E4**	III	**Live chicken**	**Poultry**	-	-	-	A	-
	E5	III	**Live chicken**	**Poultry**	-	-	-	A	-
	E6	III+95	**Live chicken**	**Poultry**	-	-	-	A	-
	E7	III	**Chicken meat**	**Poultry**	-	-	-	A	-
	E8	III	**Live chicken**	**Poultry**	-	-	-	A	-
	F1	II	**Butcher`s nare**	Abattoir	-	-	-	A	+
	F2	II	**Chicken meat**	**Poultry**	-	-	-	A	-
	F3	**II**	Wild rabbit meat	Abattoir	-	-	-	A	+
	F3	**III**	Pork meat	Abattoir	-	-	-	A	+
F	**F3**	**II**	**Chicken meat**	**Poultry**	-	-	-	A	-
	F4	II	**Butcher`s nare**	Abattoir	-	-	-	A	+
	F4	NT	Pork meat	Abattoir	-	-	-	A	+
	F5	II	**Butcher`s nare**	Abattoir	-	-	-	A	+
	F5	NT	Pork meat	Abattoir	-	-	-	A	+
	F6	NT	**Butcher`s nare**	Abattoir	-	-	-	A	+
G	G1	**III**	**Chicken meat**	**Human**	+	+ or -	-	A or C	
	H1	95	**Veterinary student`s nare**	**Human**	+	+ or -	-	A or C	
	H2	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	H3	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	H4	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
H	**H5**	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	H5	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	H6	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	H7	ND	**Bovine mastitis**	**Bovine**	-	+	+	A	
	I1	ND	Pig nare	NHS3	-	+	-	A	
	I1	ND	Pig nare	NHS3	-	+	-	A	
I	**I1**	ND	Pig nare	NHS3	-	+	-	A	
	I1	ND	Pig nare	NHS3	-	+	-	A	
	I2	**II**	Quail meat	NHS5	-	-	-	C	
	J1	I+III	Goat`s milk	Ovine	-	+	+	C	
	K1	**II**	Quail meat	**Human**	+	+ or -	-	A or C	
K	K2	ND	**Bovine mastitis**	NHS3	-	+	-	A	
	K3	I+II+III+V	**Fromage frais**[2]	NHS4	-	+	-	C	
	L1	NT	Goat`s milk	Ovine	-	+	+	C	
	L2	NT	Goat`s milk	Ovine	-	+	+	C	
	L3	NT	Goat`s milk	Ovine	-	+	+	C	
	L4	NT	Goat`s milk	Ovine	-	+	+	C	
L	L5	NT	Goat`s milk	Ovine	-	+	+	C	
	L6	NT	Goat`s milk	Ovine	-	+	+	C	
	L7	NT	Goat`s milk	Ovine	-	+	+	C	
	L8	NT	Goat`s milk	Ovine	-	+	+	C	
	L9	NT	Goat`s milk	Ovine	-	+	+	C	

[1] The cut-off in the PFGE-dendrogram for the corresponding clusters was at 50 % similarity. The bold marked strains belonged to the same PFGE pattern or close related strains with a similarity of 80%.

[2]This strains were involved in food poisoning outbreaks.

Table 42: Epidemiological details and analysis of 31 food poisoning outbreaks from France according to Kerouanton et al. (2007) (see Appendix: Figure 25 next page)

PFGE[1]		Outbreak			Food	*S. aureus* CFU/g of food	Biotype[2]	Resistance[3]	SE detected in food sample[4]	RFPA[5]
Cluster	Pattern	Number	Year	No.of strains						
A	p46	29b	2001	1	Raw milk semi-hard cheese[6,7]	2.9×10^4	NHS3	Str	SED[8]	negative
	p1	17	1998	12	Raw milk semi-hard cheese[7]	5.7×10^6	NHS3	**Pen**, Cd, As	negative	negative
	p29	5[a]	1983	2	Raw milk soft cheese[6,7]	1.0×10^4	NHS4	**Pen**	negative	negative
B	p39	12	1989	1	Chicken [7]	1.5×10^6	Human	Susceptible	ND	SEC
C	p10	29a	2001	5	Raw milk semi-hard cheese[6,7]	2.9×10^4	NHS6	Af, Eb	SED[8]	negative
D	p23	6	1983	1	Meat[7]	not kown	Human	**Pen**, Cd	ND	SEA, SED
	p22	10	1987	2	Cake	5.0×10^7	Human	Cd, As	SEA	SEA
	p6	3	1983	2	Raw milk semi-hard cheese	2.0×10^6	Human	**Pen**, Str, Cd, As	SEA, SED	SEA, SED
	p7	1[a]	1981	1	Raw milk semi-hard cheese	3.0×10^7	Human	**Pen**, Cd, As	SEA	SEA, SED
	p27	27[a]	2001	4	Sliced soft cheese	$>1.5\times10^5$	Human	**Pen**, Cd, As	SEA	SEA, SED
	p25	24[a]	2001	2	Chocolate cake	1.0×10^4	Human	Cd, As, Eb	SEA	SEA, SED
	p16	13	1992	2	Potato and rice salads	5.0×10^9	Human	**Pen**, Fus, Cd	SEA	SEA
	p28	26[a]	2001	4	Roast lamb	3.0×10^7	Human	**Pen**, Fus, Cd, As, Af, Eb	SEA	SEA
	p26	23a	2001	3	Panacakes	1.5×10^6	Human	**Met,** Kan, Tob, Neo, PEF, Fus, Cd, As, Hg	SEA	SEA, SED
	p2	21	2000	3	Sliced pork	1.8×10^6	Human	**Met**, Kan, Tob, Neo, LSgA, PEF, Cd, As	SEA, SED	SEA, SED
	p45	15	1997	10	Raw milk cheese	1.0×10^7	Human	Cd, As, Hg	+	SEA, SED
	p45	16	1998	24	Raw milk cheese	3.0×10^7	Human	Cd, As, AF, Eb	+	SEA, SED
E	p35	25	2001	6	Cream	2.2×10^7	Human	**Pen,** Fus, Cd, As, Af, Hg	SEA	SEA
F1	p31	31	2002	3	pottet meat	2.3×10^6	Human	Tet	SEA	SEA
	p4	18	1999	11	Chocolate milk	7.6×10^2	Human	**Pen**, MLSTi, Cd, Eb	SEA	SEA
	p24	2[a]	1983	2	Cooked beef	7.5×10^9	Human	Cd, As	SEA	SEA
	p50	14	1997	10	Nougatine[6]	2.0×10^3	Human	MLSTi, Cd	negative	SEA
	p5	4	1983	1	Rice and tuna salad	3.0×10^7	Human	Cd, As	SEA	SEA
F2	p8	19	2000	27	Mixed salad	High	Human	Tet, Cd, As	SEC	SEC
	p47	22	2001	1	Rice[6]	2.9×10^4	Human	Tet, Cd, Eb	negative	negative
G	p11	28	2001	5	Roasted pork	4.5×10^6	Human	**Pen**, Cd, Eb	SEA	SED
	p21	23b	2001	2	Panacakes	1.5×10^6	Human	**Pen**, Cd, As, Hg	SEA	SEA, SED
	p19	11	1988	1	Spaghettis	3.0×10^8	Human	**Pen,** Tet, Min	SEA	SEA
H	p32	8[a]	1985	3	Soft cheese	3.0×10^7	Human	**Pen**, MLSTi, Cd	SEB	SEB
	p12	7[b]	1985	13	Soft cheese	3.0×10^8	Human	**Pen**, MLSTi, Cd	SEB	SEB
	p3	9	1986	1	Sheep´s milk cheese	1.0×10^6	Human	**Pen**	SEB	SEB
I	p44	30	2002	5	Raw sheep´s milk cheese	2.8×10^5	Ovine	Susceptible	SEA	SEA
	p9	20	2000	7	Raw sheep´s milk cheese	2.6×10^4	Ovine	**Pen**, Str, Cd	SEA	SEA

