Molecular and mathematical epidemiology of *Staphylococcus aureus* and *Streptococcus uberis* mastitis in dairy herds

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To family, friends,
farmers, and the future.

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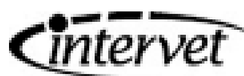

PHARMACIA Animal Health

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General introduction

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Introduction

Mastitis is the most common and costly production disease affecting dairy cows. Many interpretations of the word "mastitis" exist in research and in farming practice. Literally, "mastitis" is "inflammation of mammary gland tissue". Inflammation of the bovine udder is usually caused by infection, mostly by bacteria, but it can also be the result of sterile inflammation due to chemical, physical or mechanical trauma. This dissertation deals with intramammary infections caused by bacteria. Intramammary infections can be accompanied by visible signs, such as clotting and discoloration of milk, swelling and discoloration of the udder, fever, anorexia and even death. When signs are discernible with the naked eye, infection has caused clinical mastitis. When no signs are visible, pathogen presence has resulted in subclinical mastitis, and laboratory techniques such as measurement of somatic cell count (SCC) and bacteriological culture are needed to detect inflammation and infection. Mastitis is a major concern to dairy producers and the food industry around the world for reasons of farm profitability, food quality and animal and public health:

1. Farm profitability is affected by clinical and subclinical mastitis, due to decrease in milk production and changes in milk composition[65, 66], costs of antibiotic treatment, extra labor and withholding of milk[29], death or culling of infected cows[45, 67], and decreased fertility[92, 124].

2. Mastitis is often associated with increase of SCC[39, 154]. When milk with increased SCC is added to the bulk tank, legal limits for bulk milk SCC (BMSCC) may be violated, or thresholds for premium bonus may not be met[12, 127].

3. Clinical mastitis can be an acute and painful condition that affects animal welfare[44].

4. Some mastitis pathogens affect food safety because they produce toxins that cause food poisoning, as in the case of *Staphylococcus aureus*[122, 152], or because the bacteria, e.g. *Listeria* or veterotoxin producing *Escherichia coli*, are zoonotic agents[31, 120, 136].

5. To prevent or cure mastitis, antibiotics are routinely used in herd management and veterinary medicine. Large-scale use of antibiotics may lead to increased prevalence of antibiotic resistance in animal pathogens or commensals, and subsequent transfer of bacteria or resistance genes to humans or human pathogens[77, 87, 143].

In this chapter, some basic concepts of mastitis research and control are introduced, including the notions of contagious and environmental mastitis, current mastitis control programs, and molecular and biometrical approaches to the study of mastitis epidemiology. At the end of the chapter, the aims and design of the project are described, and an outline of the remainder of this dissertation is given.

Contagious and environmental pathogens

Infection can be defined at the level of the individual host, and at the level of the host population. At the individual level, infection has been defined as “a disease process that is the result of multiplication and dissemination of an infectious agent in a host”[28]. At the population level, infectious disease is characterized by “transmission of the agent from one host to another”[58]. In the latter interpretation, "infectious" can be equated to "communicable" (able to be passed on, especially of a disease[1]) or "contagious" (transmitted by contact between individuals[1]). An organism can be infectious at the level of the individual host, while it is not infectious at population level.

Mastitis can be caused by a large variety of pathogens[22, 154]. Some are infectious at the host level only, and others are infectious at both host and population level. The type of infectiousness (host level, or host and population level) of the main bacterial pathogens that cause mastitis in dairy cattle is summarized below.

Escherichia coli and other coliforms such as *Klebsiella spp*. are examples of pathogens that are infectious at the host level but not at the population level. Those Gram-negative bacteria are called "environmental pathogens" and they cause "environmental mastitis"[132]. The primary reservoir of environmental pathogens is the environment of the dairy cow. Exposure of udder quarters to the pathogen may occur at any time during the life of a cow, independent of the presence of infections in herd mates. Because the bacteria are present or even ubiquitous in the environment of the cow, eradication of disease through prevention of cow-to-cow transmission or elimination of reservoirs of bacteria is not possible.

Environmental mastitis is contrasted to "contagious mastitis", which is caused by "contagious pathogens"[50]. Bacteria that cause contagious mastitis, such as *Streptococcus agalactiae* and *Staphylococcus aureus*, are infectious at the individual level and at the population level. The primary reservoir of the contagious pathogens is the infected animal or udder quarter and transmission is largely limited to the milking process[50]. For contagious pathogens, the number of new infections in a population depends on the number of infected individuals that is already present[79]. Eradication of contagious disease is possible when reservoirs of infection are eliminated and routes of cow-to-cow transmission are cut off[93].

For *Streptococcus dysgalactiae* and *Streptococcus uberis*, there is disagreement with respect to their classification. In some laboratories, the two species are grouped together as "environmental streptococci"[144, 154]. However, *Streptococcus dysgalactiae* and *Streptococcus uberis* differ in many bacteriological and epidemiological characteristics[15, 88, 150]. In this dissertation, they will be dealt with as distinct species. *Streptococcus dysgalactiae* largely conforms to the description of contagious pathogens[50, 88], while *Streptococcus uberis* has many characteristics of an environmental pathogen[88, 132].

Understanding the epidemiology of a disease, including disease distribution and transmission, is important for the development of prevention and control programs[52]. Procedures that may be very successful in control or eradication of contagious mastitis,

may not be effective in the control of environmental mastitis, and vice versa[110, 132]. Based on our current understanding of mastitis epidemiology, a large number of control measures has been developed[108, 110].

Mastitis control programs

In the 1960s, Neave, Dodd, Kingwill and Westgarth laid the foundation for current mastitis control programs[38, 110]. The basic program is now known as the "five point mastitis control plan"[62], and encompasses:

1. good husbandry and milking practice with regular testing and maintenance of the milking machine
2. application of a teat disinfectant immediately after removal of the milking machine unit
3. routine antibiotic therapy for all cows after the last milking of each lactation (dry cow therapy)
4. treatment and documentation of all quarters with clinical mastitis
5. culling of cows with chronic or recurrent mastitis.

The goal of this program is to reduce the number of new infections (measures 1, 2 and 3), and to limit the duration of existing infections (measures 3, 4 and 5)[109]. For contagious mastitis, decrease in prevalence (number of existing infections) will contribute to a decrease in incidence (number of new infections).

Implementation of the five point mastitis control plan, also known as "standard mastitis prevention program", has led to control of *Strep. agalactiae* mastitis and, to a lesser extent, *Staph. aureus* and *Strep. dysgalactiae* mastitis[62, 93, 109]. The decrease in prevalence and incidence of contagious mastitis is reflected in a decrease of BMSCC in many countries around the world[22, 112]. The program is only partially successful in control of *Strep. uberis* mastitis[23, 110, 121], while coliform mastitis is not controlled[63, 132]. Measures for control of environmental mastitis aim at reduction of exposure through improved environmental hygiene, and at increase of host resistance, for example through vaccination[156] and adequate nutrition[8, 64, 133, 139]. Recommendations for control of contagious as well as environmental pathogens have been combined in the newly introduced ten-point mastitis control plan, issued by the National Mastitis Council[108]. In addition to measures mentioned above, it includes segregation of infected and non-infected animals[155] and recommendations with respect to biosecurity[47].

Mastitis control in The Netherlands

In The Netherlands, control schemes have been successful in reduction of BMSCC levels from 400,000 cells/ml in 1982 to 221,000 cells/ml in 1995[15, 127] and in elimination of *Strep. agalactiae* infections[16, 105, 115]. As average BMSCC decreased and the number of farms with low BMSCC increased[22], research started to focus on the epidemiology and dynamics of mastitis in low BMSCC herds[82, 125].

From 1997 to 2000, mean levels of unweighted BMSCC in The Netherlands hovered around 200,000 cells/ml (Figure 1). Although much lower than levels in the '80s and early '90s, this level of SCC is indicative of intramammary infection[39] and decrease in milk production[65], implying that mastitis continues to be a problem in the Dutch dairy industry, and that the problem is not restricted to low BMSCC herds. Since April 2000, average BMSCC has shown an increasing trend, indicating aggravation of the udder health situation. In addition, clinical mastitis is a problem in herds with low, medium and high BMSCC[16]. Reduction of BMSCC and clinical mastitis will become more important as quality demands increase with respect to BMSCC, bacterial content of milk and health of cows used for food production.

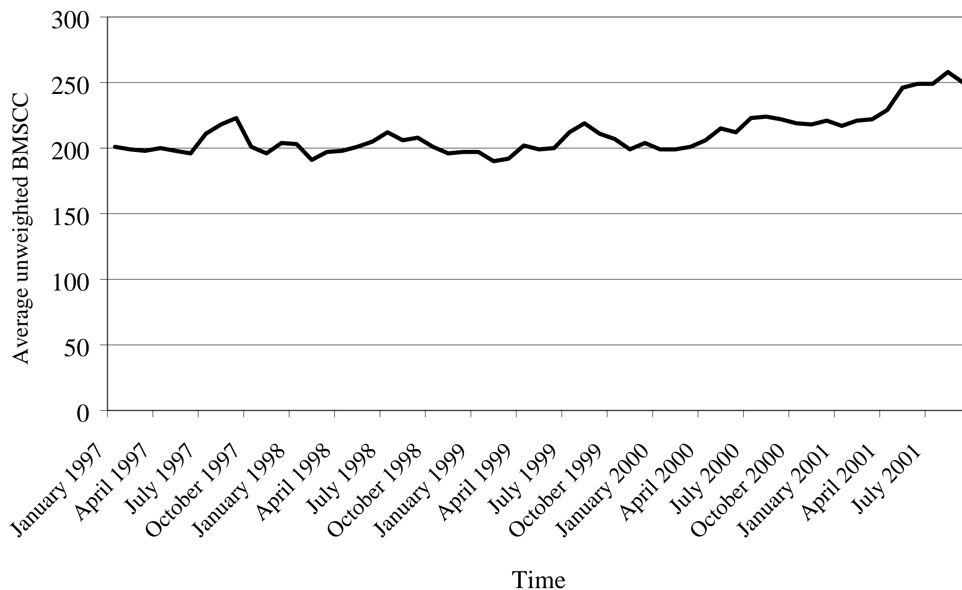

Figure 1. Development of mean unweighted bulk milk somatic cell counts in The Netherlands from January 1997 to September 2001 (Source: Milk Quality Control Station, Zutphen, The Netherlands).

The most frequently isolated agents of subclinical mastitis in The Netherlands are *Staph. aureus* and *Strep. uberis* [115]. Both pathogens are associated with increase in SCC at cow level[88, 153] and at bulk tank level[47]. In addition, *Staph. aureus* and *Strep. uberis* are among the most frequent causes of clinical mastitis in The Netherlands[15, 105]. Obviously, implementation of control measures has thus far failed to reduce the importance of *Staph. aureus* and *Strep. uberis* as causes of bovine mastitis in the Dutch dairy industry. This failure implies that known methods are not implemented vigorously or appropriately, or that our understanding of mastitis epidemiology has not been sufficient to develop adequate control measures.

It is often said that the main problem with control of *Staph. aureus* mastitis is the low cure rate of infected animals[50]. If low cure rates were the only problem, *S. aureus* mastitis would eventually be eliminated by prevention of cow-to-cow transmission. However, *Staph. aureus* occurs in the environment of dairy cows[99, 119], raising the possibility that some infections originate from the environment rather than from infected herd mates. Environmental *Staph. aureus* would explain why measures aimed at control of contagious transmission alone do not result in eradication of the disease. In *Strep. uberis* control, the main problem is considered to be that it is an environmental pathogen. Although this is indeed a major problem, it disregards the fact that a certain level of *Strep. uberis* control is achieved through strategies that prevent cow-to-cow transmission of the pathogen[110, 121]. Neglect of the control of contagious transmission of *Strep. uberis* may aggravate herd problems due to this pathogen.

To quantify contagiousness of *Staph. aureus* and *Strep. uberis*, and to study the hypothesis that each pathogen may originate from multiple sources and may use multiple routes of transmission, molecular epidemiologic methods and biometrical approaches will be used in this dissertation. The combination of molecular models and mathematical epidemiology can advance our understanding of infectious diseases and the development of methods for infection control more than either method alone could do[90]. Before the aims and design of this project are explained, some basic principles and methods used in molecular epidemiology and biometry will be introduced.

Molecular approaches to epidemiology

Molecular epidemiology is the use of molecular techniques for the study of epidemiology. “Molecular” refers to the use of techniques to characterize amino-acid or nucleic acid content[52]. Molecular techniques may be applied to the measurement of host or agent factors. In this dissertation the focus will be on bacterial agents. When applied in infectious disease epidemiology, the goals of molecular epidemiology include identification of micro-organisms and their sources, their biological relations, their routes of transmission, and genes responsible for specific characteristics such as virulence[52, 90]. In mastitis research, molecular techniques have been used to differentiate between persistent and new

infections[40, 111], to study strain-differences in virulence[100, 134], to identify possible targets for vaccine development[89, 135], and to monitor sources of food borne pathogens or pathogens that are resistant to antibiotics[37, 147].

The number of molecular techniques for identification of bacteria at the species and sub-species level is vast, and continues to grow (for review: see [137] or [148]). Techniques can be classified as phenotypic or genotypic, depending on whether they detect variability characteristics that result from the expression of genes (phenotypic), or the genetic material itself (genotypic). Phenotypic methods include determination of antibiotic susceptibility patterns, serotyping, biotyping, phage typing, and multilocus enzyme electrophoresis (MLEE). Genotypic methods include plasmid typing, single gene typing, whole genome typing, and DNA sequencing[148].

Table 1. Overview of phenotypic and genotypic techniques used for strain typing of *Staphylococcus aureus* and *Streptococcus uberis* isolates from dairy cattle.

Typing technique	*Staphylococcus aureus*	*Streptococcus uberis*
Phenotypic		
• antibiotic susceptibility	5, 20, 34, 84, 94, 95, 97, 102, 117, 118, 140, 147	72, 114
• serotyping	56, 57, 116, 135, 145, 146	55, 71, 83
• biotyping	5, 36, 46, 68, 94, 107, 145	71, 72, 83
• inhibitor typing		24, 72, 141
• toxin typing	49, 76, 85, 106, 145	
• phage typing	3-7, 46, 51, 68, 86, 91, 94, 96, 140	60
• immunoblotting		55
• MLEE[a]	49, 75, 145	
Genotypic		
• plasmid profile	5, 20, 84, 101	
• plasmid REA[b]	25	
• chromosomal REA[b]	102	55, 61, 70, 72, 73
• ribotyping	3-5, 25, 26, 49, 86, 107, 118	
• coagulase typing	2, 14, 84, 117, 158	
• *spa* typing	14, 84, 158	
• rRNA spacer typing	14, 84	
• toxin gene typing	85, 129	
• PFGE[c]	14, 26, 74, 84, 135, 147, 157, 158	19, 41, 114, 151
• RAPD[d]	48, 49, 54, 80, 91, 103, 107	53, 70, 111
• binary typing	157	

a) MLEE = multilocus enzyme electrophoresis
b) REA = restriction enzyme analysis
c) PFGE = pulsed-field gel electrophoresis
d) RAPD = random amplified polymorphic DNA typing

Oversimplifying grossly, it can be said that single gene or whole genome typing is largely based on cutting or copying of DNA and ingenious combinations thereof, followed by size-based separation of the DNA fragments that are produced. Cutting of DNA is used in restriction enzyme analysis (REA) and pulsed-field gel electrophoresis (PFGE). Copying is achieved by many variations of the polymerase chain reaction (PCR), for example for production of random amplified polymorphic DNA (RAPD) fingerprints. Cutting and copying are combined in amplified fragment length polymorphism (AFLP) typing[123]. Binding of strands of complementary DNA (hybridization) is another mechanism used to identify strains. This principle is applied in binary typing[149]. With the development of more advanced equipment, the use of DNA sequencing of selected genes becomes increasingly important, targeting single genes such as the protein A gene or the coagulase gene of *Staph. aureus*[130, 131] or multiple genes, as in multilocus sequence typing (MLST) of staphylococci and streptococci[42, 43]. Which technique is appropriate depends on the availability of facilities and on the questions at hand[137, 148]. An overview of typing techniques that have been used to study *Staph. aureus* and *Strep. uberis* mastitis in dairy cattle is given in Table 1. Due to the abundance of literature on typing of bacteria, and the publication of new material while this dissertation is processed, the overview cannot be complete.

There is no standardized nomenclature for bacterial strains, as there is for bacterial species. Any technique, or combination of techniques, that discriminates between isolates can be used to identify strains, and nomenclature depends on the way a typing laboratory assigns names to different isolates[148]. With many techniques, identification is only meaningful in a comparative analysis, i.e. within the frame of reference of one typing study (within-study comparison or intra-center comparison)[138]. Use of standardized protocols and reference strains allows for expansion of the frame of reference from a single typing study to multiple typing studies (between-study comparison, or inter-center comparison)[27]. In the future, the use of library typing techniques, in which any typing result has a universal many, may overcome current problems in strain identification and nomenclature[138]. Meanwhile, international consensus guidelines for appropriate use and interpretation of typing systems have been proposed[137, 142]. In accordance with those guidelines, the following definitions will be used in this dissertation. To clarify concepts, an example is given for each definition.

1. Isolate = collection of cells derived as a monoculture from a primary colony growing on a solid medium on which the source of the isolate was inoculated. Example: after bacteriological culture of a milk sample on an agar plate, a colony can be picked off the plate and streaked onto a new plate to obtain a pure culture of that isolate which can then be used for storage and further research.

2. Strain = an isolate or group of isolates that display specific phenotypic or genotypic characteristics that set it apart from other isolates belonging to the same species.

Example: *Staph. aureus* isolates can be subdivided into methicillin-susceptible and methicillin-resistant *Staph. aureus* strains (MSSA and MRSA, respectively).

3. Clone = progeny of a common ancestor and the result of a direct chain of replication of that ancestor. Identification of clones is based on monitoring of multiple genetic markers of sufficient discriminatory power. Example: Most MRSA strains belong to a limited number of clones, such as the Iberian clone and the New York clone[30].

Different strains exist among isolates of *Staph. aureus* and *Strep. uberis* from bovine mastitis (Table 1). If different strains within those bacterial species differ in pathogenicity and transmission patterns, classification of mastitis pathogens at the species level may lead to erroneous simplification of mastitis epidemiology and subsequent recommendation of inappropriate control measures. When the project described in this dissertation was initiated, molecular data were abundant, but little was known about the epidemiology of different bacterial strains within dairy herds.

Biometrical approaches to epidemiology

Molecular techniques are indispensable tools for sensitive and specific study of infectious disease epidemiology[52]. Another essential toolkit for the study of mastitis epidemiology is comprised of biometrical techniques. Biometry is “a numerical approach to biology that includes study design, data collection, data analysis, data display and the drawing of appropriate conclusions”[113]. It includes the use of statistics, defined more narrowly as “the skills of data manipulation and data analysis”[113], and mathematical epidemiology, i.e. "the application of mathematics to the study of infectious disease"[13]. Statistical methods are routinely used in biomedical research to collect and analyze data in such a way that conclusions drawn from them can subsequently be applied in disease prevention and control[33]. Current mastitis control programs, as described earlier in this chapter, are largely based on such studies. Because the use of statistical methods is inherent to experiments, field trials and observational epidemiology, it is impossible and irrelevant to give an overview of all mastitis research that is based on this approach.