[a] Family outbreak.

[1] PFGE-dendogramm shown in Appendix Figure 27

[2] NHS3, NHS4, and NHS6 are non- host-specific profiles described in Hennekinne et al. (2003); Two distinct profiles were observed in outbreak 23

[3] Cd, cadmium; As, arsenate; Hg, mercury; Eb, ethidium bromide; Str, streptomycin; **Pen**, penicillin G; Mlsti, macrolide – lincosamide-steptogramin inducible; KAN, kanamycin; Tob, tobramycin; Neo, Neomycin; Fus, fusidic acid; Tet, Tetracycline; LSgA, lincomycin- streptogramin A; Pef, pefloxacin; **Met,** methicillin; Min, Minocycline; AF, agriflavine.

[4] Staphylococcal enterotoxins (SEA, SEB, SEC, SED) tested by radio-immunoassay until 1986; By Elisa after 1986; +, positive results using the global detection SEA-E Tansia kit; ND, not determined.

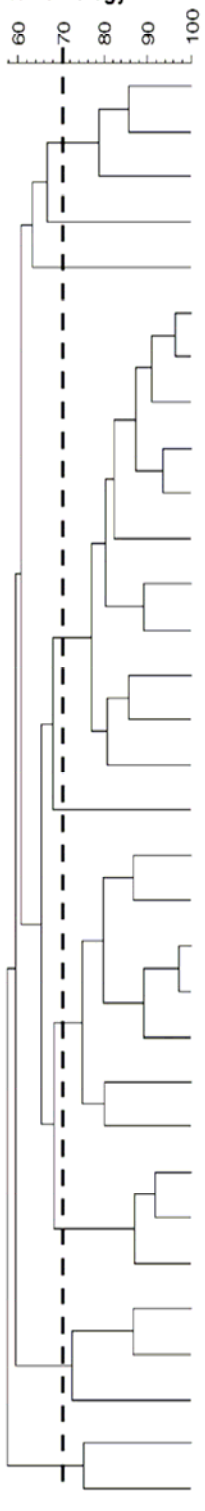
[5] Tested for SEA, SEB, SEC and SED

[6] Outbreak only suspected and not confirmed

[7] Strong epidemiological evidence implicating this food in outbreak

[8] Results not confirmed by strain characterization.

Figure 25: Dendrogram showing the genetic relatedness among the 32 PFGE patterns from food poisoning out breaks in France according to Kerouanton et al. (2007) (in more detail see Appendix: Table 49). NHS, non-host specific; ND, not determined; NT, not typeable; se_v variant *se* gene (p12 variant *sed*; p9 variant *sea*) detected by real-time PCR (Letertre et al., 2003).



PFGE pattern	Biotype	*se* genes **	Phage group *	Cluster
p46	NHS3	negative	ND	
p1	NHS3	negative	I/III/NC/exp	A
p29	NHS4	negative	I/III/NC/exp	
p39	Human	*sec, seg, sei*	NC	B
p10	NHS6	*seg, sei*	NT	C
p23	Human	*sea, sed*	III/exp	
p22	Human	*sea*	III/exp	
p6	Human	*sea, sed*	III/exp	
p7	Human	*sea, sed*	III/NC/exp	
p27	Human	*sea, sed*	III/exp	
p25	Human	*sea, sed*	III	D
p16	Human	*sea*	I/III/NC/exp	
p28	Human	*sea*	III/exp	
p26	Human	*sea, sed*	III/exp	
p2	Human	*sea, sed*	III/exp	
p45	Human	*sea, sed*	III/exp	
p35	Human	*sea*	III/exp	E
p31	Human	*sea, seh*	ND	
p4	Human	*sea, seh*	III	
p24	Human	*sea, seh*	III	F1
p50	Human	*sea, seh*	exp	
p5	Human	*sea, seh*	III/exp	
p8	Human	*sec*	I/III/NC/exp	F2
p47	Human	negative	NT	
p11	Human	*sed, seg, sei*	I/III/NC/exp	
p21	Human	*sea, sed, seg, sei*	I	G
p19	Human	*sea, seg, sei*	exp	
p32	Human	*seb, seg, sei*	V	
p12	Human	*sea, seb, sed_v seg, sei*	V	H
p3	Human	*seb, seg, sei*	V	
p44	Ovine	*sea*	ND	I
p9	Ovine	*sea_v*	NT	

*[a]The HPS and experimental phages (exp) of the 83a-Complex (89, 93); NT, not typable; ND, not determined; NC, phage 95,

** Tested by PCR for genes: *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh* and *sei*.

Table 43: Statistically[1] significant differences in the distribution of strains with specific characteristics between ALFP (Amplified fragment length polymorphism) according to Gonano et al. (2007)

Cluster	No. of isolates	Characterization of clusters			
		Over-representation	*P* value	Under-representation	*P* value
A	8		0.048	Clumping factor- positive	0.014
C	2	Human isolates	0.048		
D	106			*se* -positive	0.012
				Penicillin G-resistant	0.042
E	3	Human isolates	0.011		
F	5	*seh*	0.012	*seg*	0.019
				sei	0.040
G	18	*sei*	0.005	Egg yolk- positive	0.020
		se -positive	0.012	Clumping factor- positive	0.034
		Veterinary isolates	0.030	Antimicrobial-resistant	0.004
				Penicillin G-resistant	0.004
				Human isolates	0.028
H	5	Human isolates	0.010		
I	31	Egg yolk- positive	0.005	*sei*	0.018
		tst	0.035	*seg/sei*	0.023
		se -positive	<0.0005	Veterinary isolates	<0.0005
		sea	0.042		
		Antimicrobial-resistant	<0.0005		
		Penicillin G-resistant	<0.0005		
		Human isolates	<0.0005		
J	10	*sec*	0.009	Egg yolk- positive	0.017
				seg	0.046

[1]Over- and under-representation of specific *se* genes was calculated with respect to the number of se-positive strains of each origin.

Table 44: The distribution of 101 *Staphylococcus aureus* isolates assigned to 22 different multilocus sequence types (**ST**).The STs are listed with respect to the allelic profile (www.mlst.net) the clonal complex (**CC**) to which the ST belonged, the number of isolates (n) assigned to each ST, the number of PT`s **(PTs)** observed among isolates from each ST, and the regions (reg) from which isolates belonging to each ST were found. Furthermore, the number of isolates from bovine bulk milk (**B**), caprine bulk milk (**C**) and raw milk products (P) are indicated, and finally the number of isolates (n) of each ST with particular SE-gene profiles **(Joergensen et al., 2005)**

ST	Allelic-profile	CC	n	PTs	Region	Source			SE-genes (n)
						B	C	P	
131*	40-66-46-2-7-50-18	1	1	1	A	1	-	-	*sei* (1)
132*	6-66-47-2-7-50-18	1	14	3	A,D,F	14	-	-	neg (10), *sed/sej* (2), *sei* (2)
133*	6-66-46-2-7-50-18	1†	45	20	A,C	15	25	6	neg (11), *sec/tst* (34)
139*	6-66-46-2-45-50-18	1	1	1	A		1	-	*ec/tst* (1)
478*	54-66-46-2-7-50-18	1	1	1	F	1	-	-	*ec/tst* (1)
481*	6-66-46-2-54-50-18	1	5	2	D	3	2	-	*sec/tst* (5)
1	1-1-1-1-1-1-1	2†	2	2	C	2	-	-	*seh* (2)
147*	1-1-1-36-1-1-1	2	1	1	C	1	-	-	*seh* (1)
477*	1-1-1-1-1-50-1	2	1	1	-	-	-	1	*seh* (1)
130*	6-57-45-2-7-58-52	3†	11	4	A,C,D	2	9	-	neg (11)
480*	6-57-45-2-7-58-18	3	1	1	D	-	-	-	neg (1)
483*	6-57-63-2-7-58-52	3	1	1	D	1	-	-	neg (1)
30	2-2-2-2-6-3-2	4	1	1	-	-	-	1	*sea/seg/tst* (1)
484*	2-2-2-2-1-3-2	4	1	1	-	-	-	1	*sea/seg/tst* (1)
479*	52-87-54 -18-56-32-65	5	7	1	D,F	7	-	-	*seg* (7)
520*	55-87-54-18-56-32-65	5	1	1	D	1	-	-	*seg* (1)
25	4 -1-4 -1-5-5-4	−−	1	1	F	1	-	-	*sei/seg* (1)
97	3 -1-1 -1-7-5-3	−−	1	1	-	-	-	1	neg (1)
135*	39-69-1 -4 -12-1-10	−−	1	1	C	1	-	-	*sej/sed/sei/seg* (1)
136*	38-55-45-18-38-14 -2	−−	1	1	C	1	-	-	neg (1)
137*	7-6-47-5-8-8-6	−−	1	1	C	-	1	-	*sei/seg* (1)
482*	59-79-66-2-62-76-71	−−	2	1	-	-	-	2	*sei* (2)

*New registration to www. mlst. Net;†Predicted ancestor/founder of clonal complex;−−Singleton STs

Figure 26: Dendrogram created from PFGE restriction profiles of 306 *Staphylococcus aureus* isolates from bovine bulk milk (B), caprine bulk milk (C), ruminant mastitis, raw milk products (P) and human blood cultures (H). The cluster cut-off was set at 80 % similarity. The information columns indicate the assigned cluster number, the number of different PT`s (PTs) observed within each cluster, the number of isolates from each source, and the multilocus sequence types (ST) (see Appendix: Table 42 above) and SE-gene profiles observed among isolates in each cluster. **(Jørgensen et al., 2005)**