A study type that does warrant mention here is the risk factor study. In dairy herds, bacterial mastitis is the result of interactions of pathogens with their hosts in a specific environment. Therefore, an understanding of host and environmental factors that affect this interaction is essential to mastitis control and prevention. Statistical methods have been used to identify risk factors for mastitis, and to quantify the magnitude of their effect at the level of the individual cow or herd (relative risk) and the impact in a population of cows or herds (attributable fraction)[98]. At herd level, risk factors have been identified for incidence of clinical mastitis[15, 126] and prevalence of subclinical staphylococcal and streptococcal infections[18, 32, 69]. In herds with staphylococcal or streptococcal mastitis, all individuals may

be exposed to the pathogens. Usually, only a fraction of the population gets infected, suggesting differences between cows or quarters in susceptibility to infection. Cow and quarter level risk factors for S*taph. aureus* mastitis have been identified in experimental studies[81]. A limited number of quarter level risk factors for staphylococcal and streptococcal infections have been studied in observational studies[128]. Differences between cows and quarters in susceptibility to naturally occurring clinical and specifically subclinical *Staph. aureus* and *Strep. uberis* mastitis are largely unexplained.

Statistical analyses are commonly based on the assumption that observations are independent. If they are not, correction for interdependence is sometimes possible[17, 104]. For pathogens that are infectious at the population level, observations are not independent by definition[58]. For adequate description and analysis of the dynamics of diseases that are infectious at the population level, mathematical models are necessary[13, 78]. A key concept in mathematical models of infectious disease epidemiology is the basic reproductive number, R_0. The concept is used to quantify the potential for spread of microparasites (viruses, bacteria, protozoa and fungi), and macroparasites (helminths and arthropods). Microparasites and macroparasites are very different groups, both from a biological and mathematical perspective. For microparasites, R_0 is the average number of secondary infections produced when one infected individual is introduced in a fully susceptible host population[13]. When R_0 is lower than one, each individual will on average produce less than one successful offspring, and infection will not spread in the population, but die out. When R_0 is larger than one, each infected individual will on average produce more than one infected offspring, and infection will spread in the population. The population as a whole can be represented by a model consisting of three compartments: susceptible hosts (S), infected hosts (I), and recovered hosts (R). The three-compartment SIR model is the archetypal compartmental model for mathematical description of infectious disease dynamics[13]. Mathematical models can be written in a deterministic manner, based on averages and ignoring random variability, or in a stochastic manner, i.e. accounting for random variability.

Mathematical models are used to test hypotheses about the mechanisms underlying disease transmission and to study the effect of control procedures[21, 35, 78]. In mastitis research, this approach has been used to quantify the effect of post-milking teat disinfection on contagious transmission of *Staph. aureus* infections[79]. Once risk factors, mechanisms of disease transmission, and the effect of control measures are known, models can be used to support decision-making, e.g. with respect to treatment or culling of cows with mastitis[67], or to examine the effect of multiple combined control measures[10]. Dairy herds are big, complex and expensive systems and it is not feasible to examine all management scenarios by means of experimentation or field trials. Models enable us to examine multiple strategies that could not be tested otherwise. Furthermore, models can be updated as new treatments or interventions become available, and they can help to identify directions for further research[11]. Mathematical models of mastitis have been reviewed by Allore and Erb[9].

Aim and design of the project

The main purposes of the project described in this dissertation were to improve our understanding of the dynamics of *Staph. aureus* and *Strep. uberis* mastitis in dairy herds that have medium level BMSCC (200,000 - 300,000 cells/ml) despite implementation of the standard mastitis prevention program, and to identify host and pathogen traits that affect the occurrence and outcome of infection, using a combination of molecular, statistical and mathematical approaches to mastitis epidemiology. More specifically, the aims of this study were:

- to implement and improve molecular typing techniques for identification of *Staph. aureus* and *Strep. uberis* strains, and to examine associations between strains and clinical and epidemiological characteristics of infection.
- to evaluate risk factors at cow and quarter level for subclinical and clinical *Staph. aureus* and *Strep. uberis* mastitis after natural exposure.
- to develop mathematical models and estimate model parameters to gain insight into the dynamics of *Staph. aureus* and *Strep. uberis* mastitis in dairy herds, with attention to the role of heifers, the effect of previous infection on susceptibility to subsequent infection, and the importance of environmental sources of infection.

The dynamics of mastitis at herd level were observed in a longitudinal prospective study in commercial dairy herds. The longitudinal design allowed for the observation of the time order of events and hence for identification of possible cause-effect relationships between host factors and susceptibility to infection. The design also allowed for measurement of duration of infections and semiquantification of severity of infections, using clinical symptoms and SCC as criteria. Herds were selected based on records that documented a history of at least a year of medium BMSCC (200,000-300,000 cells/ml) despite implementation of most if not all points of the standard mastitis prevention program during that period, and on willingness of farmers to cooperate in the study. *Staphylococcus aureus*, *Strep. uberis*, and *Strep. dysgalactiae* had been the main causes of infection in each herd.

In statistical terms, infection is a rare event relative to the number of days at risk. Therefore, a large number of quarters and cows needed to be observed over a long period of time. The precision of post-hoc analyses was dependent on the actual numbers of infection events that were recorded. Whole populations rather than random samples from populations were observed for accurate estimation of transmission parameters. Earlier studies had indicated that short-term infections might be important in the population dynamics of mastitis, and that such infections went unnoticed with sampling at 5 to 6 week intervals. Therefore, a shorter sampling interval was necessary. At the same time, it was acknowledged that different herds needed to be included because dynamics in an individual herd can be very different from the average dynamics in herds with similar management, especially when disease outbreaks occur[82]. To strike a balance between the need for

frequent sampling and the need to include multiple herds, and within the logistic constraints of the project, a 3-week sampling interval and inclusion of three herds were chosen.
Quarter milk samples from all lactating animals were routinely collected at 3-week intervals from May 1997 to December 1998. Throughout the study period, additional quarter milk samples from all cows and heifers were collected by farmers at calving or purchase (entry into the milking herd), dry-off or culling (exit from the milking herd), and when clinical mastitis was observed. The farmers recorded calving dates, treatment dates, dry-off dates and cull dates. From October 1997 to December 1998, body condition scores and teat end callosity scores were recorded at 3-week intervals by technicians that were trained for the purpose. All milk samples were used for bacteriological culture according to NMC standards[59]. Samples taken during routine visits were also used for determination of quarter milk SCC. All *Staph. aureus* isolates, and a selection of *Strep. uberis* isolates, were stored in pure culture for molecular typing studies. A collection of 225 *Staph. aureus* isolates from an American study on reservoirs and fomites of *Staph.. aureus* mastitis in dairy herds was also made available for strain typing [51].

Outline of this dissertation

After this general introduction (Chapter 1), the use of genotypic methods for typing of bovine *Staph. aureus* is explored. Chapter 2 is a pilot study on the use of PFGE and binary typing, and on associations between *Staph. aureus* strains and virulence, sources of infection, and host species (comparison of bovine and human strains). In Chapter 3, PFGE and binary typing are used on a larger scale, to re-assess associations between reservoirs of *Staph. aureus* and routes of transmission that had previously been examined by phenotypic but not genotypic methods, and to explore the usefulness of binary typing as a routine typing method in mastitis microbiology. The dynamics of *Staph. aureus* mastitis are quantified in Chapter 4, using an elaborate version of the basic SIR model. The herd is described as a population of uninfected, subclinically infected, clinically infected and recovered-uninfected udder quarters. Model parameters are estimated from field data. In addition, changes in herd management that would affect mastitis dynamics are simulated. The homogeneity of the population with respect to susceptibility to infection, that is assumed in the compartmental model, is scrutinized in Chapter 5. In that chapter, risk factors for *Staph. aureus* mastitis and for *Strep. uberis* mastitis are identified at the level of the cow and at the level of the udder quarter. An outbreak of *Strep. uberis* mastitis is described in Chapter 6, and the SIR model is expanded again, this time to incorporate the absence or presence of other pathogens, or recovery from earlier episodes of *Strep. uberis* mastitis. Epidemiological, clinical and molecular characteristics of *Strep. uberis* infections are examined in more detail in Chapter 7. Finally, in Chapter 8, the results from molecular and biometrical studies are integrated at population, host and pathogen level for both pathogens, and implications for herd health and future research are discussed.

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- Chapter 2 -

Application of pulsed-field gel electorphoresis and binary typing as tools in veterinary clinical microbiology and molecular epidemiology of bovine and human *Staphylococcus aureus*

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Abstract

Thirty eight bovine mammary *Staphylococcus aureus* isolates from diverse clinical, temporal and geographical origins were genotyped by pulsed-field gel electrophoresis (PFGE) after *Sma*I digestion of prokaryotic DNA, and by means of binary typing using 15 strain-specific DNA probes. Seven pulsed-field types and four subtypes were identified, as were 16 binary types. Concordant delineation of genetic relatedness was documented by both techniques, yet based on practical and epidemiological considerations, binary typing was the preferable method. Genotypes of bovine isolates were compared to 55 previously characterized human *S. aureus* isolates through cluster analysis of binary types. Genetic clusters containing strains from both human and bovine origin were found, but bacterial genotypes were predominantly associated with a single host species. Binary typing proved an excellent tool for comparison of *S. aureus* strains, including methicillin resistant *S. aureus*, derived from different host species and from different databases. For 28 bovine *S. aureus* isolates, detailed clinical observations *in vivo* were compared to strain typing results *in vitro*. Associations were found between distinct genotypes and severity of disease, suggesting strain specific bacterial virulence. Circumstantial evidence furthermore supports strain specific routes of bacterial dissemination. We conclude that PFGE and binary typing can be successfully applied for genetic analysis of *S. aureus* isolates from bovine mammary secretions. Binary typing in particular is a robust and simple method and promises to become a powerful tool for strain characterization, for resolution of clonal relationships of bacteria within and between host species, and for identification of sources and transmission routes of bovine *S. aureus*.

Abbreviation key: BT = binary type, **MRSA** = methicillin resistant *S. aureus*, **MSSA** = methicillin susceptible *S. aureus*, **PFGE** = pulsed-field gel electrophoresis, **SCC** = somatic cell count.

Introduction

Infections due to staphylococci are of major importance to veterinary and human medicine. *Staphylococcus aureus* is one of the most significant pathogens causing intramammary infections in dairy cattle worldwide[6, 15, 34]. In humans, *S. aureus* is a major cause of community acquired as well as nosocomial morbidity and mortality. In the last decades increasing prevalence of methicillin-resistant *S. aureus* strains has become an additional infection control problem in human medicine[4, 7, 25]. Staphylococcal strains may vary considerably in virulence and epidemiological potential. To control spread of staphylococcal infections, sources of contamination and mechanisms of transmission must be identified. Detailed pathogenetic and epidemiological studies depend on the availability of typing systems that differentiate between strains belonging to the same bacterial species.

In veterinary microbiology, many techniques have been applied for characterization of bovine *S. aureus* strains. Phenotypic methods include phage typing[3, 13, 37], biotyping[11, 23] and multilocus enzyme electrophoresis[12, 17]. Genotypic methods include single gene typing systems, such as detection of coagulase gene polymorphism[2, 36] and ribotyping[3, 12, 29], and whole genome typing systems, such as arbitrarily primed PCR[12, 21, 24]. Furthermore, plasmid pattern analysis has been used to differentiate among *S. aureus* isolates of bovine origin, based on diversity of extrachromosomal DNA[3]. In human microbiology, most of these procedures have been superseded by newer methods that have enhanced resolving powers, including pulsed-field gel electrophoresis (PFGE) of DNA macrorestriction fragments[5, 28, 32] and, more recently, binary typing[40]. PFGE is a reliable and reproducible method with high discriminatory power. Drawbacks of this method are that it is laborious and expensive, and that complex DNA patterns may be difficult to interpret, especially for large collections of isolates[38, 39]. For clinical laboratories processing great numbers of samples, these limitations may be impediments to routine use. Binary typing is a highly reproducible and stable library typing method with excellent discriminatory abilities. It has the additional advantage of producing a simple binary output, facilitating interpretation and comparison of typing results, and it lacks experimentally unstable parameters such as electrophoretic conditions[42]. Recently, several authors have reported on the use of PFGE for characterization of bovine isolates[3a, 21a], but so far binary typing has not been applied to *S. aureus* isolates of bovine origin.

The purpose of this study was to determine whether PFGE and binary typing are suitable techniques for differentiation of isolates of *S. aureus* recovered from bovine mammary secretions. In addition, a collection of bovine isolates was compared to a collection of human isolates, including methicillin-resistant strains, to explore clonal relatedness of isolates from cattle and humans as determined by binary typing. Finally, associations of bacterial strains with clinical observations in cattle were examined to identify possible relations between genotypes and bacterial virulence or routes of spread.

Materials and Methods

Bacterial isolates

This study included 38 bovine *S. aureus* isolates collected from eight dairy herds in The Netherlands between May 1997 and February 1999. Three herds (I, II and III) were involved in a longitudinal survey on population dynamics of intramammary infections. In those herds, milk samples were routinely collected from all four udder quarters of each cow at three-weekly intervals for 81 weeks. Samples from the other five herds were submitted to the diagnostic laboratory of the Animal Health Service, Deventer, The Netherlands, as part of a dairy health improvement scheme. Bacteria were cultured from milk samples according to National Mastitis Council standards[16] and identified at the species level as described previously[20]. Isolates were stored frozen until further use. Isolates were selected to represent different geographical, temporal and clinical origins (Table 1).

Binary typing data on 55 human *S. aureus* isolates from diverse geographic and temporal origins in the United States and The Netherlands were used (Table 2). Human collections include methicillin resistant *S. aureus* strains (MRSA, n=37) and methicillin susceptible *S. aureus* strains (MSSA, n=18) and have been described in detail before[38, 41, 42].

Clinical and subclinical disease characteristics

Detailed records of clinical observations were available for isolates 1 to 33 (Table 1). In addition, somatic cell counts (SCC) of milk samples yielding isolates 1 to 33, with exception of isolates 8-10, were determined by means of a Fossomatic cell counter (Foss Electric, Hillerød, Denmark). SCC is a measure of the leucocyte content of milk and is used as an indicator for infection. The threshold between non-infected and infected is commonly set at 200.000 cells/ml [10]. Isolates 34 and 35 were cultured from bulk milk samples from farm III and disease classifications do not apply. For samples yielding isolates 36-40, SCC was determined but detailed clinical data were not available.

Based on clinical symptoms and SCC, isolates 1-33 were classified as belonging to one of four clinical groups in order of increasing severity of infection; (1) subclinical infection with non-elevated SCC (in 1000 cells/ml: median 97, range 11-152), (2) chronic subclinical infection with elevated SCC (in 1000 cells/ml: median 1278, range 210-7821), (3) short duration mild clinical disease or short duration subclinical disease with high SCC (in 1000 cells/ml: median 4560, range 411-8710), and (4) acute severe clinical disease (Table 1). SCC was not determined for group 4 samples, because clot formation in mammary secretions interfered with SCC measurement.

Table 1. Summary of epidemiologic data, PFGE typing data and binary typing results for 38 bovine *Staphylococcus aureus* isolates.

Herd	Isolate	Cow-quarter[a]	Collection period	Clinical characteristics	Clin. group[b]	PFGE type	Binary code[c]	Binary type[d]
I	1	75-1	1997 (Aug)	long, high SCC	2	A	001111111111101	8189
	2	75-2	1997 (Aug)	long, high SCC	2	B	001111111111101	8189
	3	75-4	1997 (Aug)	long, high SCC	2	B	001111111111101	8189
	5	78-3	1997 (Aug)	long, high SCC	2	B.1	001111111111101	8189
	6	63-3	1997 (June)	short, high SCC	3	B	001111111111111	8191
	7	77-4	1997 (Oct)	short, low SCC	1	C	000010011000001	1217
	8	67-3	1998 (Jan)	severe disease	4	D	101010011110011	21747
	9	74-4	1997 (Dec)	severe disease	4	D	101010011110011	21747
	10	76-4	1997 (Dec)	severe disease	4	D	101010011110001	21745
	12	25-4	1997 (May)	short, high SCC	3	D	101010011110011	21747
	13	25-4	1998 (May)	short, high SCC	3	D	101010011110001	21745
	14	18-4	1998 (Nov)	long, high SCC	2	B.2	001111111110001	8177
	15	53-2	1998 (Nov)	long, high SCC[e]	2	B	001111111110001	8177
	16	90-3	1998 (Nov)	long, high SCC[e]	2	B	001111111110011	8179
II	17	11-3	1997 (July)	long, high SCC	2	E	000110011010011	3283
	18	18-3	1997 (July)	long, high SCC	2	E.1	010110011010011	11475
	19	99-2	1997 (July)	long, high SCC	2	E	000110011010001	3281
	20	108-3	1997 (July)	long, high SCC	2	F	001011011111111	5887
	21	47-3	1998 (May)	mild clinical	3	D	101010011010001	21713
	22	29-4	1997 (Aug)	mild clinical	3	D	101010011010011	21715
	23	70-3	1998 (Apr)	mild clinical	3	D	101010011010011	21715
	24	21-4	1998 (May)	mild clinical	3	E	000110011010011	3283
III	25	25-3	1997 (July)	long, low SCC	1	C	000010011010011	1235
	26	95-1	1997 (July)	mild clinical	3	E	000110011010011	3283
	27	46-4	1997 (Dec)	long, low SCC	1	C	000010011010011	1235
	28	29-1	1998 (May)	long, high SCC	2	E	000110011010011	3283
	29	29-1	1998 (July)	long, high SCC	2	E	000110011010011	3283
	30	31-3	1998 (May)	long, high SCC	2	E	000110011010011	3283
	31	31-3	1998 (July)	long, high SCC	2	E	000110011010011	3283
	32	86-2	1998 (May)	long, high SCC	2	E	000110011010011	3283
	33	86-2	1998 (July)	long, high SCC	2	E	000110011010011	3283
	34	n.a.	1998 (May)	bulk milk sample	-	E	000110011010011	3283
	35	n.a.	1998 (July)	bulk milk sample	-	E	000110011010011	3283
IV	36	Ada 126	1999 (Feb)	unknown	-	E	000110011010011	3283
V	37	9363-3	1999 (Feb)	unknown	-	E.2	000111111110011	4083
VI	38	205-4	1999 (Feb)	unknown	-	E	000110011010011	3283
VII	39	68	1999 (Feb)	unknown	-	D	101010011010011	21715
VIII	40	Klara-4	1999 (Feb)	unknown	-	G	001011111111101	6141

a) udder quarter position: 1 = right front; 2 = left front; 3 = right rear; 4 = left rear

Continued overleaf

[b)] 1 = subclinical infection with SCC ≤ 152,000 cells/ml; 2 = chronic subclinical infection with SCC ≥ 210,000 cells/ml; 3 = mild clinical disease or short subclinical disease with high SCC; 4 = acute severe clinical disease.
[c)] Overall results after hybridization with 15 strain specific DNA probes (AW-1 trough AW-15)[42].
[d)] Binary type is the binary code transformed to a decimal number.
[e)] Subclinical disease with occasional mild clinical flare-ups (clots in milk).

PFGE

PFGE was carried out as described by Struelens *et al.*[35]. *Sma*I (Boerhinger, Mannheim, Germany) was used for digestion of genomic DNA. PFGE of DNA digests was performed with a CHEF Mapper (BioRad, Veenendaal, The Netherlands) through a 1% SeaKem agarose gel (FMC, SanverTECH, Heerhugowaard, The Netherlands) under the following conditions: initial switch time 5 s to final switch time 15 s, run time 10 hr, followed by initial switch time 15 s to final switch time 45 s for 14 hr; linear ramping; 6 V cm^{-1}; 120° angle (60°/-60°); 14°C; 0.5x TBE. A lambda DNA polymer (BioRad, Veenendaal, The Netherlands) was used as molecular size marker. Gels were stained with ethidium bromide for 1 hr, destained in water and photographed under UV light with a charged-coupled device (CCD) camera.

Macrorestriction patterns were analyzed both visually and by computer-aided methods. Visual interpretation of banding patterns was done following guidelines suggested by Bannerman *et al.*[5] and Tenover *et al.*[38, 39]. Isolates with identical restriction profiles were assigned the same type and identified with a capital letter. Isolates that differed from main types by one to three band shifts consistent with a limited number of genetic events were assigned subtypes, indicated with a numeral suffix. Isolates with more than three such differences were considered to be different types. Banding patterns were digitized with a Hewlett-Packard Scanjet IIcx/T scanner and stored as TIFF files. Patterns were analyzed using GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium) to calculate Dice coefficients of correlation and to generate a dendrogram by UPGMA clustering (Unweighted Pair Group Method using Arithmetic averages).

Binary typing

Macrorestriction fragments obtained through PFGE were Southern blotted onto Hybond N^+ membranes (Amersham, Buckinghamshire, United Kingdom). Cloned DNA fragments designed for binary typing of human *S. aureus* strains were used as probes[42]. Labeling, hybridization and detection of the probes were performed with enhanced chemiluminescence (ECL) direct labeling and detection systems, according to the manufacturer's protocols (Amersham Life Science, Buckinghamshire, United Kingdom). Hybridization of 15 DNA probes was scored with a 1 or a 0 according to the presence or

absence of a hybridization signal, resulting in a 15 digit binary code for each *S. aureus* isolate. Binary codes were transformed into decimal numbers to define binary types (BT) and a dendrogram was constructed using hierarchical cluster analysis (SPSS 8.0 for Windows, SPSS Inc.).

Table 2. Characterization of human *Staphylococcus aureus* collections from which binary types are used in this study.

Collection	Geographic origin	Isolate numbers[a]	Description of collection	Reference or source
1	United States	41-66	Community acquired MRSA strains from a New York City hospital (n=26)	41
2	United States (CDC)[b]	67-80	Selection of geographically diverse strains from multicenter collection of MRSA strains(n=5) and MSSA strains (n=9)	38, 42
3	The Netherlands	81-85	MSSA strains isolated from healthy persistent nasal carriers (n=5)	42
4	The Netherlands	86-95	MRSA strains (n=6) and MSSA strains (n=4) from outbreaks in Dutch hospitals and nursing homes	42

a) Isolate numbers as used in figure 4.
b) Center for Disease Control and Prevention.

Statistical analysis

Log-normalized SCC for clinical groups 1, 2 and 3 was compared by means of One-way ANOVA (SPSS 8.0 for Windows, SPSS Inc.)

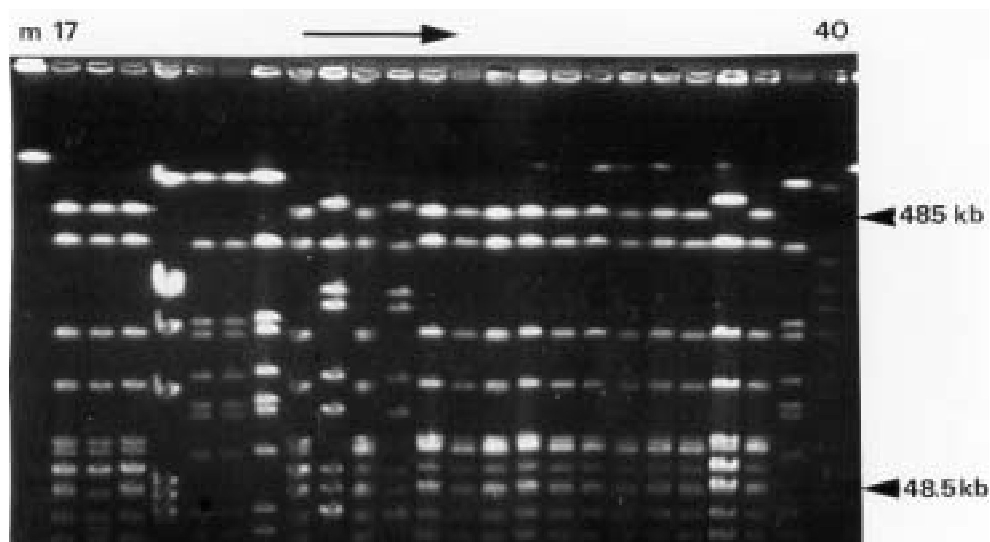
Fisher's exact test of the relationship between clinical groups of origin and strains was performed using analytical software (StatXact version 2.05, CYTEL Software Corporation, Cambridge, MA). Isolates 34-40 were excluded from this analysis because insufficient clinical data were available. Isolates 29, 31 and 33 were excluded because they represent the same infectious episodes as isolates 28, 30, and 32, respectively. For analysis of the association between clinical groups and PFGE types, types that occurred only once (A, F) were excluded from analysis and subtypes (B.1, B.2, and E.1) were grouped together with their respective main types. For analysis of the association between clinical groups and binary types, BT-clustering at 90% genetic similarity was used to define separate groups.

Results

PFGE

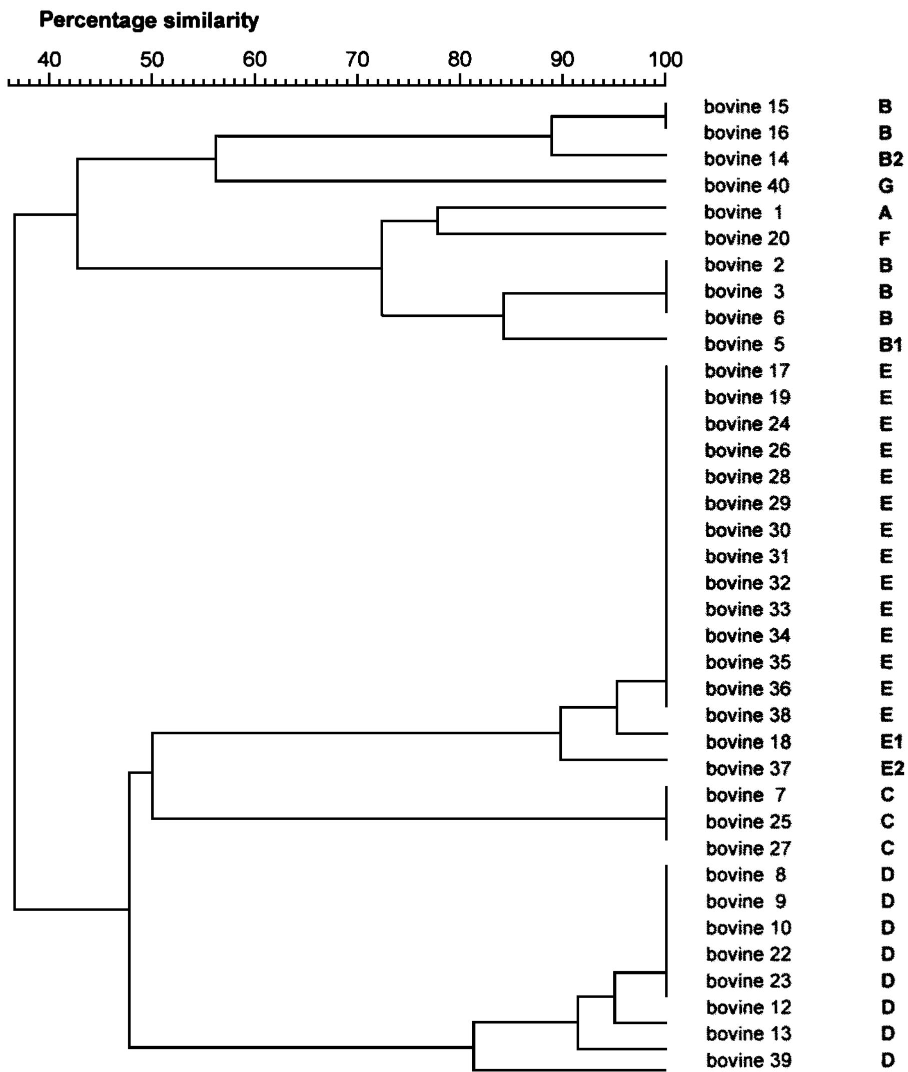
All bovine isolates were typable by PFGE. Among 38 isolates seven pulsed-field types and four subtypes were identified through visual interpretation of gels (Figure 1, Table 1). Three PFGE types (A, F and G) and all subtypes (B.1, B.2, E.1 and E.2) were identified only once, while PFGE types C, D and E were found in two, three and four herds, respectively.

Figure 1. Example of pulsed-field gel electrophoresis of *Sma*I macrorestriction fragments of bovine *Staphylococcus aureus* isolates, showing isolates 17 to 40. Molecular sizes are indicated on the right.



GelCompar analysis of PFGE results defined more clusters than visual interpretation. Depending on the level of genetic relatedness 13, 11 or 8 clusters were identified for 95%, 90%, and 80% similarity, respectively (Figure 2). The visually identified PFGE type B was divided in four (95%) or two (80%) separate clusters, while PFGE type D was divided into three (95%), two (90%), and one (80%) cluster(s). In the GelCompar analysis, visual PFGE types E and E.1 were grouped together at 95% similarity, and E, E.1 and E.2 at 90% genetic similarity.

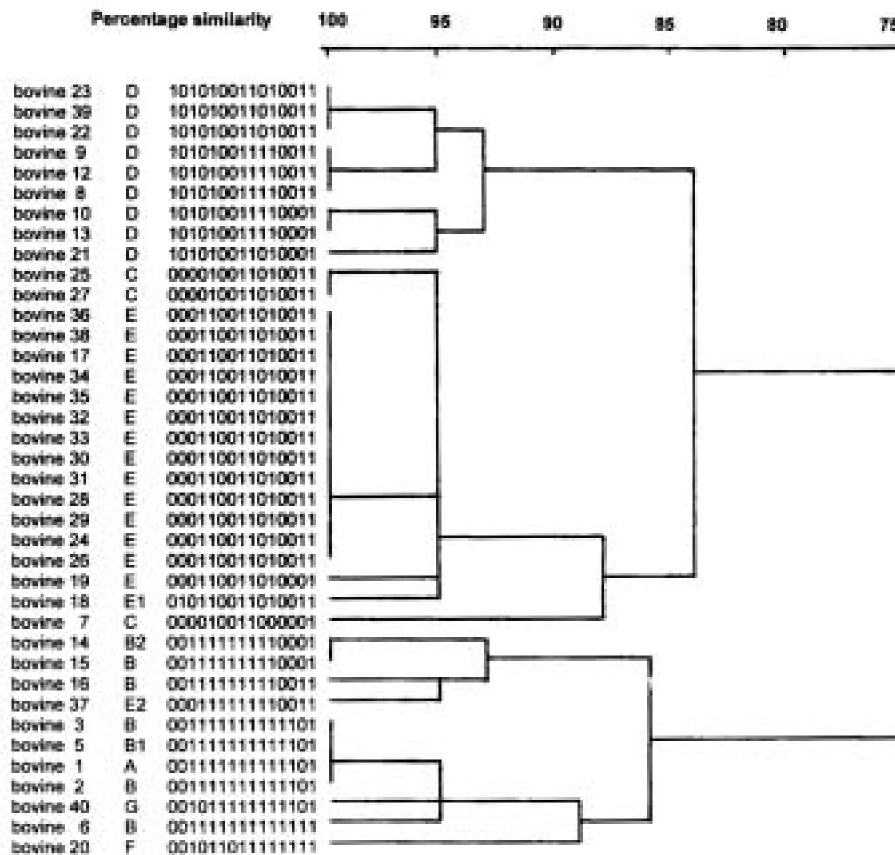
Figure 2. Dendrogram showing the level of similarity between *Sma*I macrorestriction patterns of 38 bovine *Staphylococcus aureus* isolates as determined by PFGE and subsequent GelCompar analysis of digitized photographs. Scale indicates level of genetic relatedness within this set of strains. Capital letters indicate PFGE types as based on visual interpretation of PFGE results.



Binary typing

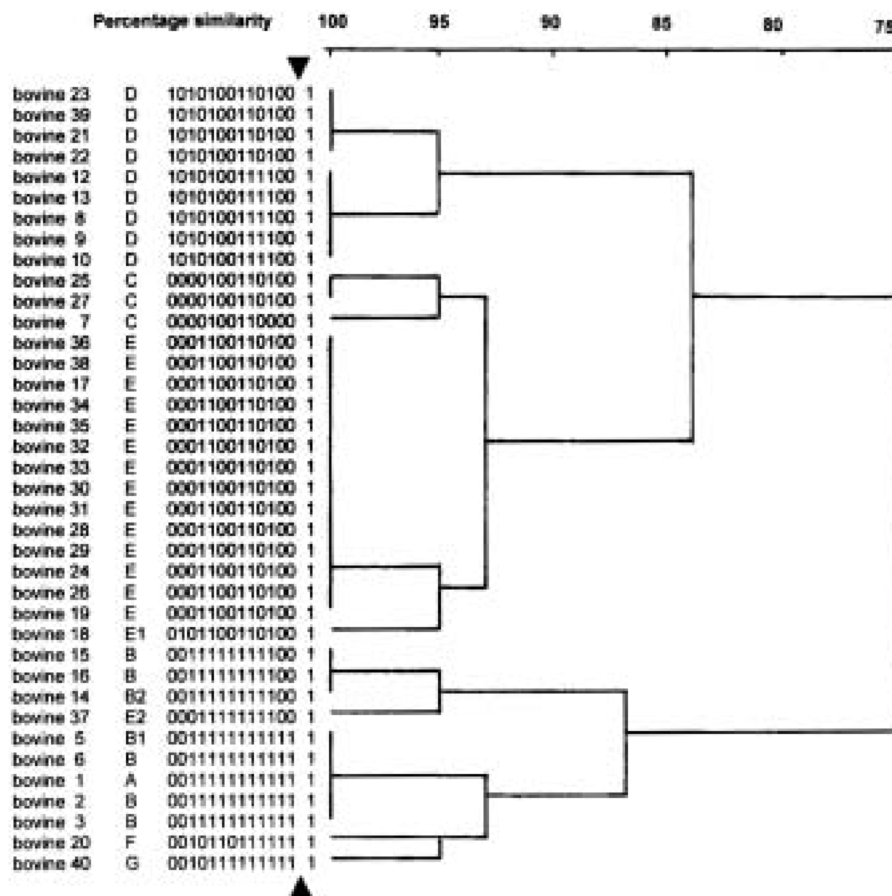
All bovine isolates were typable using the binary method (Table 1). Out of 15 probes designed for typing of human *S. aureus* strains, four hybridized to all bovine isolates (AW-5, AW-8, AW-9 and AW-15), while all other probes hybridized to at least one bovine isolate. Genetic relatedness of isolates based on binary typing was depicted in a dendrogram (Figure 3.a).

Figure 3a. Dendrogram showing the grouping of 38 bovine *Staphylococcus aureus* strains on the basis of hybridization scores after binary typing with probes AW-1 to AW-15. Isolate number, visual PFGE type and binary code are given for all isolates. Scale indicates level of genetic relatedness within this set of strains.



For 95%, 90%, and 85% genetic similarity, respectively, eight, six and three clusters of strains were identified. Binding of probe AW-14 showed a low level of reproducibility among epidemiologically related isolates. Therefore, a separate dendrogram was constructed excluding AW-14 (Figure 3.b), reducing the number of clusters to six at 95% similarity.

Figure 3b. Dendrogram showing the grouping of 38 bovine *S. aureus* strains after omission of probe AW-14 that is associated with hypervariable regions on the bovine staphylococcal genome. Isolate number, visual PFGE type and binary code are given for all isolates. Scale indicates level of genetic relatedness within this set of strains.



Concordance between PFGE and binary typing

PFGE types assigned were compared with binary types. General agreement was found between the techniques, but with some discrepancies. Several visually identified PFGE types were grouped together by binary typing (e.g. A, three B isolates, B.1 and G at 95% binary similarity; E, E.1 and two out of three C isolates at 95% similarity; B, B.2 and E.2 at 90% similarity). Other PFGE types were divided into multiple binary clusters that differed by two or three probes (e.g. B into two binary clusters at 90% similarity) (Figure 3.a). Concordance of delineation of genotypically related clusters as determined by PFGE and binary typing improved when probe AW-14 was excluded (Figure 3.b).

Within-herd and between-herd heterogeneity

Genetic heterogeneity among *S. aureus* isolates recovered from bovine mammary secretions was observed within and between herds. Isolates from herd I (n=16) were divided into four PFGE types (A-D) and subclonal variation was observed in PFGE type B (subtypes B.1 and B.2). In herd II (n=8) three PFGE types (D-F) were identified, with subclonal variation in PFGE type E (subtype E.1). In herd III (n=11) two PFGE types (C, E) occurred. In isolates obtained from five herds that were not related to each other or herd I, II and III, three PFGE types and one subtype were identified (D, E, E.2, G), demonstrating that both heterogeneity and homogeneity between herds exists. Heterogeneity based on binary typing parallels heterogeneity of PFGE types for all herds.

Comparison of bovine and human strains

Binary types of bovine isolates from this study were compared to binary types from human *S. aureus* isolates that had been typed before with the same method[41, 42]. Most isolates clustered as host specific clones and full identity of the 15 digit binary code of bovine and human isolates was never observed (Figure 4). At 95% similarity, human isolate 61 clustered together with bovine isolate 6 (one-digit difference at probe AW-11), and human isolate 62 clustered with bovine isolates 1-5 (one-digit difference at probe AW-1) and bovine isolate 40 (two-digit difference). At 90% similarity, these bovine and human isolates formed one cluster that also included human isolate 53. Human isolates within this cluster differed from bovine isolates in the same cluster by three digits at most, with differences associated with six out of 15 DNA probes used. Human isolate 82 clustered together with all bovine type D isolates at 90% similarity, as did human isolate 50 with bovine isolate 20. Human isolates 50, 53, 61 and 62 were community acquired MRSA strains from a New York City hospital (Table 2). Human isolate 82 was an MSSA strain isolated from a persistent nasal carrier in The Netherlands. Bovine isolates that clustered with human isolates originated from four Dutch dairy herds that were epidemiologically unrelated to each other or the human sources of *S. aureus* included in the comparison.