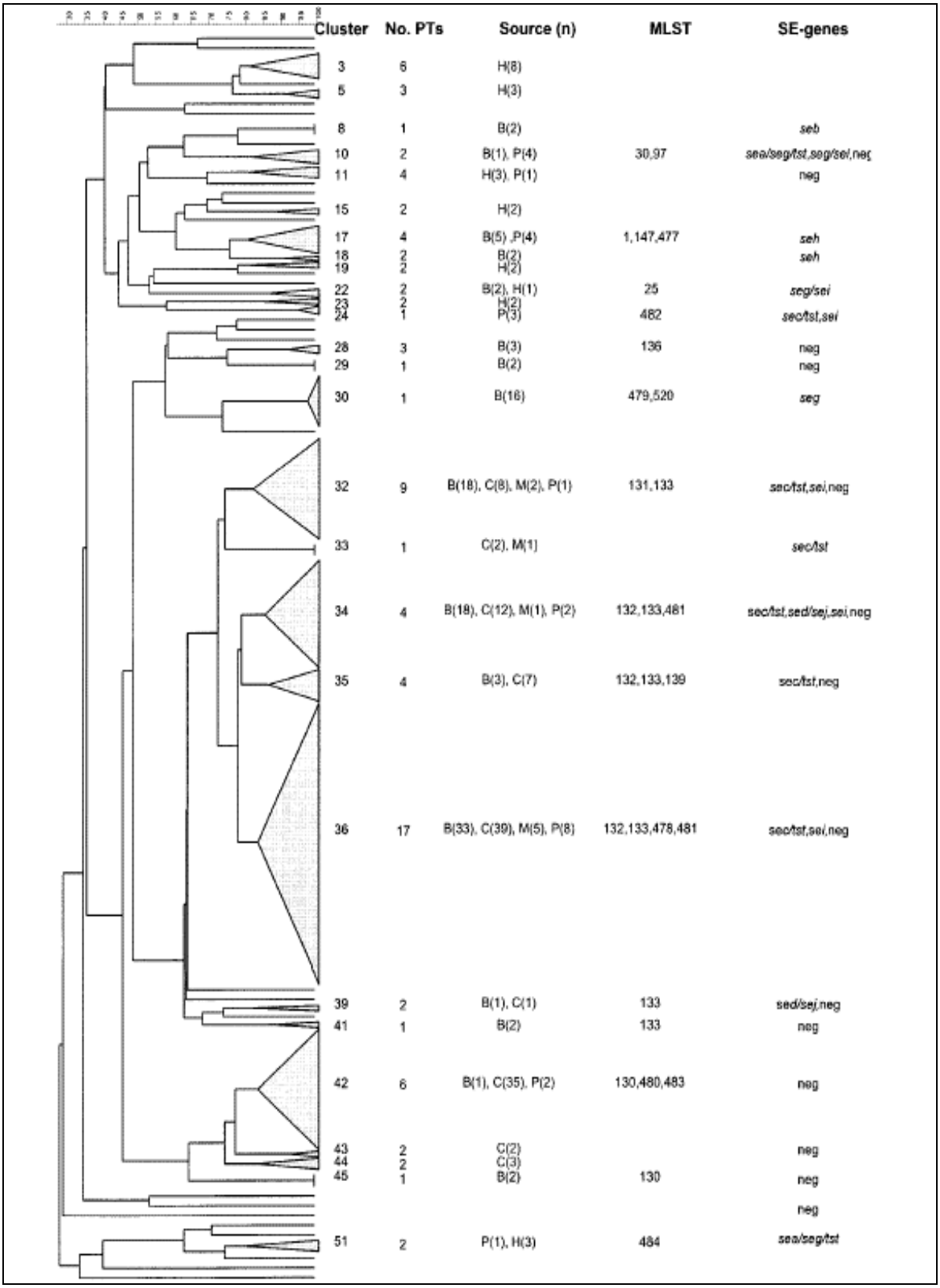


Table 45: SE genes (*se*) in *S. aureus* isolates obtained from raw milk cheese on BP-RPF and BA detected by use of multiplex PCR according to Loncarevic et al. (2005)

Origin	No. of Samples	*S. aureus* positive Samples	No. of isolates		PCR (No of positive isoaltes with SE genes)												
					BB-RPF							BA					
			BB-RPF	BA	*seb*	*sec*	*sed*	*seg*	*sei*	*sej*	*seh*	*seb*	*sec*	*sed*	*seg*	*sei*	*sej*	*seh*
Caprine milk	8 (6)*	1	5	5		**4**[†]												
		1	5	5		5							5					
		1	5	5		5							5					
		1	5	5		5							5					
		1	4	5		4							5					
		1	5	3		5							3					
Bovine milk	10 (4)	1	5	5		5							5					
		1	5	5				1	5						3	5		
		1	5	5								**1**						
		1	5	5		**2**[b]												
Raw milk Cheese	26 (24)	1	5	5				5	**5**						1			
		1	5	4				**5**	**5**									
		1	5	5				**5**	**4**									
		1	5	5				5	**5**						2			
		1	5	5				5	**5**						1			
		1	5	5			**1**	4	**4**	**1**		**2**			2			
		1	5	5									**1**[a]					
		1	5	5		3							3					
		1	3	5		**2**[b]									**4**			
		1	5	5					5				**1**[a]			4		
		1	3	2					3							2		
		1	5	1				**1**	**1**									
		1	5	5					2						**3**	3		
		1	5	5		5							4					
		1	1	3		1							2					
		1	**0**[a]	2									**2**[a]					
		1	5	3		5							2					
		1	3	5		2							5					
		1	2	5							2							5
		1	5	5							2							3
		1	1	5		1							5					
		1	4	**0**[b]		**4**[b]												
		1	**0**[a]	6									**6**					
		1	4	10		4							8					

* Number of positive samples in brackets

[†] Bold marked numbers indicate differences in detectable isolates with *se.*

[a] Probably phage type 119 with *sec and* αβ- haemoylsis on BA with no or only weak coagulase reaction (Table 9)

[b] Strains with *sec* but without α- or β haemolysis but as stong coagulase reaction according to Table 21(Profile 22) and Appendix Table 26

Table 46: *Staphylococcus aureus* isolated from milk, skin and milker`s hands from all 40 dairies under study (Frost et al., 1991) (see Appendix Table 47 and 49, Figure 26 under it)

Source	No. Samples	No. *S.aureus* isolates (%)
Milk	1703	144 (8.4%)
Skin	1528	72 (4.7%)
Liner	371	34 (9.2%)
Hands[1]	80	4 (5.0%)

[1]Milker`s hand were according to Zadoks et al. (2002) sampled before milking

Table 47: Contrast of four different methods for isolation of *S. aureus* according to Frost et al. (1992) from teat skin and milking unit liners (isolation methods for the strains shown in Appendix Tables 46, 48 and 49, Figure 26 under it)

Test	Reservoir		Average (%)
	Skin (n=72)	Liner (n=34)	
Blood agar	12.5%[a]	44.4%[a]	23.2%[a]
Modified Baird-Parker agar	20.8%[ab]	16.6%[b]	19.4%[a]
Liquid Baird-Parker	29.1%[ab]	16.6%[b]	25.0%[a]
Liquid Vogel-Johnson	45.8%[c]	38.9%[a]	43.5%[a]

[1]Means within a column with a different superscript and means within a row underscored with the same line, were significantly different ($P < .05$). Standard errors of the mean within colums were .051, 0.072 and.044.