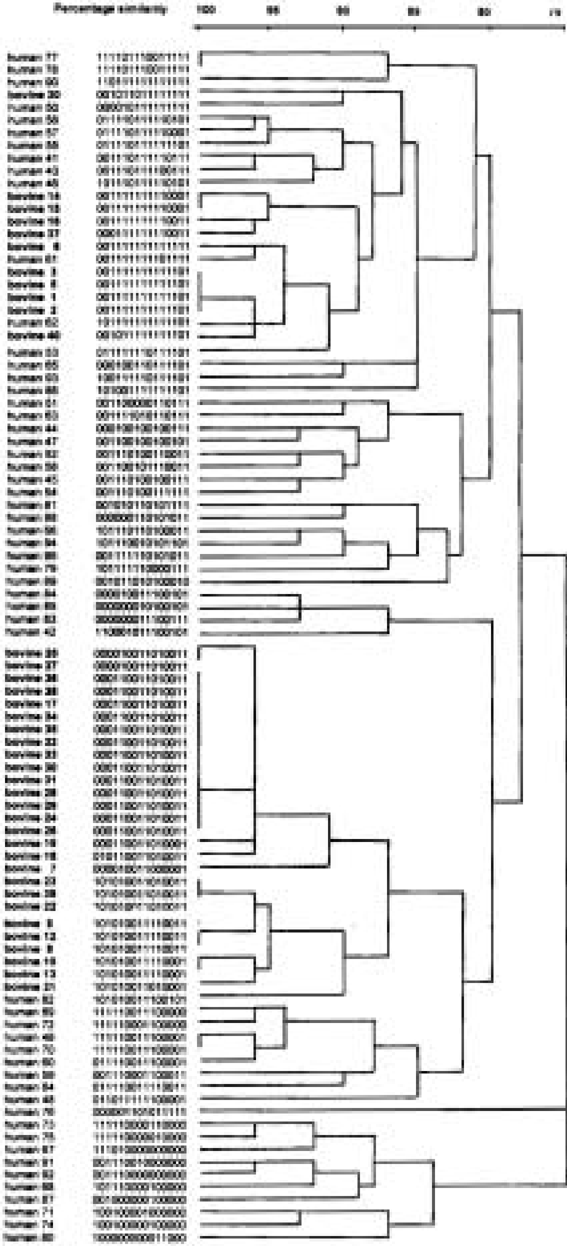


Figure 4. Dendrogram showing the grouping of 55 unrelated human *Staphylococcus aureus* strains described previously[42] and 38 bovine *S. aureus* strains on the basis of hybridization scores after binary typing with 15 DNA probes. Isolate numbers and binary codes are shown for all isolates. Scale indicates level of genetic relatedness within this collection of strains

Association with clinical characteristics

Mean SCC of quarter milk samples differed with clinical group for groups 1, 2 and 3 (F-value=45.63, p<0.001, df=2). Subclinical infection with low SCC (clinical group 1) was associated with PFGE type C (Table 1; three C isolates in three group 1 samples). Binary typing discriminated between type C isolated from herd I (BT 1217) and herd III (BT 1235), in agreement with geographical clustering. Chronic subclinical infection with high SCC (clinical group 2) was associated with PFGE types A and B in herd I. PFGE types A and B were not isolated from any samples belonging to clinical groups 1 or 4 and only once from group 3. In herds II and III, clinical group 2 was associated with PFGE type E. Type E was also isolated from a group 3 sample, while one group 2 sample yielded PFGE type F. Clinical group 3 yielded several strains, categorized as B (herd I), D (herd I and II) or E (herd II and III). Acute severe clinical mastitis (clinical group 4) was associated with PFGE type D.

Associations between clinical groups and visually identified PFGE types were statistically significant (Fisher statistic=26.00, p=0.002, df=9). Associations between clinical groups and binary clusters were of borderline statistical significance when all probes were included in the analysis (Fisher statistic=24.70, p=0.05, df=15). Associations were significant after exclusion of probe AW-14 (Fisher statistic=19.10, p-value=0.02, df=15).

Discussion

PFGE and binary typing

Variation in gene content of staphylococcal chromosomes may be associated with presence of non-essential but clinically or epidemiologically relevant genes (e.g. virulence genes, resistance genes)[23, 28]. PFGE is a well-known and powerful method for detection of genetic variation in *S. aureus* populations[5, 35]. Binary typing is a recently developed high-resolution molecular typing system that produces simple binary output and has the potential to become a technically simple and fast library typing system for *S. aureus* strains[42]. To our knowledge, PFGE and binary typing had not been applied to *S. aureus* isolates of bovine origin. In this study, PFGE-profiles and binary codes for 38 isolates derived from bovine mammary secretions were determined and compared. After PFGE of *Sma*I macrorestriction fragments, seven main PFGE types and four subtypes were identified visually. Computer-aided cluster analysis identified more distinct types, depending on the level of genetic similarity chosen as cut-off value. What level of discrimination between clusters of strains is desired depends on the purpose of genotyping and results of PFGE must be analyzed in light of the epidemiological background[5, 35]. One visually identified PFGE type, type B, was subdivided over multiple clusters after UPGMA analysis, even at low genetic similarity levels (Figure 2). Isolates were from similar clinical and geographical

background, but subdivision may be related to different temporal origins of the samples (Table 1). Discrepancies between visual and computer aided interpretation are a drawback of pulsed-field typing, and limit its usefulness as a routine diagnostic technique for large numbers of samples.

In this study binary typing was preceded by PFGE typing, but binary typing can be performed as a single typing technique [42]. Binary typing yielded 16 binary codes, clustered in three to eight clones, depending on levels of genomic similarity. The relevant level of discrimination and suitability of individual probes are subject of further study. However, interpretation of probe binding results is unequivocal. Probes AW-12 and AW-13 gave identical results, while probes AW-5, 8, 9 and 15 hybridized to all bovine strains and did not contribute to the discriminatory power of the typing system. A larger collection of bovine isolates should be studied to determine the informative value of these probes for differentiation of bovine *S. aureus* strains.

Concordance between PFGE and binary typing

Several PFGE types were subdivided by binary typing. Binary codes within a PFGE type often differ by no more than one digit and in many cases this is the digit associated with probe AW-14 (Table 1, Figure 3.a). The observed discrimination within PFGE types may therefore be related to the detection of hypervariable domains on the genome of bovine *S. aureus* strains with probe AW-14. Similar "hypervariability" or inconsistent presence of probe-binding sequences has been described for epidemiologically and genetically related human *S. aureus* strains[40]. The results could imply that probe AW-14, that is stable for typing of human *S. aureus* strains, is not stable for typing of *S. aureus* of bovine origin. On the other hand, probe AW-14 could be used to study short-term genome evolution in bacterial populations from bovine origin[41].

When ignoring binary code differences caused by probe AW-14, closer agreement between binary typing and pulsed-field typing is obtained, but some one-digit differences within PFGE types remain (Figure 3.b). PFGE types C isolated from herds I and III differ by one digit, associated with probe AW-11. This genotypic difference can be related to different geographical origins, but not to a difference in clinical course of infection. For PFGE type D, differences exist in geographical origin and in clinical course. Whether severity of disease is a herd effect (herd I vs. herd II), a strain effect (BT 21745/21747 vs. BT 21713/21715), a cow effect (mild cases in older animals, severe cases in heifers) or a chance effect is unknown.

Some binary clones are subdivided by PFGE (e.g. B and B.2 within BT 8177 and A, B and B.1 within BT 8189). Since isolates within these binary types where from similar geographical, temporal and clinical origin, binary typing seems the epidemiologically superior technique in these cases.

Within-herd and between-herd heterogeneity

PFGE and binary typing differentiated strains within and between herds. Similar results were obtained by means of PCR-based DNA-fingerprinting in the USA[24] and The Netherlands[21], through multilocus enzyme electrophoresis of a worldwide collection of strains[17], by coagulase gene typing of European, American and Asian isolates[2, 36], with a combination of techniques for bovine isolates from the USA and Ireland[12], and by PFGE of German isolates[3a]. In all studies, including the present one, a limited number of predominant types was found both within herds, in agreement with the contagious nature of *S. aureus* mastitis[21], and between herds, suggesting that certain variants present in the environment may have predilection for causing intramammary infections[2, 12, 36].

Subclonal heterogeneity within herds may be due to temporal evolution. Herds were selected for inclusion in the longitudinal survey, based on a history of presence of the pathogen in the herd for more than one year. The study period covered an additional 18 months, allowing for further genetic diversification[41]. Similar subclonal genetic variation over time has been described for DNA macrorestriction patterns from human *S. aureus* isolates[27].

Comparison of bovine and human strains

Out of 55 human isolates and 38 bovine isolates, five human and 16 bovine isolates belonged to clusters sharing 90% to 95% similarity as determined by binary typing. At higher similarity levels, all clones were host species specific. Similar results were obtained by Kapur *et al.*[17] and by Lopes *et al.*[22]. The results are consistent with the concept of host specificity among *S. aureus* clones and imply that successful transfer of bacteria between humans and cattle is not a frequent event[17]. However, several studies are available that suggest that transfer of bacteria between humans and cows is possible[13, 30, 37]. Those studies mostly focus on the role of humans as source of infection for dairy cattle. Another reason to be concerned about interspecies transfer of *S. aureus* is the routine use of antibiotics in dairy herd management[15, 33, 34]. In farms with *S. aureus* mastitis problems oxacilline is used as dry cow treatment for all animals[8]. Resistance to the closely related antibiotic methicillin is rare in bovine *S. aureus*[22] but has been reported from New York State[29], Europe[9] and Japan (cited in [18]). Widespread use of oxacillin could promote selection of resistant clones[7]. If interspecies transfer occurs, methicillin resistance in bovine strains may contribute to increasing prevalence of MRSA in humans. Since binary typing is a library system that can be applied to *S. aureus* isolates originating from humans and cattle, it is a useful tool in monitoring origins of MRSA strains and interspecies transfer of *S. aureus*. Addition of probes to test for presence of mecA gene in the bovine typing system would furthermore allow monitoring of MRSA prevalence in veterinary diagnostic laboratories.

Association with clinical characteristics

A limited number of isolates were included in statistical analyses and interdependence of within-herd observations was not taken into account. Thus, results of the analyses must be interpreted with care. However, in this study there was a significant correlation between *S. aureus* strains and disease characteristics observed *in vivo*. Such information is rarely available because most studies focus on clinical or subclinical mastitis only[21, 21a, 37], or don't contain information on clinical background of samples[2, 12, 36]. Matthews *et al.*[24] observed heterogeneity between subclinical and clinical isolates based on arbitrarily primed PCR, but heterogeneity within the group of subclinical isolates and overlap between genotypes isolated from both groups precluded firm associations. Kenny *et al.*[19] reported enterotoxin production by bovine mammary isolates of *S. aureus* and suggest that enterotoxin production may be associated with clinical course of disease. Matsunaga *et al.*[23] attempted to relate toxin production and other virulence factors to severity of clinical disease. They concluded that the properties of *S. aureus* isolated from peracute cases were different from those of acute and chronic isolates. No obvious differences between acute and chronic isolates were observed. The first conclusion is in agreement with our finding that all group 4 cases (peracute cases) are attributable to a specific PFGE type and binary type. In addition, our results suggest a difference between acute (clinical) and chronic (subclinical) cases, as shown by the associations between PFGE type C and clinical group 1, PFGE types B and E and group 2, and PFGE type D and group 3, respectively.

PFGE types C and E differed in binding of probe AW-4 only, but were associated with clearly distinguishable leucocyte response *in vivo* (low vs. high SCC). Differences in leucocyte response *in vitro* have been described by Aarestrup *et al.*[1] for different coagulase types isolated from cases of subclinical mastitis. Probe AW-4 has been shown to be homologous to a mobile genetic element, IS*257*[42]. IS*257*, also known as IS*431*, is a common insertion sequence in the staphylococcal chromosome and plasmids and can be associated with several resistance determinants, including methicillin resistance[7].

It must be emphasized that associations between clinical outcome of disease, PFGE types, binary types and specific probes in the binary typing system are as yet speculative and more typing needs to be done. If associations are confirmed, binary typing can be used for identification of unusual and more virulent strains, allowing for further pathogenetic studies and for tailored advice to farmers on management of specific cases.

An aspect of the association between genotype and epidemiological background that merits attention is the relation between PFGE type D and its origin. PFGE type D was isolated from all group 4 samples, all of which were obtained from heifers before first calving. Occurrence of *S. aureus* mastitis in preparturient heifers is a widely reported phenomenon[14, 26]. Based on biotyping, antibiograms and phage typing, Roberson *et al.*[31] concluded that milk from the dairy herd and heifer body sites are the most likely sources of infections. In their study, the environment was a possible source of infection in 17 out of 61 cases but never the sole possible source. In contrast, our results show that the predominant *S. aureus* genotype in the milking herd (PFGE type B, BT 8177/8189) is different from the

genotype found in heifer mastitis isolates (PFGE type D, BT 21745/7). This implies that the dairy herd is not the most likely source of heifer infections. In herd II, all type D cases occurred at a time that no other infected animals were present in the milking herd, as determined by three-weekly routine samplings (data not shown). Though not conclusive, this observation also suggests the environment as a more likely source of infection than the dairy herd. Determination of reservoirs, including environmental sources, is considered an important step when attempting to control *S. aureus* in a dairy herd[30, 33]. The genotyping techniques presented in this paper can be helpful in elucidating the relative importance of environmental sources in the farm level ecology of *S. aureus*.

Conclusion and future developments

This study shows that both PFGE and binary typing can be successfully applied to characterize *S. aureus* isolates of bovine mammary origin. Binary output was easier to interpret than banding patterns generated by PFGE and binary typing seemed superior to PFGE in clustering isolates from similar epidemiological background. As a library typing system, binary typing facilitates comparison of *S. aureus* isolates from bovine and human origin from world wide collections, analysis of clonal relatedness and host specificity, and monitoring of interspecies transfer. In this study, genetically related clusters of strains from human, bovine and mixed origin occurred. For isolates obtained from bovine mammary secretions, associations between bacterial strains and clinical characteristics of infection *in vivo* were observed, as was a tentative association between strains and sources of infection. Those observations need further validation through the study of larger strain collections or infection experiments. We conclude that binary typing in particular is a technique that is suitable for use in veterinary clinical microbiology and may contribute to development of case specific and farm specific recommendations for management of *S. aureus* problems in bovine medicine.

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- Chapter 3 -

Comparison of *Staphylococcus aureus* from skin, milking equipment and bovine milk by phage typing, PFGE and binary typing

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Abstract

Staphylococcus aureus isolates (n = 225) from healthy bovine teat skin, human skin, milking equipment, and bovine milk were fingerprinted by pulsed-field gel electrophoresis (PFGE). Strains were compared to assess the role of skin and milking equipment as source and fomite for bovine mastitis. PFGE of SmaI digested genomic DNA identified 24 main types and 17 subtypes among isolates from 42 herds, and discriminated between isolates from bovine teat skin and isolates from milk. Human skin isolates belonged to the same PFGE types as bovine skin isolates. Milking equipment harbored strains from teat skin and from milk. Phage typing (Fox, L. K., M. Gershmann, D. D. Hancock, and C. T. Hutton. 1991. Cornell Vet. 81:183-193) was less discriminatory than PFGE and failed to differentiate between isolates from skin and milk. A subset of 142 isolates was identified by binary typing with probes for human *S. aureus*. Typeability, discriminatory power and concordance with site of isolation were lower than for PFGE. Within several PFGE types, binary typing discriminated between main types and subtypes, isolates from different herds, or isolates from different sources. Binary typing is not suitable as a replacement for PFGE but may be used in combination with PFGE to refine strain differentiation. Based on PFGE, it is concluded that the milking machine is a fomite for the spread of *S. aureus* strains from bovine teat skin and for strains from milk. Bovine teat skin and skin of milkers' hands are not important sources of bovine intramammary *S. aureus* infections.

Abbreviation key: MLST = multilocus sequence typing, **PFGE**= pulsed-field gel electrophoresis.

Introduction

Staphylococcus aureus is an important cause of mastitis in dairy cows. Infected cows' udders are the main reservoir from which *S. aureus* is transmitted to other cows in the herd[9]. Prevention of pathogen transmission from cow to cow reduces mastitis incidence[21]. However, when mastitis control measures are implemented, new infections continue to occur and eradication of *S. aureus* intramammary infection is difficult to achieve. Infections that originate from sources outside of the mammary gland may contribute to the infection control problem[25]. Many sources of *S. aureus* exist, including housing materials and fodder, equipment and air, bovine skin, non-bovine animals, and humans[23]. Quantitative evidence suggests that teat skin is an important reservoir for intramammary infection[23]. Human-to-bovine transmission has also been proposed[5, 33]

Because many strains of *S. aureus* exist, isolates must be typed at the subspecies level to pinpoint the sources and routes of spread of the pathogen in a population[32]. For decades, bacteriophage typing was the standard method for typing of *S. aureus*. Phage typing is still widely used today, despite a number of drawbacks[30, 36, 42]. Drawbacks include limited typeability of isolates, limited technical reproducibility of results, and variable expression of epidemiological determinants, resulting in limited biological reproducibility. Pulsed-field gel electrophoresis (PFGE) is a DNA-based typing technique that has higher typeability, within-laboratory reproducibility and discriminatory power than phage typing[1, 30]. In the past decade, PFGE has replaced bacteriophage typing as the gold standard for typing of *S. aureus*[1, 3].

Although well suited for outbreak analysis, PFGE lacks between-center reproducibility because results depend on experimental conditions, and because interpretation of banding patterns is open to subjective differences[3, 4, 38]. To overcome such differences, library typing systems are developed in which any typing result has universal meaning[31]. Examples of library typing systems for *S. aureus* are binary typing[41] and multilocus sequence typing (MLST)[6]. Binary typing does not require DNA sequencing equipment and is more likely to be available to peripheral microbiology laboratories than MLST[39]. Binary typing was developed for human *S. aureus*. In a pilot study, it appeared to be a promising tool for typing of bovine *S. aureus*[43] but large-scale experiments have not yet been performed. To validate the new typing technique, knowledge of the origin of typed isolates and comparison to known typing methods is necessary[32, 34].

The aim of the current study was to examine a collection of *S. aureus* isolates from bovine and human skin, milking machine unit liners and bovine milk to assess the role of skin and the milking machine as reservoirs and fomites of mastitis. The collection had previously been characterized by means of phage typing[10]. It is anticipated that typing by means of the current gold standard, PFGE, may reveal more detailed information and additional insight. Secondly, the usefulness of binary typing for large-scale studies of bovine *S. aureus* was evaluated, based on comparison with epidemiological data and results from PFGE.

Materials and Methods

Bacterial isolates

A collection of 225 isolates from bovine teat skin (n =70), milkers' hands (n = 4), milking machine unit liners (n = 34), and bovine milk (n = 117) was studied. Isolates originated from a cross-sectional survey in 42 dairy herds in Washington State, USA. Teat skin isolates and milk isolates were obtained from a random selection of cows in each herd. All milkers' hands were sampled before milking, and all milking equipment was sampled after milking of the herd[10].

Phage typing

Isolates had been typed using a set of 18 phages, as described by Fox et al.[10] Phage types were identified by a six-digit code, where every digit represents the combined results for three phages. Per triplet of phages, eight permutations of susceptibility and resistance exist, indicated by 0 (---), 1 (+++), 2 (++-), 3 (+-+), 4 (-++), 5 (+--), 6 (-+-) or 7 (--+). Thus, an isolate with phage type 006000 was only susceptible to the third triplet, which represents phages 7, 8 and 9, and within the triplet only phage 8 lysed the bacteria. Phage types that differed by one digit could differ in susceptibility to one, two or three phages.

PFGE

PFGE of *Sma*I digests was performed as described before[32]. DNA macrorestriction fragments were separated in a 1% SeaKem agarose gel (FMC, SanverTech, Heerhugowaard, The Netherlands) using a CHEF mapper (Bio-Rad, Veenendaal, The Netherlands)[43]. A four-band difference between macrorestriction patterns was interpreted as a different PFGE type, indicated by a capital letter. If isolates differed by up to three bands, they were classified as subtypes of a PFGE type, and indicated by a numeral suffix[11, 35].

Binary typing

Binary typing of a convenience sample of 142 isolates was performed with probes AW-1, 2, 3, 5, 6, 9, 11, 14 and 15 developed by Van Leeuwen et al.[41], using the method described for typing of bovine *S. aureus*[43]. Macrorestriction fragments obtained through PFGE were Southern blotted onto Hybond N^+ membranes (Amersham Life Science, Buckinghamshire, United Kingdom) and hybridized with each probe using ECL direct labeling and detection, according to the manufacturer's recommendations (Amersham). Presence or absence of a

hybridization signal was scored with one or zero, resulting in a nine-digit binary code for each isolate. Binary codes were converted into decimal numbers shown as binary types[41].

Statistical analysis

For isolates from bovine teat skin and milk (n=187 for full collection and n = 113 for binary typed collection), associations between site of isolation (teat skin vs. milk) and phage type, PFGE type or binary type (specified type vs. all other types) were examined. Statistical significance of associations was tested by Chi square analysis or 2-sided Fisher Exact test, as appropriate (Statistix for Windows, Version 1.0, Analytical Software Co., La Jolla, CA, USA). Human skin isolates were excluded from the analysis because the number of isolates was too limited to warrant the use of statistical testing. Liner isolates were excluded from the analysis because liners could be contaminated by *S. aureus* originating from teat skin or milk. Analyses were performed across herds. Numbers of isolates from individual herds were too small to permit significance testing within herds. Statistical significance was declared at $P < 0.05$.

Results

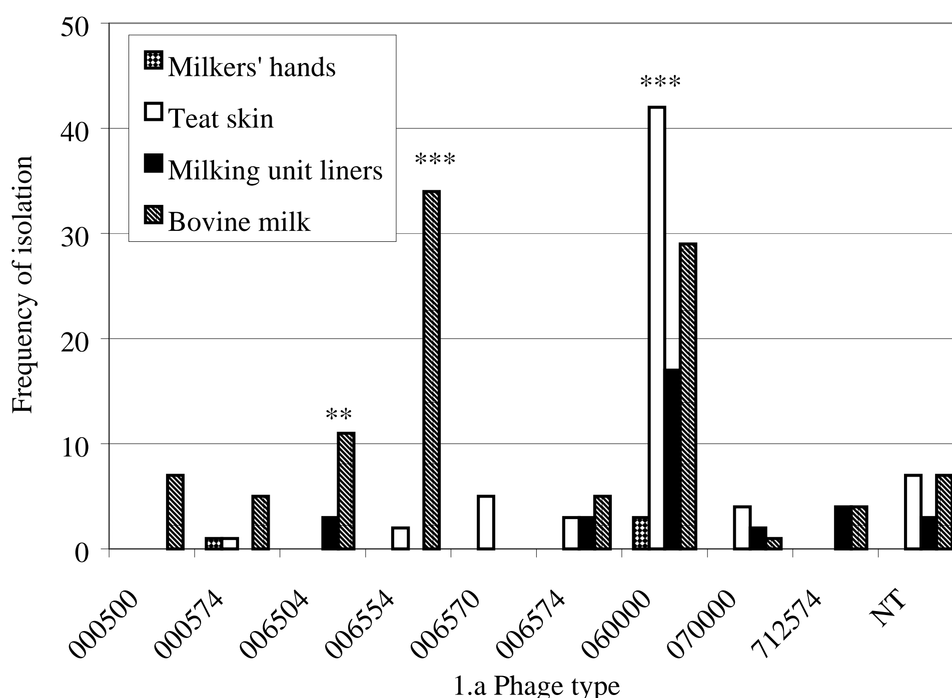
Of 225 PFGE-typed isolates, 208 were successfully typed by phage typing. Ten isolates were not lysed by any phage and seven yielded conflicting patterns upon repeated phage typing. Three phages (11, 12 and 16) did not lyse any isolates, and two phages (1 and 2) both lysed one (the same) isolate only. In total, 21 phage types were identified (Table 1).

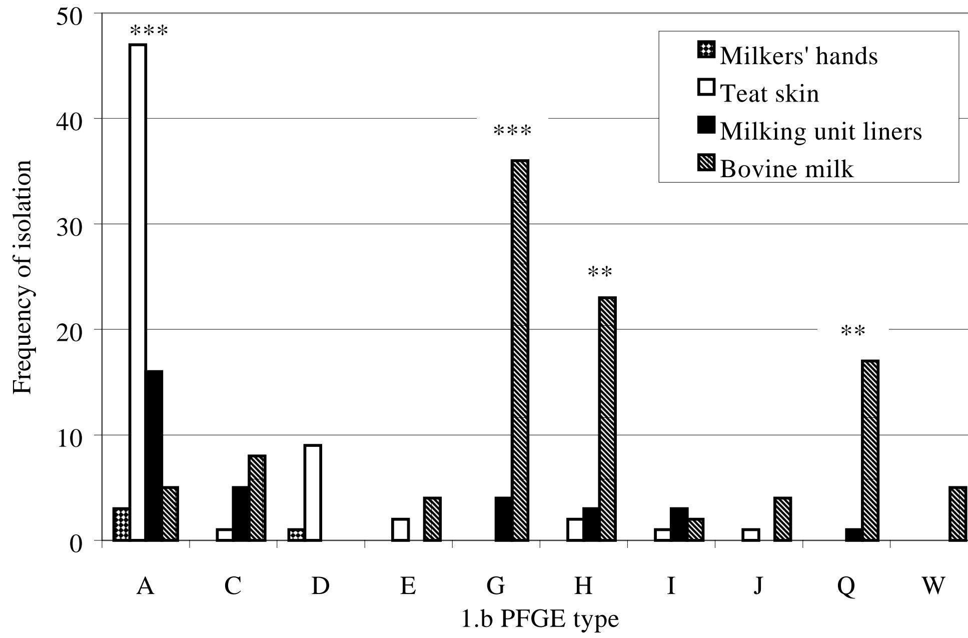
Table 1. Number of strains identified with phage typing, PFGE and binary typing among *Staphylococcus aureus* isolates from bovine teat skin, milkers' hands, milking machine liners, and bovine milk in different herds. Numbers in brackets apply to isolates that were binary typed.

Site of isolation	Isolates	Herds	Phage types	PFGE types main types + subtypes	Binary types
Teat skin	70 (57)	27 (20)	9 (6)	12 + 2 (8+2)	(13)
Milkers' hands	4 (3)	4 (3)	2 (1)	2 + 2 (1+2)	(3)
Liners	34 (26)	16 (13)	8 (6)	9 + 3 (7+2)	(9)
Milk	117 (62)	29 (24)	18 (13)	20 + 13 (18+8)	(15)
Total	225 (142)	42 (37)	21 (16)	24 + 17 (20+13)	(20)

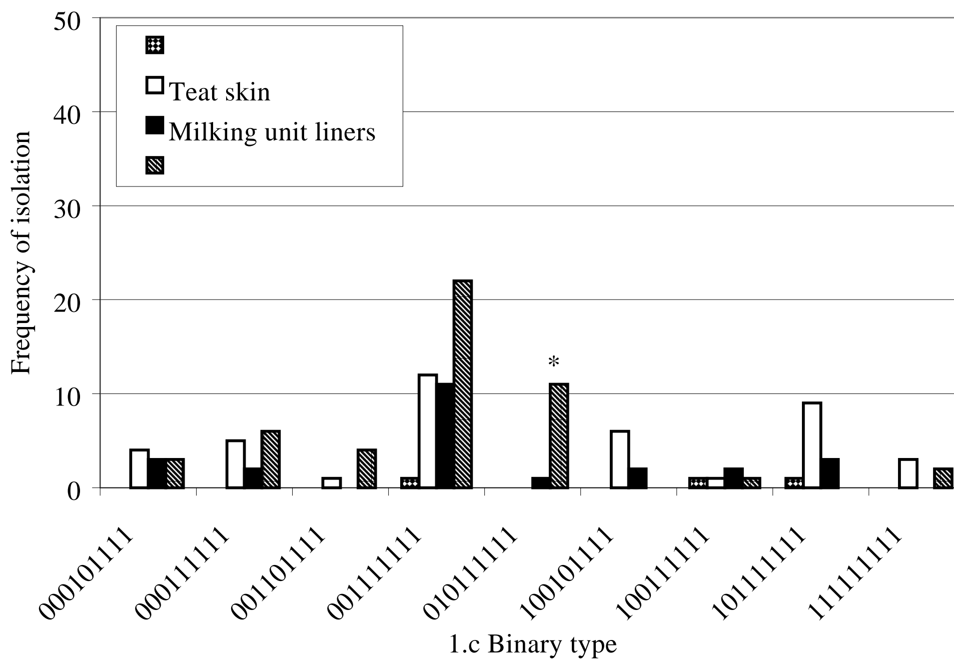
Twelve phage types were represented by four or fewer isolates. The remaining nine phage types were represented by five or more isolates. The distribution over teat skin, hands, liners and milk for the nine predominant phage types and for the group of isolates with undetermined phage type is shown in Figure 1.a. Among phage types represented by five or more isolates, type 0005000 was only isolated from milk, while types 000570 and 006570 were not isolated from milk. Phage type 060000 was significantly associated with isolation from teat skin (Chi-square = 23.1; 1 df; $P < 0.0001$), and phage types 006554 and 006504 were significantly associated with isolation from milk (Chi-square = 19.3; df = 1; $P < 0.0001$, and Chi-square = 6.99; df = 1; $P < 0.01$, respectively). Four phage types represented 68% of all isolates. Ten of 13 phage types (77%) that where represented by more than one isolate were found in more than one herd.

Figure 1. Distribution of phage types (1.a), PFGE types (1.b) and binary types (1.c) over sites of isolation. Only types that were represented by five or more isolates are shown. NT = not typeable. Asterixes indicate significant difference in frequency of isolation between milk and teat skin (* $P \leq 0.01$; ** $P \leq 0.001$; *** $P \leq 0.0001$). When nothing is indicated, no significance testing was performed. Binary types were converted to decimal numbers.





1.b PFGE type

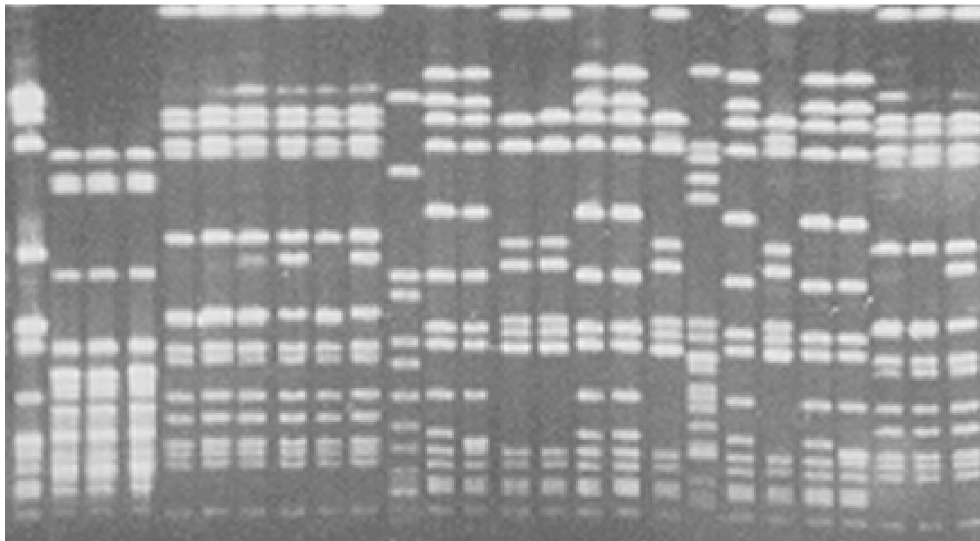


1.c Binary type

PFGE identified 24 main types and 17 subtypes (A to A5, G to G6, H to H2, I and I1, J and J1, Q and Q1, W and W1). Representative examples are shown in Figure 2. Fourteen PFGE types were represented by four or fewer isolates. Ten PFGE types were represented by five or more isolates. The distribution over teat skin, hands, liners and milk for the ten predominant PFGE types is shown in Figure 1.b.

Figure 2. Example of PFGE profiles of *Sma*I macrorestriction fragments of *Staphylococcus aureus* isolates from bovine teat skin, milking machine unit liners, and bovine milk. PFGE types are indicated with capital letter, and subtypes with numeral suffix.

V U U U A1 A1 A A A1 A T G G Q Q G G Q S G Q G G A1 A1 A



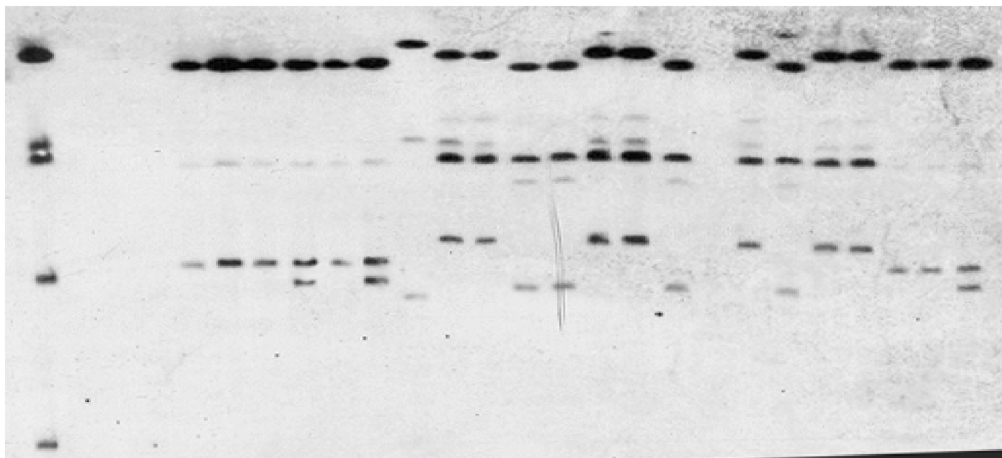
Among PFGE types represented by five or more isolates, type W was only isolated from milk, while type D was only isolated from skin. PFGE type A was significantly associated with isolation from teat skin (Chi-square = 86.2; df = 1; $P < 0.0001$), while types G, H, and Q were significantly associated with isolation from milk (Chi-square = 26.7, 10.7 and 11.2, respectively; df = 1; $P < 0.0001$, $P = 0.001$ and $P < 0.001$). When main types and subtypes were considered separately, associations for main type A, subtype A.1, main type G, main type H and main type Q were significant at $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, $P < 0.05$, and $P = 0.001$, respectively. Main type A and subtype A.1 were both associated with isolation from skin, and main types G, H, and Q with isolation from milk. No other subtypes were represented by ten or more isolates.

Within the subset of binary typed isolates, associations between site of isolation and types A, A.1, G, H and Q were significant at $P < 0.0001$, $P < 0.0001$, $P < 0.01$, $P < 0.01$, and $P < 0.001$, respectively. The five main PFGE types represented 76% of isolates. Nine of 13 PFGE types (69%) that were represented by multiple isolate were found in multiple herds. When considering all main types and subtypes separately, 20 were represented by only one isolate. For (sub)types represented by more than one isolate, 15 of 21 (71%) were found in multiple herds.

Of 142 isolates that were binary typed, 138 were typeable. Four isolates did not hybridize with any of the probes used. The proportion of isolates that hybridized with probe ranged from 16.2% for probe AW-2 to 96.5% for probe AW-15 with a median of 92.3%. Binary typing identified 20 binary codes among 142 isolates. The majority (n = 11) were represented by four or fewer isolates. The distribution over teat skin, hands, liners and milk for the nine binary types that were represented by five or more isolates is shown in Figure 1.c. Binary type 9811 (code 010111111) was significantly associated with isolation from milk (Chi-square = 10.0; df = 1; $P < 0.01$). This binary type was found in one herd only. No other binary types were significantly associated with milk or teat skin as site of isolation. For many isolates, hybridization of probes to multiple PFGE fragments was observed, indicating the presence of multiple probe complement copies (Figure 3).

Figure 3. Example of binary typing of *Staphylococcus aureus* isolates from bovine teat skin, milking machine unit liners and milk. Hybridization of probe AW-14 after PFGE and Southern blotting of DNA macrorestriction fragments is shown. Numbers and letters indicate strains as identified by PFGE typing (shown in Figure 2). Apart from strains U and S, all strains show hybridization of probe AW 14, and all positive isolates show binding to multiple probe complement copies.

V U U U A1 A1 A A A1 A T G G Q Q G G Q S G Q G G A1 A1 A



The nine types shown in Figure 1.c represented 82% of the binary typed isolates. Of 15 binary types that were represented by more than one isolate, 12 (80%) were found in multiple herds.

Within sites of isolation, phage typing and PFGE were largely concordant (Table 2). For bovine teat skin, liners, and bovine milk, phage typing identified phage type 060000 as the most or second most frequently isolated strain. PFGE divided phage type 060000 into PFGE types A, G, Q and W. PFGE type A and its subtype A.1 were the predominant strains among teat skin and liner isolates, but they were rarely isolated from milk. By contrast, PFGE types G, Q, and W were isolated from milk, rarely from liners, and never from teat skin. Thus, PFGE typing was more discriminatory than phage-typing and subdivided the phage type into PFGE types in accordance with site of *S. aureus* isolation.

Some PFGE types were subdivided into two types by phage typing (Table 2). PFGE type D was subdivided into phage types 000570 and 006570 that differed in susceptibility to one phage (phage 8). PFGE types C, G and H were subdivided into phage types 006504 and 006554, that also differed in susceptibility to one phage (phage 13). Considering that a one-phage difference in phage susceptibility is not necessarily indicative of genetically different strains[30], and that phage type 0065x4 (where x represents 0 or 5) was divided into three pulsed-field types, PFGE can still be considered more discriminatory than phage typing.