Figure 27: Distribution of phage types (a) PFGE type (b), and binary types (c) over sources of isolation from teat skin and milk. Only types that were represented by five or more *S. aureus* isolates are shown (Zadoks et al. 2002). NT - not typable

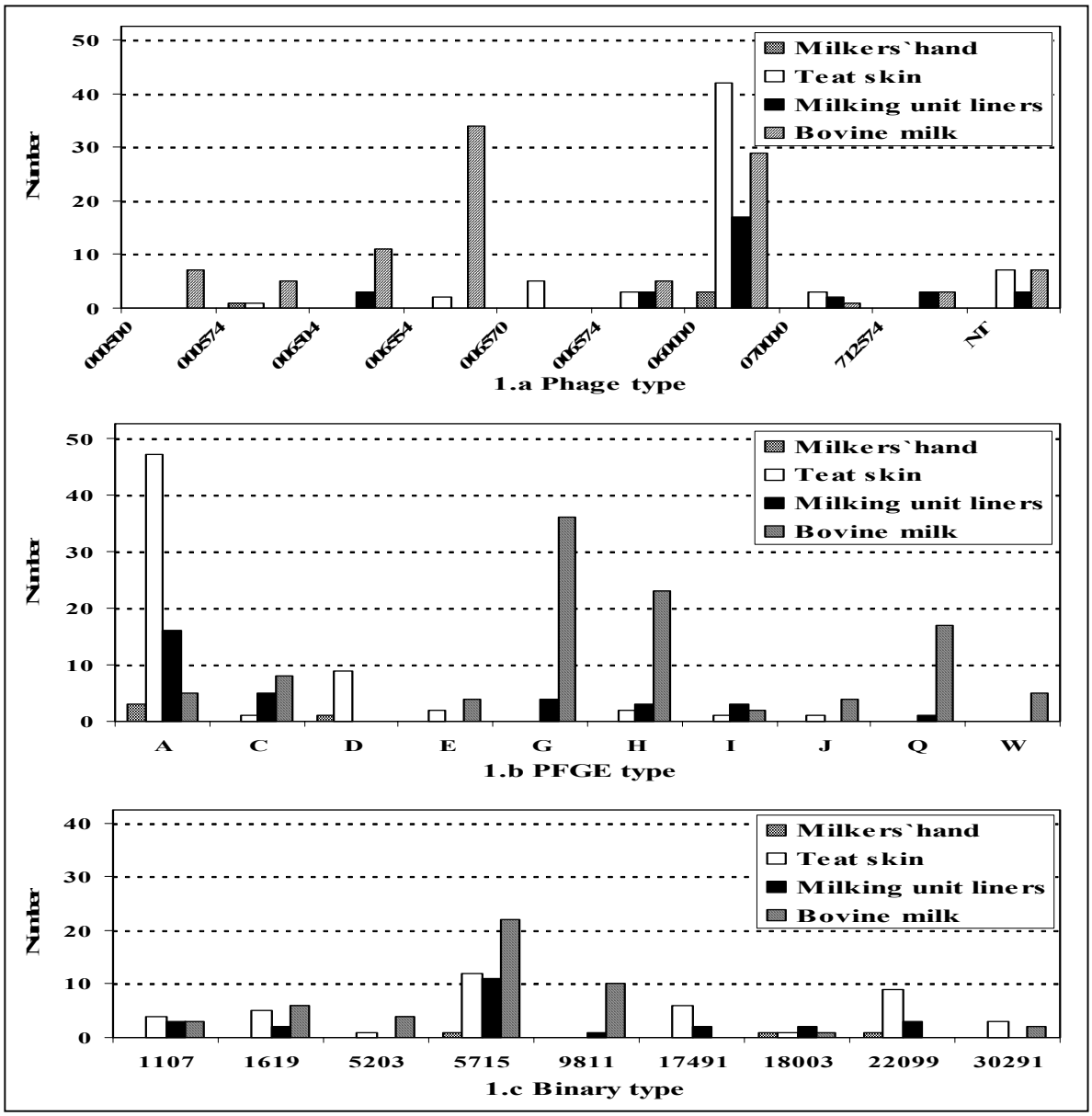


Table 48: Distribution of *S. aureus* phage types from bovine milk and swabbings from milking unit liners and teat skin (Frost et al., 1991)

Phage type	No. Farms	No. From Each Source			Total no. of isolates
		Milk	Liner	Skin	
000500	5	8	0	0	8
000570	2	0	1	5	6
000574	4	10	1	3	14
006000	2	2	0	3	5
006050	1	2	0	0	2
006070	1	1	0	0	1
006504	6	16	4	0	20
006534	1	1	0	0	1
006554	10	39	0	2	41
006570	1	0	0	5	5
006574	3	13	3	2	18
036504	1	1	0	0	1
060000	30	38	15	43	96
070000	4	1	2	4	7
070060	1	1	0	0	1
070567	1	1	0	0	1
072574	1	0	1	0	1
076574	1	3	0	0	3
200060	1	1	0	0	1
711560	2	1	0	1	2
712060	1	0	0	1	1
712574	1	0	0	1	1
712574	6	4	5	0	9
714534	2	1	2	0	3
	Total	144	34	70	248

Table 49: Cross tabulation of main PFGE types and phage types or binary types according to Zadoks et al. (2002)

Type	No. of isolates with PFGE type												Total no. of isolates
	A	C	D	E	G	H	I	J	Q	W	X	MX	
Phage													
000500					1	1		**4**				1	7
000574			1	**4**			2						7
006504		4			*7*	2						1	14
006554	1	2			**20**	*10*						3	36
006570			**5**										5
006574						**8**	2					1	11
06000	**57**	2	1		4				*14*	4	2	8	92
07000	5											1	6
712574		2				4	2						8
MX	3	3	3	1	4	3				1	**2**	2	22
NT	5	1		1	4			1	4			1	17
Total	71	14	10	6	40	28	6	5	18	5	4	18	225
Binary													
1107	7			1						1		1	10
1619	5	2	1					**3**		2		1	14
5203				**3**	1								4
5715	*11*	1		2	*10*	**13**	*6*	1				2	46
9811[a]	1								**10**				11
17491	*8*												8
18003	5												5
22099	**13**												13
30291											**4**	1	5
MX	8	**3**			3				4			**8**	26
Total	58	6	1	6	14	13	6	4	14	3	4	13	142

Data in boldface type are predominant PFGE and phage types. Data in italic type are considered to be in agreement under lenient classification scheme. Abbreviations: MX miscellaneous; NT, not typeable;
A Binary type 9811 was found in one herd only.

Figure 28: Dendrogram containing PFGE patterns of 85 strains collected from bovine with corresponng *spa*-types and MLST- *types* (ST) according to Ikawaty et al. (2009). At the 50 % similarity level, seven branches are distinguished

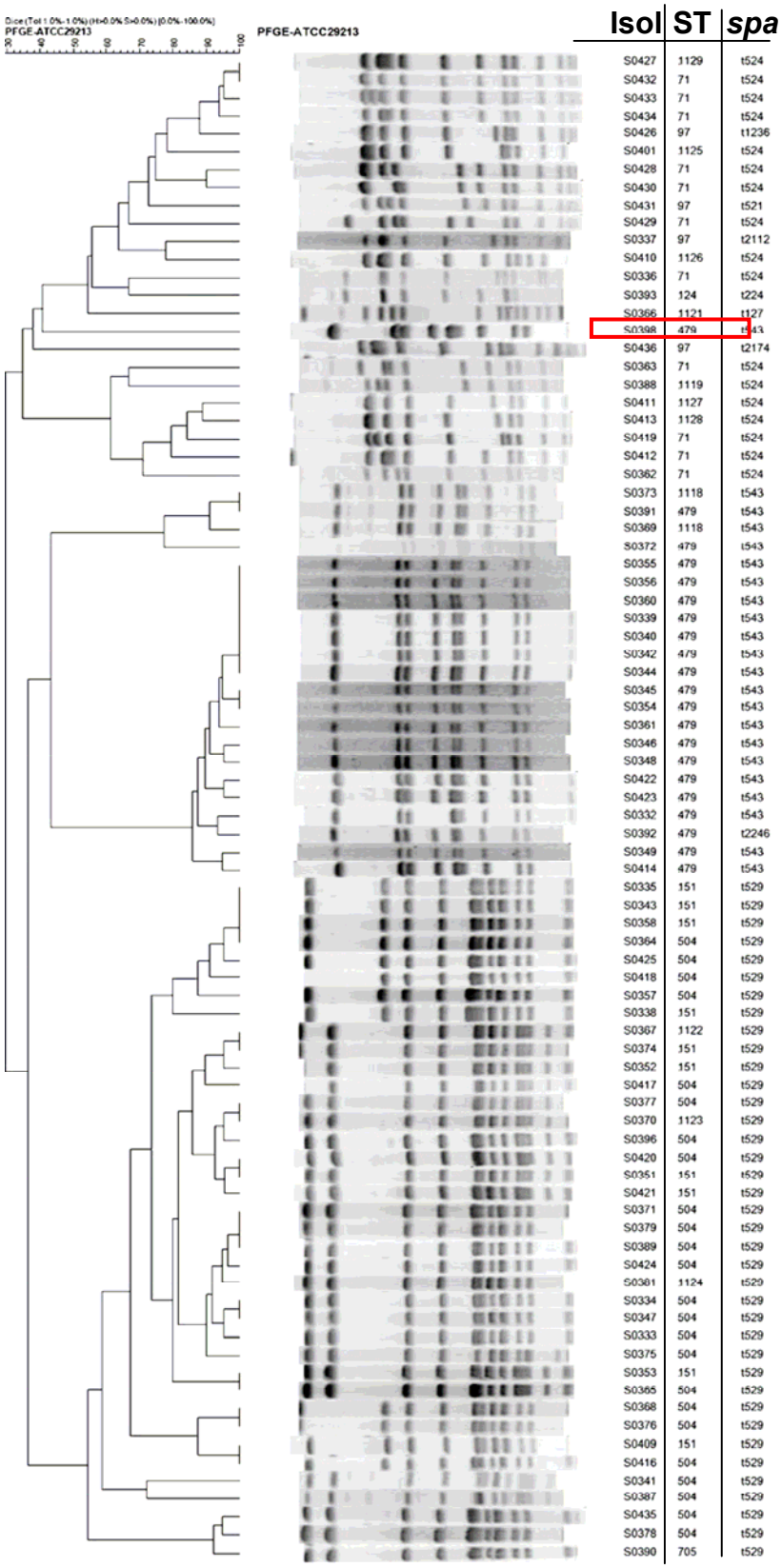


Table 50: Molecular characteristics of ostrich colonization and bovine, caprine, ovine, and Buffalo milk *S. aureus* isolates from Rio de Janeiro State, Brazil according to Aires-de-Sousa et al. (2007)

PFGE[1] Type	No. of isolates	No. of isolates animal species					*spa* typing data			MLST data	
		Bovine (10)[a]	Caprine (2)	Ovine (1)	Bufallo (5)	Ostrich[2] (1)	Repeat sucession	Type	Lineage	Allelic profile	ST
A1	7	1			4	2	r07r23r12r21r17r34r34r33r34	t359	A	3-1-1-1-1-5-96[a]	747[b]
A2	1					1	"	"	A	"	
A3	3	2			1		"	"	A	"	
A4	6	1	2	2	1		r07r23r12r21r17r34r34**r34r**[†]33r34	t267	A	"	
A5	1	1					"	"	A	"	
A6	1		1				r07r23r12r21r17r34r34r34**r34r34r34r34**r33r34	t1182	A	"	
B1	3			3			r03r16r12r21r17r16r17r17r17r23r24	t1180	B	89[a]-66-46-2-7-50-18	750[b]
B2	2				2		r03r16**r21r17r23r13r17r17**r17r17r23r24	t1181	B	"	
C	1	1					r07r23	t605	A	3-68-1-4-1-5-40	126
D1	2				2		r07r23r21r16r34r33r13	t127	A	1-1-1-73[a]-1-1-1	751[b]
D2	1				1		"	"	A	"	
F	2				2		r26r23r17r34r17r20r17r12r17r16	t002	C	1-4-1-4-12-1-10	5

[1]The resulting band patterns were analyzed by visual inspection, followed by analysis with BioNumerics software. Profiles with more than 80% similarity were considered closely related.

[2]anal samples were randomly collected from five ostriches created in an breeding ostrich

[a]Number of herds in brackets

[b]Allele or ST newly registered at www.mlst.net.

[†]Differences in repeat successions of one clonal ST type are bold marked.

Table 51: Distribution of combined phage- and ribotypes of 404 *S. aureus* isolated from clinical and subclinical mastitis in nine Danish dairy cattle herds according to Larsen et al. (2000) (NT, Not typable)

Ribotype	Phage type	Herd								
		A	B	C	D	E	F	G	H	I
1	52/52A/80+	54[a]	26[a]	22[a]	17[a]	10[a]	6	6	22	15[a]
3	52/52A/80+							2		
9	52/52A/80+				1				1[a]	
23	52/52A/80+					5				
19	29/52+		2				25[a]		11	6
26	29/52+			2						
52	29/52/6/42E/47/54/75/81+								2	
57	29/52/6/42E/47/54/75/81+							1		
11	29/52/52A/79/80/95	1								
6	3A/55+			1						
17	75/85+							94[a]		
17	47					30				
18	6/42E/47/54/75/77/81+	5								
22	94/96					5			2	
4	95+		2						2	
2	NT		1		13			1		
50	NT									11

[a] indicate the ribo-and phage type of the *S.aureus* strain isolated from a virtually acute, clinical case of bovine mastitis

Table 52: Staphylococcal strain typing results by 12 methods[a] according to Tenover et al. (1994)