Binary typing showed partial agreement with PFGE (Table 3). Some binary types were divided into multiple types by PFGE. For example, binary type 5715 (code 001111111) encompassed PFGE types A, G, H and I. Conversely, several PFGE were divided into multiple binary types. Within PFGE type A, binary typing discriminated between herds of origin, and between main type A and subtype A.1 (Table 4). For eight of nine herds that harbored type A as well as A.1, binary types of A and A.1 isolates differed in binding of probe AW-1, while main type A and its accompanying subtype A.1 could differ between herds by more than one probe. Binary types also differed between main type W (binary type 1619, code 000111111, two isolates, two herds) and subtype W.1 (binary type 1107, code 000101111, one isolate). Within PFGE types C and Q, binary typing differentiated between herds of origin. Within PFGE type E, all originating from one herd, binary typing differentiated between isolates from milk and isolates from skin. Finally, for PFGE type J, differences in binary types were concordant with herd of origin, site of isolation and/or main type/subtype distinction (Table 5).

Table 2. PFGE types and phage types of *Staphylococcus aureus* isolates from bovine teat skin, milking machine unit liners, and bovine milk. MX= miscellaneous types. NT = not typeable or conflicting typing results. Boldface: predominant types.

Source and	PFGE type											Total
Phage type	A	C	D	E	G	H	I	J	Q	W	MX	
Teat skin												
000500	-	-	-	-	-	-	-	-	-	-	-	-
000570	1	-	3	-	-	-	-	-	-	-	-	4
000574	-	-	-	1	-	-	-	-	-	-	-	1
006504	-	-	-	-	-	-	-	-	-	-	-	-
006554	1	-	-	-	-	-	-	-	-	-	1	2
006570	-	-	5	-	-	-	-	-	-	-	-	5
006574	-	-	-	-	-	2	1	-	-	-	-	3
060000	**36**	1	1	-	-	-	-	-	-	-	4	**42**
070000	4	-	-	-	-	-	-	-	-	-	-	4
712574	-	-	-	-	-	-	-	-	-	-	-	-
MX	-	-	-	-	-	-	-	-	-	-	2	2
NT	5	-	-	1	-	-	-	1	-	-	-	7
Liner												
000500	-	-	-	-	-	-	-	-	-	-	-	-
000570	-	-	-	-	-	-	-	-	-	-	-	-
000574	-	-	-	-	-	-	-	-	-	-	-	-
006504	-	1	-	-	1	-	-	-	-	-	1	3
006554	-	-	-	-	-	-	-	-	-	-	-	-
006570	-	-	-	-	-	-	-	-	-	-	-	-
006574	-	-	-	-	-	2	1	-	-	-	-	3
060000	**13**	1	-	-	1	-	-	-	1	-	1	**17**
070000	2	-	-	-	-	-	-	-	-	-	-	2
712574	-	2	-	-	-	-	2	-	-	-	-	2
MX	1	-	-	-	-	1	-	-	-	-	-	2
NT	-	1	-	-	2	-	-	-	-	-	-	5
Milk												
000500	-	-	-	-	1	1	-	4	-	-	1	7
000570	-	-	-	-	-	-	-	-	-	-	-	-
000574	-	-	-	3	-	-	2	-	-	-	-	5
006504	-	3	-	-	**6**	**2**	-	-	-	-	-	**11**
006554	-	2	-	-	**20**	**10**	-	-	-	-	2	**34**
006570	-	-	-	-	-	-	-	-	-	-	-	-
006574	-	-	-	-	-	4	-	-	-	-	1	5
060000	4	-	-	-	3	-	-	-	**13**	4	5	**29**
070000	-	-	-	-	-	-	-	-	-	-	1	1
712574	-	-	-	-	-	4	-	-	-	-	-	4
MX	1	3	-	1	4	2	-	-	-	1	2	14
NT	-	-	-	-	2	-	-	-	4	-	1	7

Table 3. PFGE types and binary codes of *Staphylococcus aureus* isolates from bovine teat skin, milking machine unit liners, and bovine milk. MX= miscellaneous types. Boldface: predominant types. Binary code indicates binding of probes AW-1, 2, 3, 5, 6, 9, 11, 14 and 15.

Source and	PFGE type													Total
Binary code	A	C	E	F	G	H	I	J	Q	U	W	X	MX	
Teat skin														
000000000	-	-	-	-	-	-	-	-	-	2	-	-	-	2
000011111	3	-	-	-	-	-	-	-	-	-	-	-	-	3
000111111	5	-	-	-	-	-	-	-	-	-	-	-	-	5
001111111	**7**	-	2	1	-	-	1	1	-	-	-	-	-	**12**
011111111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
111111111	-	-	-	-	-	-	-	-	-	-	-	3	-	3
101111111	**9**	-	-	-	-	-	-	-	-	-	-	-	-	**9**
100111111	1	-	-	-	-	-	-	-	-	-	-	-	-	1
100101111	6	-	-	-	-	-	-	-	-	-	-	-	-	6
000101111	4	-	-	-	-	-	-	-	-	-	-	-	-	4
001101111	-	-	-	-	-	-	-	-	-	-	-	-	1	1
010111111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MX	5	-	-	-	-	-	-	-	-	-	-	-	-	5
Liner														
000000000	-	-	-	-	-	-	-	-	-	1	-	-	-	1
000011111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
000111111	-	2	-	-	-	-	-	-	-	-	-	-	-	2
001111111	**3**	-	-	-	**3**	**2**	**3**	-	-	-	-	-	-	**11**
011111111	-	1	-	-	-	-	-	-	-	-	-	-	-	1
111111111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101111111	3	-	-	-	-	-	-	-	-	-	-	-	-	3
100111111	2	-	-	-	-	-	-	-	-	-	-	-	-	2
100101111	2	-	-	-	-	-	-	-	-	-	-	-	-	2
000101111	3	-	-	-	-	-	-	-	-	-	-	-	-	3
001101111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
010111111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MX	-	-	-	-	-	-	-	-	1	-	-	-	-	1
Milk														
000000000	-	-	-	-	-	-	-	-	-	-	-	-	1	1
000011111	-	-	-	-	-	-	-	-	1	-	-	-	-	1
000111111	-	-	-	1	-	-	-	3	-	-	2	-	-	6
001111111	-	1	-	-	**7**	**11**	**2**	-	-	-	-	-	1	22
011111111	-	2	-	-	1	-	-	-	-	-	-	-	-	3
111111111	-	-	-	-	-	-	-	-	-	-	-	1	1	2
101111111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100111111	1	-	-	-	-	-	-	-	-	-	-	-	-	1
100101111	-	-	-	-	-	-	-	-	-	-	-	-	-	-
000101111	-	-	1	1	-	-	-	-	-	-	1	-	-	3
001101111	-	-	3	-	1	-	-	-	-	-	-	-	-	4
010111111	1	-	-	-	-	-	-	-	**10**	-	-	-	-	11
MX	-	-	-	-	2	-	-	-	2	-	-	-	4	8

Table 4. Division of PFGE types A and A.1 by herd of origin, site of isolation and binary type. All herds that harbored main type A as well as subtype A.1 are included. *Staphylococcus aureus* isolates belonging to other subtypes of A, and results from herds harboring only main type A or only subtype A.1 are not shown. Boldface: hybridization of probe AW-1 for type A/subtype A.1 combinations.

Herd	Site	PFGE type	Binary type and code	Number of isolates
7	teat skin	A	1619 = 000111111	1
	teat skin	A.1	1619 = 000111111	1
	teat skin	A.1	1091 = 010101011	1
8	liner	A	22099 = **1**01111111	2
	liner	A.1	5715 = **0**01111111	1
14	liner	A	22099 = **1**01111111	1
	teat skin	A	22099 = **1**01111111	4
	liner	A.1	57215 = **0**01111111	2
23	teat skin	A	17491 = **1**00101111	2
	teat skin	A	1107 = 000101111	1
	teat skin	A.1	1107 = **0**00101111	2
30	teat skin	A	22099 = **1**01111111	1
	teat skin	A.1	5715 = **0**01111111	1
	liner	A.1	1107 = 000101111	1
33	teat skin	A	22099 = **1**01111111	2
	teat skin	A.1	5715 = **0**01111111	1
34	teat skin	A	22099 = **1**01111111	1
	teat skin	A.1	5715 = **0**01111111	2
36	teat skin	A	17491 = **1**00101111	1
	teat skin	A.1	1107 = **0**00101111	1
37	liner	A	18003 = **1**00111111	1
	teat skin	A.1	1619 = **0**00111111	3

Discussion

The first purpose of this study was to compare *S. aureus* isolates from skin and milk. PFGE differentiates between *S. aureus* strains that are predominantly isolated from healthy bovine teat skin and *S. aureus* strains that are predominantly isolated from milk. The association between site of isolation and strain is highly significant for the most frequently isolated PFGE types and subtypes in this study. It is concluded that teat skin is not an important reservoir for intramammary infection. This is in contrast to conclusions from earlier studies, that were based on phage typing of *S. aureus*[10] or quantative analysis[23]. A discrepancy between conclusions based on PFGE typing and phage typing is easily explained, because the predominant phage type was divided into multiple distinct PFGE types in accordance with epidemiological data.

This is not surprising, as it is generally accepted that PFGE is more discriminatory for typing of *S. aureus* than phage typing[11, 26, 36, 42].

Table 5. Division of PFGE types C, E, F, J and Q by herd of origin, site of isolation and binary type.

PFGE type	Site	Herd	Binary type and code	Number of isolates
C	liner	7	1619 = 000111111	2
C		8	13907 = 011111111	1
C	milk	8	13907 = 011111111	2
C		15	5715 = 001111111	1
E	milk	13	1107 = 000101111	1
E		13	5203 = 001101111	3
E	teat skin	13	5715 = 001111111	2
F	teat skin	4	5715 = 001111111	1
F	milk	12	1107 = 000101111	1
F		24	1619 = 000111111	1
J	milk	1	1619 = 000111111	3
J.1	teat skin	13	5715 = 001111111	1
Q	milk	5	9811 = 010111111	10
Q		19	595 = 000011111	1
Q		20	9283 = 010101011	2
Q	liner	20	9283 = 010101011	1

The discrepancy between our results and those obtained by quantitative analysis may be due epidemiological differences between the populations under study, and to differences in the interpretation of data. Our study deals with lactating cows, while Roberson *et al.*[23] studied non-lactating young stock. Roberson and his coworkers found heifers with *S. aureus* "on teats" (i.e. on teat skin, on the teat orifice or in the teat canal) to be more likely to have *S. aureus* in milk samples at parturition than heifers without *S. aureus* on teats. The odds ratio was estimated at 3.34 and the statistical significance of this result was reported as $P = 0.07$. Because the number of intramammary infections that was observed in their study was limited, an exact test was more appropriate than the Chi-square analysis reported in the paper. Reanalysis of the data supplied in the original paper by means of logistic regression and an exact test (LogXact, version 1.2, Cytel Software Corporation, Cambridge, MA, USA) resulted in an estimated odds ratio of 3.33 with $P = 0.14$. Thus, the results are suggestive of a role of teat skin as reservoir for intramammary infection in heifers, but not conclusive. In a subsequent study on heifers, phenotypic methods were used to determine whether teat skin could be the primary source of infection for intramammary *S. aureus* at parturition. This was the case in one of eight heifers. Genotypic (non-)identity of isolates was not established[24].

Both with phenotypic and with genotypic methods, some overlap between isolates from skin and milk was observed in our study. This could indicate that skin may incidentally be a source of intramammary infection, that typing methods did not have sufficient discriminatory power to distinguish fully between skin and milk isolates, or that some samples from teat skin and milk were cross-contaminated. Care was taken to collect samples aseptically, but swabbing of the teat skin and teat orifice may result in contact of the swab with milk, especially if milk letdown occurs. Similarly, milk samples may get contaminated with bacteria from other sources. To avoid misclassification of contamination and infection, multiple milk samples are used to define infection status of the mammary gland in many studies[20]. In our study, single milk samples were used. Only four PFGE type A isolates were identified among *S. aureus* isolates from 1703 milk samples (0.2%). Even if type A isolates in milk originated from contamination, the level of contamination of milk samples was very low.

The discriminatory power of PFGE depends in part on the interpretation of typing results. Tenover and colleagues[35] formulated guidelines for interpretation of restriction patterns. Their categorization of isolates as “indistinguishable” (no band differences), “closely related” (1-3 band differences), “possibly related” (4-6 band differences) or “different” (>6 band differences) was developed to classify strains from disease outbreaks. It has been applied in many other settings, e.g. to study large collections of staphylococcal isolates from multiple hospitals[42], or isolates collected over long periods of time[11]. Often, the classification is simplified, and isolates that differ from a main type by 1-3 bands are classified as subtypes of the main type, while isolates with four or more band differences are considered different main types[11, 26]. In large-scale studies of dairy herds, more extreme classifications have been used. On the one hand, “splitters” considered any band difference indicative of a strain difference, arguing that isolates were selected from multiple herds and not from a limited outbreak[12, 14]. On the other hand, “lumpers” have classified isolates as subtypes when they differed from the main type by six bands[2, 29]. In our study, subtypes were usually identified in herds where the main type was also present (14 of 17 subtypes). Hence, subtypes and main types could be epidemiologically related, which would justify interpretation of a 1-3 band difference as indicative of a subtype. However, binary typing differentiated between main types and subtypes for several PFGE types. This would support interpretation of any band difference as indicative of a new type. The conclusion that skin isolates are different from milk isolates held true when PFGE subtypes were included with main types, and when subtypes were treated as separate types.

The majority of phage types, PFGE types and binary types were found in multiple herds. This is in contrast with results from Korea[12] and Denmark[16] where the majority of types was unique to a herd, but it is in agreement with many other studies. Based on phage typing[16, 18], ribotyping[16, 22], multilocus enzyme electrophoresis[7, 13], random amplified polymorphic DNA typing[7], PFGE[2, 29], coagulase gene typing[19] and binary typing[43] a number of strains and clones of *S. aureus* were found to be common to multiple herds, multiple regions within countries, or even to multiple countries and continents. The

predominance of a limited number of *S. aureus* strains may be the result of an increased resistance to the host immune response[19]. The differences between studies partly depend on data interpretation, as discussed, and partly on the choice of typing techniques. Different techniques target different characteristics of the bacteria, resulting in different outcomes[34]. Furthermore, molecular markers have a variety of molecular clock speeds[28, 37]. As a result, *S. aureus* isolates that appear identical when typed with one method may be discriminated when typed with a different technique. The choice of technique or combination of techniques should depend on spatial and temporal aspects of the epidemiological question at hand, and on availability of laboratory facilities and expertise[30, 36].

The number of human skin isolates in our study was limited. The isolates were obtained through swabbing of milkers' hands before milking of the herd. PFGE types were akin to bovine skin isolates but different from bovine milk isolates. Phage typing and binary typing did not discriminate between human skin isolates and isolates from other sources. Several studies have compared bovine and human isolates. Based on phage typing and ribotyping, a proportion of *S. aureus* strains were found to be similar between human skin and bovine milk or skin[10, 15, 33]. PFGE and binary typing mostly revealed differences between human *S. aureus* isolates and bovine mammary isolates[17, 43]. Results from an MLEE typing study have originally been interpreted as "consistent with the concept of host specialization"[13], and subsequently as "indicating that many bovine isolates are more closely allied to human isolates than to other bovine isolates"[8]. In most studies, the focus is on host specificity, i.e. on the comparison of human and bovine strains. Our data, though limited, suggest that there may be "organ specificity" and that this organ specificity may run across lines of host specificity. Adaptation of pathogenic clones to specialized niches, e.g. the mammary gland, has also been proposed based on DNA micro-array analysis of *S. aureus* isolates[8].

Machine milking unit liners are important fomites for transmission of *S. aureus* in dairy herds and the use of liner backflush reduces contamination with bacteria[10]. PFGE analysis revealed that liners could be contaminated with *S. aureus* from teat skin, and with *S. aureus* from milk. This implies that liners are fomites for skin flora and for intramammary infections. Although liners can be contaminated with skin and udder flora, transmission from skin flora to the mammary gland and vice versa seems rare, as indicated by the site-specific PFGE types that were observed in our study.

The second purpose of the current study was to determine whether binary typing was suited for large-scale molecular epidemiological studies of bovine *S. aureus*. Binary typing had lower typeability, discriminatory power and concordance with epidemiological data than the current gold standard, PFGE. The combination of PFGE, followed by Southern blotting, and binary typing has technical drawbacks. In this procedure, DNA concentrations can not be standardized. As a result, several isolates gave weak hybridization signals, making interpretation difficult and defeating one of the main purposes of a library typing technique, i.e. unequivocal interpretation of typing results.

During PFGE, DNA fragments smaller than 25 kb may have been lost, which could account for non-typeability of isolates. Although most probe binding occurred to large macrorestriction fragments, hybridization to smaller fragments was observed (Figure 3, strain V). Because bovine and human strains largely belong to different *S. aureus* lineages, non-typeability may also be the result of the failure of bovine strains to bind probes that were developed for typing of human *S. aureus.* To improve binary typing for bovine *S. aureus*, development of DNA probes for host-specific strains may be needed. Binary typing differentiated between isolates within PFGE types, often in agreement with herd of origin or site of isolation. The combined use of multiple methods is known to be more discriminatory than the use of PFGE alone[27, 40]. In the future, other techniques may supplement PFGE or replace it as gold standard for typing of *S. aureus*[6, 8, 27, 28, 39]. Even then, the choice of typing methods should be driven by the level of discrimination that is needed for a specified goal and the availability of resources.

Summarizing, PFGE showed that *S. aureus* isolates from bovine teat skin were different from isolates from bovine milk, and similar to a small number of isolates from human skin. Milking machine unit liners may act as fomites for transmission of strains from skin and milk. PFGE had better typeability, discriminatory power, and concordance with site of isolation than phage typing and binary typing. Binary typing is not suited yet as stand-alone technique for large-scale molecular studies of bovine *S. aureus* but it is a useful addition to PFGE for refinement of strain identification.

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- Chapter 4 -

A mathematical model of *Staphylococcus aureus* control in dairy herds

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Submitted

Abstract

An ordinary differential equation model was developed to simulate the dynamics of *Staphylococcus aureus* mastitis in dairy herds. Herds were described as populations of udder quarters that were never infected, subclinically infected, clinically infected, or recovered from infection. Data to estimate model parameters were obtained from an 18-month observational study in three commercial dairy herds with sampling of milk from all udder quarters upon entry into the lactating population, routinely at 3-week intervals, when clinical mastitis was observed, and upon exit from the lactating population. A deterministic simulation model was constructed to estimate the value of the basic (R_0) and effective (R_t) reproductive number in each herd, and to examine the effect of changes in herd management on mastitis control. In all herds, R_0 was below the threshold value 1, indicating that control of contagious transmission was achieved. R_t was higher than R_0 because recovered individuals were more susceptible to infection than individuals without prior infection history. Simulation showed that dynamics in two herds were well described by the model, and that treatment of subclinical mastitis and prevention of influx of infected individuals into the population contribute to fast decrease of *S. aureus* prevalence to low levels. For one herd, the model failed to mimic field observations. Explanations for the discrepancy are given in a discussion of current knowledge and assumptions underlying the model.