Strain	Outbr	Ox	Phage type	Antibio-gram	Biotype	Plasmid	*Hind* / Ribo	*Cla* / Ribo	IS type	RFLP type	PCR	SmaI/ PFGE	SmaI/ FIGE	Immuno	MLEE
SA-16	NO	S	NR	I	INTER	NP	D	e	NH	NH:NH:NH:NH	0.0	I	VII	K	F
SA-04	NO	R	6/47/54/75	B	A-2b	B	F	i	NH	NH:X:4:NH	2.1	E	IV	D	E
SA-12	NO	R	47/54/75/77/83A	G	A-3b	NP	B	b	C	I:A:1:NH	9.0	J	IC2	A	A5
SA-18	NO	R	47/54/75/77/83A	J	A-3b	I	B	b	C	I:A:1:NH	9.0	J	IC3	A2	A3
SA-20	NO	R	47/54/75/77/83A	K	A-3b	J	B	b	C	I:A:1:NH	9.0	J	IC1	A1	A1
SA-06	NO	I	NR	C	A-3b	C	A	a	B	II:NH:1:a	9.0	C	III	A4	A4
SA-07	NO	S	53/+	D	H-4	D	B	c	NH	NH:NH:1:NH	9.0	B	V	C	A2
SA-08	NO	R	54/75/77/81	E	I-2b	E	E	d	D	I:NH:6:NH	7.0	G	IIA	E1	D1
SA-11	NO	R	NR	E	A-2b	E	G	d	G	II:NH:6:NH	7.0	F	IIB	E2	D2
SA-01	NH1	R	54/77	A1	A-1b	A	A	a.1	A	I:A:5:a	9.0	K.1	IB	A1	A1
SA-09	NH1	R	54/77	A	A-1b	NP	A	a.1	A	I:A:5:a	9.0	K.2	IB	A1	A1
SA-03	NH1	R	47/54/75/77	A2	A-3b	NP	A	a	C	I:A:1:NH	9.0	A	IA	A	A1
SA-13	NH1	R	54/77	A3	A-1b	G	A	a	A	I:A:1:a	9.0	A	IA	A3	A2
SA-14	NH1	S	54/75/77	H	B-1b	H	C	i	NH	NH:NH:1:NH	9.0	H	VI	E3	C
SA-19	NH1	R	54/77	A4	G-1b	A	A	a.1	A	I:A:1:a	9.0	K.3	IB	A1	A1
SA-17	NH2	R	54/75/77	A	C-3b	A	A	a	A	I:A:1:a	9.0	A	IA	A	A1
SA-02	NH2	R	75/77	A	A-3b	A	A	a	A1	I:A:1:b	9.0	A	IA	A	A1
SA-15	NH2	R	77	A	A-3b	A	A	a	A1	I:A:1:a	9.0	A	IA	A1	A5
SA-05	NH2	R	77	A	A-3b	A	A	a	A	I:A:1:a	9.0	A	IA	A	A1
SA-10	NH2	R	77	A	A-3b	A	A	a	A	I:A:1:a	9.0	D	ID	A1	B
SB-07	NO	S	6/47/54/75	C	A-2b	D	C	i	NH	NH:X:4:NH	2.1	D	IIB3	D	B3
SB-03	I	R	75/+	A	C-4	C	A	a	E	I:A:1:a	9.0	A	IA	A6	A1
SB-05	I	R	75/+	A	A-4	C	A	a	E	I:A:1:a	9.0	A	IA	A6	A1
SB-10	I	R	75/+	A	A-4	C	A	a	E	I:A:1:a	9.0	A	IA	A6	A1
SB-12	I	R	75/+	A	C-4	C	A	a	E	I:A:1:a	9.0	A.1	IA	A6	A1
SB-15	I	R	75/77/83A	A	C-4	C	A	a	E	I:A:1:a	9.0	A	IA	A6	A1
SB-19	I	R	75/+	A	A-4	C	A	a	E	I:A:1:a	9.0	A	IA	A5	A1
SB-20	I	R	75/+	A	A-4	C	A	a	E	I:A:1:a	9.0	A	IA	A5	A1
SB-01	NO	R	75/77	A	A-4	A	A	a	E	I:Y:1:a	9.0	A.1	IB1	A5	A1
SB-16	NO	R	75/77/83A	A	A-4	A	A	a	E	I:Y:1:a	9.0	A.1	IB1	A5	A1
SB-18	NO	R	75/+	A	C-4	J	A	a	E1	I:A:1:a	9.0	A	IA	A7	A1
SB-17	NO	I	96	E	B-3b	I	F	j	NH	NH:NH:1:NH	6.0	E	IV	G	A2
SB-14	NO	R	47/54/75/77/83A	A1	A-3b	H	E	a	D	I:A:1:NH	9.0	A.2	IB2	A5	A3
SB-08	NO	S	95	B1	C-4	E	D	d.1	NH	NH:NH:1:NH	2.0	F	III	E5	C
SB-02	II	S	3A/55	B	B-1b	B	B	b	NH	NH:NH:7:NH	6.0	B	IIA	D1	B1
SB-04	II	S	3A/55	B	D-1b	B	B	b	NH	NH:NH:7:NH	6.0	B	IIA	D1	B1
SB-06	II	S	3A/55	B	B-1b	B	B	b	NH	NH:NH:7:NH	6.0	B	IIA	D1	B1
SB-11	II	S	3A/55	B	B-3b	G	B1	b	NH	NH:NH:7:NH	14.0	C	IIB2	D2	B1
SB-09	NO	S	3A	D	D-3b	F	B	b	NH	NH:Z:7:NH	6.0	B	IIA	D1	B1
SB-13	NO	S	3A	B2	D-3b	G	B	b	NH	NH:NH:7:NH	6.0	B.1	IIB1	E6	B2
SC-03	NO	S	6/47/54/75	C	A-2b	C	A	i	NH	NH:NH:4:NH	2.1	C	III	D	B
SC-01	III	R	75	A	A-1b	A	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-04	III	R	75	A	A-1b	D	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-05	III	R	NR	A1	A-1b	D	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-09	III	R	75	A	A-1b	D	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-11	III	R	75	E	A-1b	NP	A	b	NH	I:A:4:NH	10.0	A	IB	F	A1
SC-12	III	R	75	A2	A-1b	A	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-14	III	R	75	A2	B-2b	A	A	b	F	I:A:4:a	10.0	A	IA	F	A2
SC-15	III	R	75	A	A-1b	D	B2	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-17	III	R	75	A	A-1b	A	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-20	III	R	75	A	A-1b	D	A	b	F	I:A:4:a	10.0	A	IA	F	A1
SC-08	NO	S	NR	B	B-3a	E	B1	g	NH	NH:NH:1:NH	2.0	B.1	II	E7	A3
SC-02	IV	S	52/52A/80/47/54/ 83A/84/95	B	E-1b	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	C1
SC-06	IV	S	95	B	J-1b	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	C1
SC-07	IV	S	95	D	I-1a	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	C1
SC-10	IV	S	52A/79/80/47/54/ 75/77/83A/95	B	I-2a	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	C1
SC-13	IV	S	95	B1	I-1b	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	C1
SC-16	IV	S	95	B1	I-1b	B	A	g	NH	NH:NH:1:NH	2.0	B	II	H	D1
SC-18	IV	S	95	F	I-3b	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	C1
SC-19	IV	S	95	B1	D-1a	B	B	g	NH	NH:NH:1:NH	2.0	B	II	E7	D2