Abbreviation key: BMSCC = bulk milk somatic cell count, **C** = clinically infected, **DCT** = dry cow therapy, **IMI** = intramammary infection, **PMTD** = post-milking teat disinfection, **R** = recovered-uninfected, $\mathbf{R_0}$ = basic reproductive number, $\mathbf{R_t}$ = effective reproductive number, **S** = subclinically infected, **SCC** = somatic cell count, **U** = uninfected.

Introduction

Staphylococcus aureus is an important cause of udder infections in dairy herds[33, 45]. Infections with *S. aureus* can result in clinical or subclinical disease and are usually associated with increase in somatic cell count (SCC)[45]. *Staphylococcus aureus* is contagious and spreads easily within dairy herds[18, 23]. When multiple cows in a herd are infected, bulk milk SCC (BMSCC) increases and legal limits for BMSCC may be violated, or thresholds for premium bonus may not be met[1, 13, 38]. Hence, control of *S. aureus* mastitis is necessary and important. The feasibility of *S. aureus* control is a matter of debate. Some authors state that *S. aureus* mastitis can be controlled[21, 36] or even eradicated[11, 44]. Goodger and Ferguson[19] showed the feasibility and economic benefit of a control program in one herd. However, others contend that it is difficult to control *S. aureus* mastitis and impossible to eradicate the disease[16, 32].

Control programs include post-milking teat disinfection (PMTD), antibiotic treatment of all cows at dry-off (dry cow therapy, DCT), culling of chronically infected animals, and segregation of infected and non-infected animals[21, 30, 44, 46]. In addition, antibiotic treatment of cows with clinical mastitis is routine practice. For cows with subclinical infections, the usefulness of treatment is disputed. In a cost/benefit analysis of treatment for the individual cow, Craven[9] claimed that treatment of subclinical mastitis is economically unjustified. Other authors look beyond the individual cow and comment on the importance of treatment of subclinical cases to prevent spread of infection in the population[7, 27, 36]. Dodd et al.[15] even state that the only practical way of increasing the rate of elimination of infections is by using antibiotics more effectively, i.e. treating clinical as well as subclinical infections.

To be successful, a control program must reduce the number of new infections and the duration of existing infections[29]. Several control programs have proven their effectiveness in field trials, but it is impractical to test all possible control scenarios. The cost of examining different combinations of control measures would be prohibitive. However, the merits of different control programs can be examined with simulation models[15]. Models are simplified representations of real systems that are presented as a set of computational rules or assumptions. The assumptions characterize the system in terms of mathematical, logical and temporal relationships[24]. Models of mastitis control were reviewed by Allore and Erb[2].

Allore and Erb[2] developed a system of ordinary differential equations (ODE) that describes a herd of dairy cows as a population of uninfected, subclinically infected, clinically infected, and recovered animals. This system of ODEs can be used to calculate a value for R_0, the basic reproductive number, for mastitis. R_0 is defined as the expected number of secondary cases produced by a primary infectious case in a wholly susceptible population[5]. When R_0 is less than one, between-animal transmission cannot maintain a disease in a population. The main input components of R_0 are the rate of new infections and the duration of infections. Thus, R_0 is a summary indicator of the efficacy of mastitis

control schemes as proposed by Neave and coworkers[29]. Calculation of R_0 has been used to estimate the efficacy of PMTD during an outbreak of *S. aureus* mastitis in a dairy herd[23]. The model that Allore and Erb[2] developed could not be run, because no estimates were available for many of the model parameters.

The purpose of the current paper is to describe the dynamics of *S. aureus* infection in three endemically infected commercial dairy herds, elaborating on the ODE model of Allore and Erb[2]. Values for model parameters and R_0 are estimated from observational data, and assumptions underlying the model and the parameter estimation procedures are discussed. The model is used in a deterministic manner to evaluate whether R_0 can be reduced to a value below 1, as would be necessary to make a control program successful in the long term.

Materials and Methods

Data collection

Data were obtained from a longitudinal observational study (from May 1997 to December 1998) in three commercial dairy herds (A, B and C) in the Netherlands. The herds were known to be endemically infected with *S. aureus*, and were considered to be illustrative for the level of management in such herds in The Netherlands[49]. Herd A consisted of 67 ± 3 lactating animals that belonged predominantly to the Meuse-Rhine-Yssel and Red Holstein breeds with 305-day milk production of 7187 ± 149 kg. Herd B consisted of 95 ± 5 lactating animals that belonged predominantly to the Holstein Friesian and Dutch Friesian breeds, with some Meuse-Rhine-Yssel crossbreeds. The 305-day milk production was 8166 ± 459 kg. Herd C consisted of 41 ± 2 lactating Holstein Friesian and Dutch Friesian animals, with 305-day milk production of 8508 ± 165 kg.

Herds were housed in free stall barns with cubicles and concrete slatted floors in winter, and mostly grazed on pasture in summer (May through October). Animals were milked twice a day. Dry udder preparation was used in all herds. In herd A, cotton towels were used for udder preparation of one or multiple cows. In herds B and C, paper towels were used and per towel only one cow was treated. At every milking, the cows, the udders and the first streams of milk from each quarter were checked for signs of clinical mastitis (any visual abnormality of milk and/or udder, with or without systemic signs of disease). During the study, farmers received information on infection status of their animals. Farmers were free to make changes in herd management using such information, as they would be if they did not participate in a study. In herd C, milking clusters were flushed with hot water (90°C) after milking of *S. aureus* infected cows, to prevent transmission of bacteria via the milking machine. Farmers supplied information on dates of calving, clinical mastitis, antibiotic treatments, dry-off and culling. For each farm, records on the infection status of

cows with subclinical or clinical mastitis in the year(s) preceding the study (2, 1 and 1.5 years for herds A, B and C, respectively) were available through the Animal Health Service, Deventer, The Netherlands.

To determine the infection status of udder quarters, foremilk samples (approximately 15 ml) were collected at 3-wk intervals from all lactating quarters in each herd. Samples were taken after the first streams of milk were discarded, and after teat ends had been disinfected with cotton swabs drenched in methylated spirits[6]. At the start of the study, duplicate samples were taken on two consecutive days to determine the initial infection status of all lactating quarters. Additional quarter milk samples (approximately 5 ml) were collected by farmers at calving (prior to first contact with the milking machine), dry-off, culling and in the case of clinical mastitis. Milk samples that were used for bacteriological culture were stored at -20°C until processing.

Within three weeks of collection, 0.01 ml of milk was cultured and bacterial species were identified according to National Mastitis Council standards[20]. A quarter was considered to have an intramammary infection (IMI) with *S. aureus* when ≥ 1000 cfu/ml of the pathogen were cultured from a single sample, when ≥ 500 cfu/ml of the pathogen were cultured from two out of three consecutive milk samples, when ≥ 100 cfu/ml were cultured from three consecutive milk samples, or when ≥ 100 cfu/ml were cultured from a clinical sample. Samples that contained more than three bacterial species were considered contaminated, and were not used to determine IMI status. Samples that were culture negative during antibiotic treatment for udder disease were not used either. A previously infected quarter was considered recovered from infection if none of the above definitions were met and the sample was free of the pathogen[48]. For statistical analysis, *S. aureus* content of milk samples was treated as a categorical variable with four levels (0-9 cfu/plate, 10-49 cfu/plate, 50-199 cfu/plate or ≥ 200 cfu/plate).

Model formulation

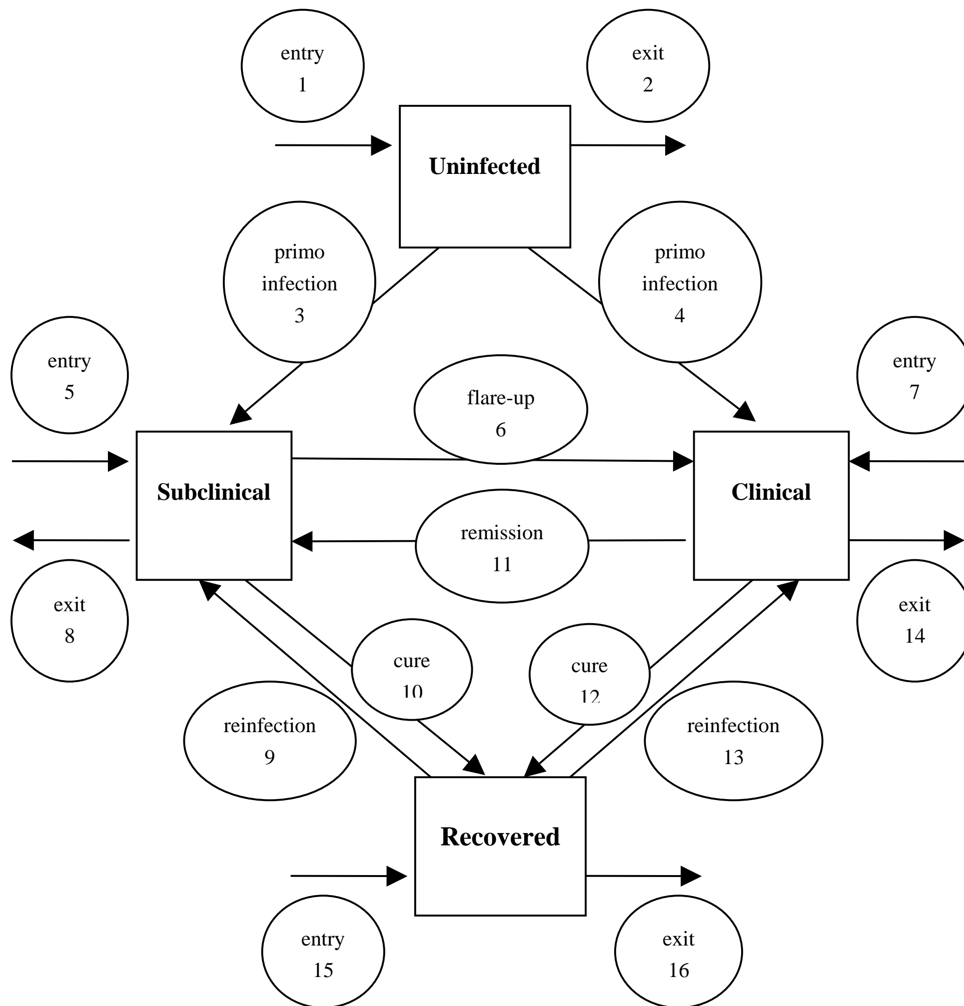
The lactating herd was described as a population of individuals that were uninfected (U), subclinically infected (S), i.e. infected with *S. aureus* but not showing any visible signs, clinically infected (C), i.e. infected with *S. aureus* and showing signs of disease, or recovered-uninfected (R). In traditional SIR models, where "S" indicates the susceptible compartment, "I" indicates the infected compartment, and "R" indicates the recovered compartment, recovered individuals are often considered to be resistant, or removed from the susceptible population. In our study, cure and reinfection of individuals was observed, showing that recovery did not confer absolute resistance to reinfection. This could be described by an SIS model, where "S" indicates "susceptible" and "I" indicates "infected", assuming that susceptibility does not differ between naive individuals and recovered individuals. We preferred to model uninfected individuals (U) and recovered-uninfected individuals (R) separately, because susceptibility differs between individuals that have not experienced infection before and individuals that have recovered from infection[49].

Individuals entered the lactating herd after purchase or at calving, and left the lactating herd at dry-off or culling. Individuals that were dried off usually re-entered the lactating population after the next calving. Entry into the population could be into any compartment. Culling or dry-off could take place for non-mastitis related reasons, e.g. infertility, lameness, or low production, or it could be because of mastitis. Uninfected and recovered-uninfected individuals could get infected (primo-infection and reinfection, respectively), and infections could be subclinical or clinical. Flare-up of subclinical infection to clinical infection, i.e. occurrence of clinical signs in a previously asymptomatic infection, and remission of clinical infection to subclinical infection, i.e. disappearance of clinical signs without disappearance of infection, were also observed. Finally, cure of subclinical and clinical infections occurred, with or without preceding antibiotic treatment. The model, a modification of the model by Allore and Erb[2], is graphically represented in Figure 1. Entry, exit and transitions rates are given in Appendix 1.

Model assumptions

In the analysis, udder quarters of cows were treated as individuals. This approach was preferred over taking cows as individual units for the following reasons. When looking at contagious mastitis, such as *S. aureus* mastitis, exposure to herd mates is a major factor in the spread of disease[18, 23, 30]. A cow with three infected quarters shedding *S. aureus* causes more exposure to herd mates than a cow with one infected quarter that sheds *S. aureus*. Also, each exposed quarter is a unit at risk for infection[23]. Therefore, the individual of interest for infectious disease modeling is quarter rather than cow. In addition, classification of individuals is clear-cut when quarters are considered as individuals. For cows, this would not be the case. For example, a cow with one subclinically infected quarter would be classified as "subclinically infected" at cow level and would not be at risk for infection, while she has three non-infected quarters that are at risk of new infection and that would justify classification of the same cow as "uninfected". Furthermore, cessation of milking in one quarter of a cow is possible, resulting in a "blind" quarter. In dairy herds, this technique is used out of necessity, or to remove an infected quarter from the population while the remaining healthy quarters of the cow continue to be productive[26]. Thus, culling is an event that may occur at quarter level, specifically for *S. aureus* infected quarters. Finally, the number of quarters is approximately four times as large as the number of cows, resulting in a larger population of individuals under study and lower impact of random events. We note that in adopting the udder quarter as the individual unit in a compartmental model, we ignore dependencies among quarters of the same cow[6, 39].

Figure 1. Flow diagram of the dynamics of *Staphylococcus aureus* mastitis in a lactating population with entries (purchase, calving), exits (dry-off, culling), occurrence of infections (primo-infections and reinfections), cure (spontaneous or after treatment) and changes in severity of infection (subclinical to clinical or vice versa). Numbers indicate rates as described in Appendix 1.



The spread of *S. aureus* in the lactating population was assumed to be the result of quarter-to-quarter transmission, and depended on the size of the susceptible compartment, the prevalence of infection, and the transmission parameter, β [23]. The transmission parameter is the probability per unit of time that an infectious quarter will infect a non-infected quarter. Because the definition of infection was based on shedding of the infectious agent, all infected quarters were assumed to be infectious albeit at possibly different levels for clinical and subclinical infections. We chose to model quarters in lactation only. Non-lactating quarters were excluded from the model, because management and contact structure differ between lactating cows and non-lactating cows (dry cows, replacement heifers, young stock). Such differences may affect pathogen transmission[25].

Separate transmission parameters were calculated for infection of uninfected and recovered-uninfected individuals, to model differences in susceptibility between the two compartments. Possible differences in infectiousness between the subclinical and clinical compartments were also incorporated in the model:

$$\text{Rate of new IMI in uninfected individuals} = \beta_U \times \left[\left(S + bC \right) / N \right] \times U \quad [1.a]$$

$$\text{Rate of new IMI in recovered-uninfected individuals} = \beta_R \times \left[\left(S + bC \right) / N \right] \times R \quad [1.b]$$

where S = size of subclinically infected compartment, b = relative change in transmission rate for clinical infections compared to subclinical infections, C = size of clinically infected compartment, N = total population size, U = size of uninfected compartment, R = size of recovered-uninfected compartment, and subscripts refer to the susceptible compartment (U = uninfected, R = recovered-uninfected). This Reed-Frost transmission function assumes homogeneous mixing of hosts. This assumption, discussed by Lam et al.[23], was considered to be an acceptable approximation of possible contacts between cows. Although contacts between individuals in herd C may have been affected by flushing of teat cup liners, homogeneous mixing was also assumed for herd C. In addition to homogeneous mixing, the model assumes homogeneity of individuals within compartments with respect to susceptibility and with respect to infectiousness[25].

Rates of entry, exit or transition between compartments were calculated as mean rates (total number of events per total time at risk) based on observational data (see: Parameter Estimation). Use of a mean rate assumes that the mean is an adequate measure of the central value for a parameter[24]. The mean was used because a better approximation of the central value was not available. Furthermore, all rates were assumed to be constant over the 18-month observation period.

Parameter estimation

To estimate rates, transmission parameters and proportions, the size of the compartments U, S, C, and R had to been known. A quarter was considered to be uninfected when there were no records of infection preceding the study, and no episodes of *S. aureus* infection during the study. A quarter was considered subclinically infected when a definition of *S. aureus* infection was met, but clinical signs were not recorded. A quarter was clinically infected when a definition of *S. aureus* infection was met and clinical signs were recorded. A quarter was considered recovered when it did not meet any of the definitions of *S. aureus* infection, and had been positive for *S. aureus* prior to the study, as documented by Animal Health Service records, or during the study. For subclinical or clinical infections that were first detected at calving, the calving date was assumed to be the date of onset of infection. For clinical infections that started during lactation, the recorded date of clinical mastitis was used as the starting date for the clinical infection. For subclinical or clinical infections that were last detected at dry-off or at culling, sample date was taken as the endpoint of infection. For other combinations, e.g. infectious episodes starting during lactation or ending between a clinical sample and a consecutive routine sample, the midpoint of the last negative and the first positive sample was taken as starting point of the episode, and the midpoint between last positive and first negative sample was taken as endpoint of the episode. The terms "positive" and "negative" apply to clinical status and to infection status of the sample. From the starting points and end points of lactations and infected episodes, the number of days that quarters contributed to a specific compartment in the population was calculated. The summation of the number of days was used as time at risk in that compartment, or compartment size. When samples were missing at dry-off or cull, the last observation with known IMI status was used as the moment of dry-off or cull.

For each herd, dry-off rates, flare-up rates, remission rates, spontaneous cure rates and cure rates after treatment were calculated as the number of observed events, divided by the time at risk in a compartment. For example, the dry-off rate for uninfected individuals was calculated as the number of dry-offs from the uninfected compartment divided by the total number of quarter days in the uninfected compartment. Reasons for culling were not recorded by farmers. Therefore, it was not possible to calculate mastitis-related culling rates directly from the data. The non-mastitis related rate of culling was assumed to be the same for all compartments. This rate was calculated for each herd based on compartment U, and was assumed to apply to compartments S and C as well. The non-mastitis related culling rate was subtracted from the total culling rate for compartments S and C to obtain the mastitis-related culling rate. Blind quarters were considered culled.

The proportion of entries for each compartment was calculated per herd based on the status (U, S, C or R) of quarters at calving or purchase. For new infections during lactation, the proportion that was subclinical or clinical was calculated for infections originating from compartments U and R, respectively, in each herd. Proportions were compared between herds, and between compartments of origin by means of two-tailed Fisher Exact test (SAS System for Windows. Version 8.01. SAS Institute Inc., Cary, NC,

USA. 1999). To examine the infectiousness of quarters in compartments S and C, levels of *S. aureus* shedding were compared between milk samples from subclinical infectious episodes and from clinical infectious episodes within each herd by means of Chi-square analysis (SAS 8.01).