[a] **Outbr:** Outbreak; **NO:** Not in epidemiologically related cluster; **OX:** Oxacillin susceptibility test results(S: susceptible; R: resistant); **INTER**: *S. intermedius* biotype; **Plasmid**: Plasmid restriction profile (NP: no plasmids); ***Hind*/Ribo**: Ribotyping result with *HindIII* ; ***Cla*/Ribo**: Ribotyping result with *ClaI* ; **IS**: Insertion sequence; **NH**: No hybridisation; **PCR**: Coagulase gene PCR typing; **PFGE**: Puls-field gel electrophoresis; **FIGE**: Field inversion gel electrophoresis; **Immuno**: Immunoblot typing; **MLEE**: Mutilocus enzyme electrophoresis

Table 53: Number of major types, subtypes, and nontypable isolates by set and number of isolates correctly identified and misclassified by each typing method according to Tenover et al. (1994) (see Appendix Table 42 above)

Method	No. of isolates in set									Total no. of types[a]	No. classified correctly[b]	No. misclassified[c]
	A			B			C					
	Types	Subtypes	Nontypeable	Types	Subtypes	Nontypeable	Types	Subtypes	Nontypeable			
Phage typing	9		3	7			5		2	18	25	4
Antibiogram	11	4		5	3		6	3		21	26	6
Biotype	6	8		4	8		6	11		23	17	2
Plasmids	9		4	10			5	2	1	20	23	0
HindIII ribotyping	7			6	1		2			16	*27*	*7*
ClaI ribotyping	6	1		5	1		3			9	*29*	*7*
IS typing	5	1	4	2	1	9	1		11	9	16	3
RLFP typing (*ClaI*)	10		1	7			4			17	28	3
Coagulase gene PCR	3		1	4	1		2	1		7	28	8
PFGE *(SmaI)*	11	3		6	3		3	1		25	28	7
FIGE *(SmaI)*	11	3		6	5		4			25	27	3
Immunoplotting	5	7		4	8		4			23	28	6
MLEE	11			7			7			21	26	4

[a] Total number of types and subtypes among the 60 isolates examinated

[b] Number of isolates identified correctly as outbreak-related (n=29)

[c] Number of unrelated isolates reported to have some strain type as outbreak-related strains

Table 54: Criteria for interpreting PFGE patterns according to Tenover et al., (1995)

Category	No. of genetic differences compared with outbreak strains	Typical no. of fragment differences compared with outbreak pattern	Epidemiologic interpretation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate is probably part of the outbreak
Possibly related	2	4-6	Isolate is possibly part of the outbreak
Different	≥3	≥7	Isolate is not part of the outbreak

Epidemiological definitions quoted by Tenover et al. (1995):

Isolate. An isolate is a general term for a pure culture of bacteria obtained by subculture of a single colony from a primary isolation plate, presumed to be derived from a single organism, for which no information is available aside from its genus and species.

Epidemiologically related isolate. Epidemiologically related isolates are isolates cultured from specimens collected from patients, fomites, or the environment during a discrete time frame or from a well-defined area as part of an epidemiologic investigation that suggests that the isolates may be derived from a common source.

Genetically related isolate. Genetically related isolates (clones) are isolates that are indistinguishable from each other by a variety of genetic tests or that they are presumed to be derived from a common parent. (Given the potential for cryptic genetic changes detectable only by DNA sequencing or other specific analyses, evidence for clonality is best considered rather than absolute).

Strain. A strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic characteristics or genotypic characteristics or both. A strain is a descriptive subdivision of a species.

Outbreak strain. Outbreak strains are isolates of the same species that are both epidemiologically related (e.g. by time, place and common source of infection) and genetically related (i. e. have indistinguishable genotypes). Such isolates are presumed to be clonly related since they have common phenotypes and were isolated within a defined periode.

Endemic strain. Endemic strains are isolates that are recovered frequently from infected patients in a particular health care setting or community and that are indistinguishable or closely related to each other by typing methods but for which no direct or epidemiologic linkage can be demonstrated. Such organisms are presumed to be clonally related, but their common origin may be more temporally distant from those of outbreak strains.

Personal data sheet

Personal information	
Name:	Klaus Mathias Gutser
Date and place of birth:	06.08.1971; Freising
Marital status:	Single
Nationality:	German
School education:	Primary school in Freising from 1978 until 1982 Secondary school in Freising from 1982 until 1991 High school diploma at 1991
Community service:	From 1991 until 1992 in Freising
Apprenticeship:	training as cheese maker from 1992 until 1995
Study of agriculture:	From 1995 until 2001 Diploma in agriculture at 2001 in TUM Weihenstephan; key area of training: the dairy chain
Research assistant in the *S. aureus*-projekt supported by IWI (foundation Baden-Württemberg):	From 2000 until 2004 on the dairy educational- und research establishment MLF Wangen (destrict Baden-Württemberg, Germany); during this time visiting different research centres of veterinary and human medicine in Germany and Austria (see acknowledgment)
Research assistant at the veterinary university of vienna:	From 2004 until 2008 on the department of milk hygienic by Prof. Martin Wagner
Product manager of traditional raw milk cheese:	From 2009 until 2011at Gebr. Baldauf & Co Lindenberg i. Allgäu; (Gebr. Baldauf & Co trade and produce of raw milk cheese since 1862)
Research assistant at the MIH: (Milk & Dairy institute Dr. Hüfner)	Collaborator by Dr. Josef Hüfner in Hergatz (Bavaria) since July 2011