For each herd, values for β_U (transmission parameter for infections from U) and β_R (transmission parameter for infections from R) were calculated from a simplified version of the generalized linear model with log-link and Poisson distributed error[23, 48]:

$$\varepsilon[\ln(IMI)] = \ln[\beta_U] + \ln[U \times (S + bC)/N] \qquad [2.a]$$

$$\varepsilon[\ln(IMI)] = \ln[\beta_R] + \ln[R \times (S + bC)/N] \qquad [2.b]$$

where ε = expected value, and IMI = the number of new IMI in the observation period. To allow for estimation of ln (β), data on number of new IMI and compartment sizes were entered for each 3-week period, and ln (U x (S + bC)/N) or ln(R x (S + bC)/N) was used as model offset. The value for b was set at one (see: Results). Analysis was done using Statistix (Statistix for Windows. Version 1.0. Analytical Software Co., La Jolla, CA, USA. 1996). A 95% confidence interval for β was calculated, taking into account the limited number of observations (25 observations for each estimation of β)[8].

Calculation of reproductive number

In previous ODE models of *S. aureus* mastitis, no distinction was made between clinical and subclinical infections, and R_0 was simply given by the product of β and the mean duration of the infectious period[23]. In the current model, the end of a clinical or subclinical episode was not necessarily the end of infectiousness, because interchange between the subclinical and clinical compartments occurred. This interchange was quantified by the flare-up rate χ and the remission rate θ, and needed to be accounted for in the calculation of R_0. Furthermore, the basic reproductive number depended on the fraction of new infections that were subclinical and clinical, respectively, as quantified by f and 1-f, and on the relative infectiousness of subclinical and clinical infections, as quantified by b. The mathematical expression for R_0 is given in Appendix 2, together with a derivation. Using the parameter estimates from this study, R_0 was estimated for each herd.

In addition to the basic reproductive number, an effective reproductive number, R_t, was calculated. This is the expected number of secondary cases produced by an infectious case in a population that is not wholly susceptible[5]. The subscript t is used to indicate the time point for which the effective reproductive number is calculated, and to differentiate between R (recovered) and R_t (effective reproductive number). R_t depends on β_U and β_R,

and on the composition of the herd at time t. The mathematical expression for R_t is given in Appendix 3.

Deterministic simulation

Using the transition rates from Appendix 1, the effective reproductive number can be calculated for any time point t, and for any composition of the population (proportion U, S, C and R) at the onset of the simulation. To this end, the mathematical model was translated into the C language and compiled with Microsoft C++™ version 1.52 as a Windows application. The model was written as a deterministic model.

To assess whether the model reflected the observed dynamics of infection, simulations were run for each herd with the parameter estimates obtained from the field study and the herd composition that was observed at the onset of the study. Next, to simulate the effect of cure of subclinical mastitis on herd dynamics, the highest cure rate that was observed for subclinical infections (cure rate from herd B) was substituted into herds with a lower cure rate of subclinical infections (herds A and C). Finally, simulations were performed using the parameter estimates from the field study but assuming zero influx into compartments S and C (p = q = 0), as would be the case if all infections in non-lactating animals and herd additions were prevented or cured before entry into the lactating population. To calculate the proportion of influx into the uninfected compartment, (1-p-q-r), and into the recovered compartment (r) in this scenario, all infections at calving in primiparous animals were assumed to have been prevented (entry into S or C substituted by entry into U), and all infections in non-lactating multiparous animals were assumed to be cured by DCT before re-entry into the population (entry into S or C substituted by entry into R).

Results

Descriptive results

During the 18-month observation period, 26,049 milk samples were collected out of which 96% could be used to determine infection status of quarters. In herd A, 15 infected quarters were present at the start of the study, 23 quarters were infected at calving, and 41 new infections with *S. aureus* were detected in lactating quarters. In herd B, eight infected quarters were present at the start of the study, 16 quarters were infected at calving, and 18 new infections were detected in lactating quarters. In herd C, three infections were present at the start of the study, six infections were detected at calving, and 40 new infections were detected during lactation. Table 1 lists the number of subclinical and clinical infections in

lactating quarters per compartment of origin for each herd. The proportion of new infections during lactation that was subclinical or clinical did not differ between herds (P = 0.10), or between compartments of origin (P = 0.29).

Table 1. Number of new infections with *Staphylococcus aureus* in lactating udder quarters observed over an 18-month period in three dairy herds. Population size expressed in number of lactating cows (n ± sd) was 67 ± 3 for herd A, 95 ± 5 for herd B, and 41 ± 2 for herd C.

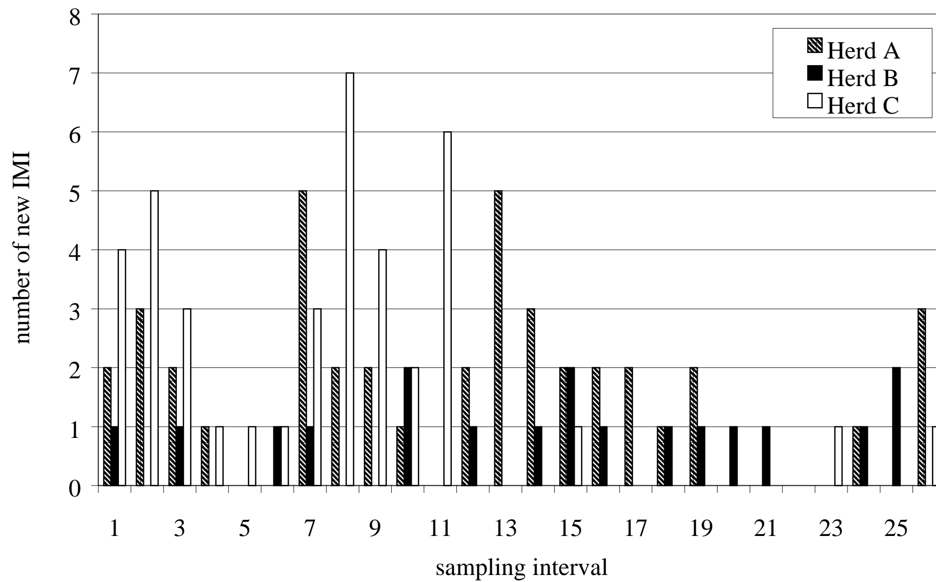
Herd	**New infections from U**		**New infections from R**		**Total**
	Subclinical	Clinical	Subclinical	Clinical	
A	24	6	10	1	41
B	12	4	2	0	18
C	29	2	9	0	40
Total	65	12	21	1	99

Number of new infections and prevalence of *S. aureus* are shown per herd in Figures 2 and 3, respectively. In herds A and C, incidence of new infections in lactating quarters was lower in the second part of the study than during the first part of the study. In herd B, incidence was approximately constant throughout the study period, with an average of one new infection per month. The number of events (cull, flare-up, cure, dry-off, remission, entry) and the number of days at risk within each compartment per herd are listed in Appendix 4.

Parameter estimates

Estimates for herd-specific cull rates, flare-up rates, cure rates, dry-off rates and remission rates are summarized in Table 2. When formulating the model, a mastitis-related cull rate, α, was incorporated for the infected compartments S and C. When calculating cull rates per compartment, cull rate from U was lower than cull rates from S and C, but also lower than cull rate from R in each herd (data in Appendix 4). Therefore, an additional mastitis-related cull rate, α_R, was introduced for compartment R. *Staphylococcus aureus* infected quarters where milking was ceased constituted two out of 20, four out of seven, and one out of five quarters that were infected upon cull in herd A, B and C, respectively.

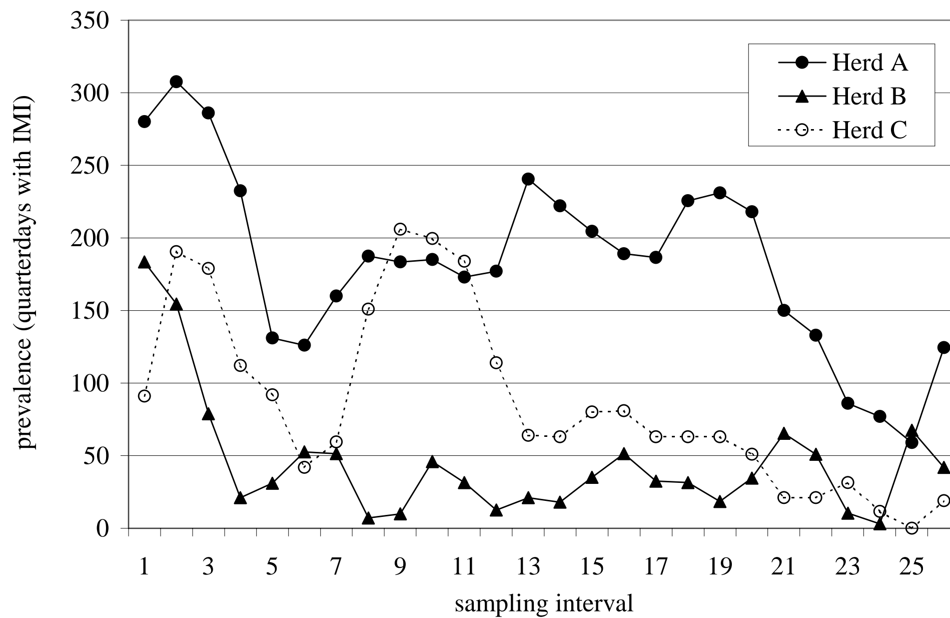
Figure 2. Number of new intramammary infections (IMI) with *Staphylococcus aureus* in lactating udder quarters during an 18-month observation period (26 intervals of three weeks) in three Dutch dairy herds. Herds consisted of 67 ± 3 (herd A), 95 ± 5 (herd B), and 41 ± 2 (herd C) lactating cows, respectively. New IMI include infections that occurred in uninfected quarters and infections that occurred in recovered-uninfected quarters.



For herd B, the number of new infections in quarters originating from R was low (n = 2). Therefore, the estimate of β_R for herd B may not be an accurate estimate of the true transmission parameter. Calculation of an overall value for β, irrespective of compartment of origin, was considered. However, estimates for β_R were considerably higher than estimates for β_U in each herd (Table 2). This is similar to results obtained for *Streptococcus uberis* in herd B[48]. Therefore, separate values for β_U and β_R were used for each herd. The observed and predicted number of infections from compartment U, as obtained from the Poisson regression model for calculation of $\beta_{U,}$ is illustrated per herd in Figure 4.

In each herd, flare-up rates (S to C), χ, were much lower than remission rates (C to S), θ. This is normal, because clinical episodes tend to be of short duration, i.e. days, while subclinical episodes may last weeks or even months. Cure rates were calculated for treated quarters, δ, and non-treated quarters, γ. In quarters with clinical infection, cure was never observed without treatment. In quarters with subclinical infection, cure was observed without treatment (spontaneous cure) and after treatment.

Figure 3. Prevalence of *Staphylococcus aureus* infection in lactating udder quarters observed over an 18-month period (26 intervals of three weeks) in three dairy herds. Population size expressed in number of lactating cows (n ± sd) was 67 ± 3 for herd A, 95 ± 5 for herd B, and 41 ± 2 for herd C.



Dry-off rates were similar between compartments in herds A and B (data in Appendix 4). In herd C, dry-off rate in compartment S was higher than in compartments U or R (5.5, 2.6 and $2.4*10^{-3}$ quarters/day-at-risk, respectively). Because the highest rate was associated with the smallest compartment, i.e. most prone to random effects, and because early dry-off of infected quarters was not consciously used as control strategy, one dry-off rate was used for all compartments within each herd. The overall dry-off rate (i.e. dry-off rate for all compartments combined) was similar between herds.

The herd specific proportions of entries into the system (p, q, and r) were determined from the infection status of quarters at calving or purchase. For calculation of the fraction of new infections in lactation that were subclinical (f) or clinical (1-f) data from all herds were combined. Proportions are included in Table 2.

Bacterial content was compared between milk samples from clinical and subclinical *S. aureus* infections within each herd. In herd A, numbers of bacteria were lower for clinically infected quarters than for subclinically infected quarters (334 samples, $P < 0.01$). In herd B (91 samples, $P = 0.26$), and herd C (142 samples, $P = 0.87$) bacterial numbers did not differ between samples from subclinically or clinically infected quarters.

Higher numbers of bacteria in milk from clinically infected quarters may lead to higher exposure from C than from S individuals, resulting in a value of b higher than 1. On the other hand, milking clusters were usually rinsed after a quarter with clinical mastitis had been milked. This affects bacterial numbers in teat cups and may result in lower exposure caused by C than by S individuals, leading to a value of b lower than one. Because the combined effect of the two phenomena could not be quantified, b was set at 1 for each herd.

Figure 4. Number of new *Staphylococcus aureus* infections originating from the uninfected compartment within each herd, as observed during an 18-month field study (26 intervals of three weeks) and as predicted by the Poisson regression model used to calculate the transmission parameter β_U in a compartmental model of *S. aureus* dynamics in lactating populations.

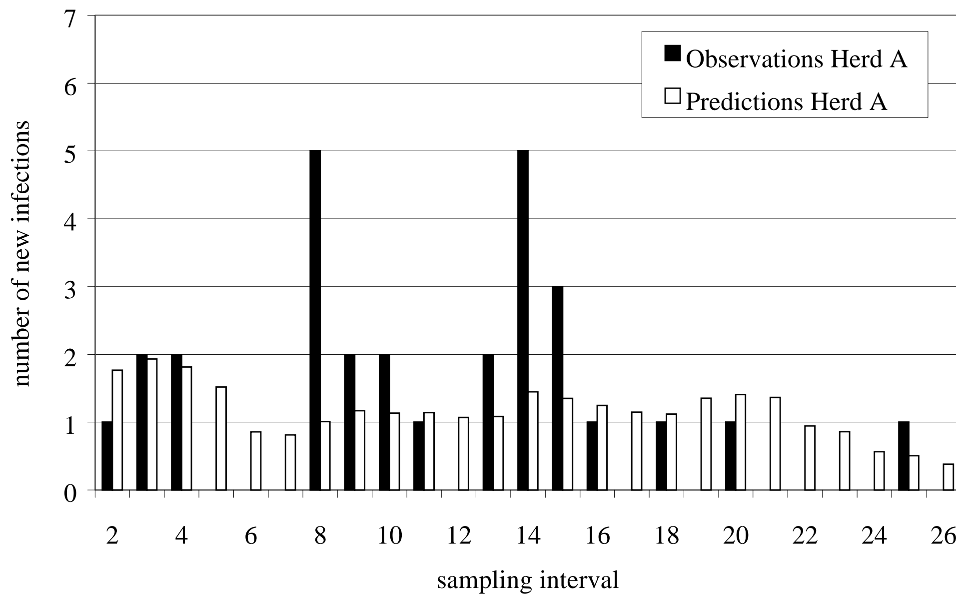


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Figure 4 (ctd). Number of new *Staphylococcus aureus* infections originating from the uninfected compartment within each herd, as observed during an 18-month field study (26 intervals of three weeks) and as predicted by the Poisson regression model used to calculate the transmission parameter β_U in a compartmental model of *S. aureus* dynamics in lactating populations.

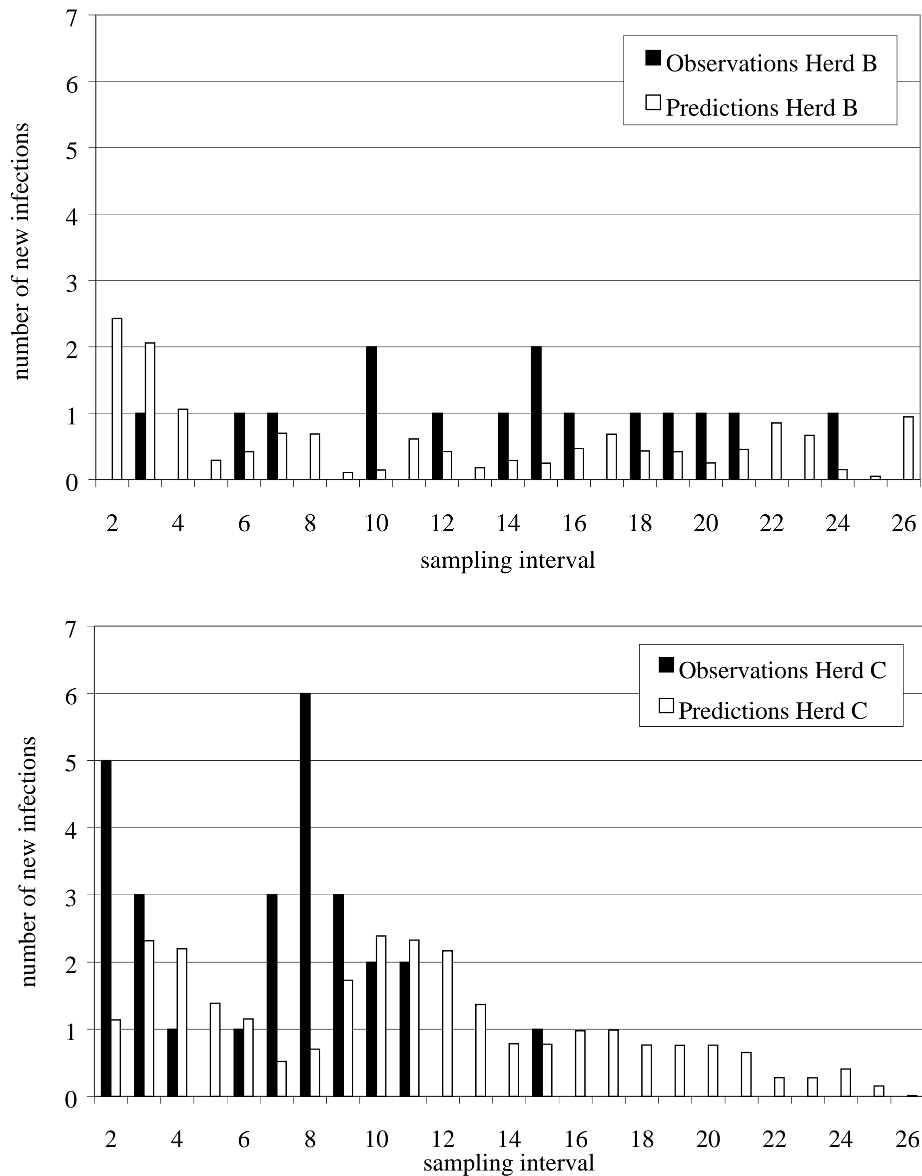


Table 2. Parameter estimates for transition rates, proportions and transmission parameters in a compartmental model that represents the dynamics of *Staphylococcus aureus* infections in a population of lactating udder quarters. U = uninfected, R = recovered-uninfected, S = subclinically infected, C = clinically infected. All rates and transmission parameters are expressed as number of events per 10^3 quarterdays at risk.

Parameter	Symbol	Herd A	Herd B	Herd C
Extra culling rate from clinical compartment	α_C	53.5	35.8	12.7
Extra culling rate from subclinical compartment	α_S	2.4	2.7	1.2
Extra culling rate from recovered compartment	α_R	1.4	0.6	0.7
Transmission parameter for new infections from U	β_U [a)]	7	14	14
Transmission parameter for new infections from R	β_R [b)]	42	52	41
Flare-up rate (S to C)	χ	2.1	4.6	4.1
Cure rate for treated clinical infections	δ_C	32.6	36.5	13.3
Cure rate for treated subclinical infections	δ_S	1.3	10.2	1.8
Spontaneous cure rate for clinical infections	γ_C	0	0	0
Spontaneous cure rate for subclinical infections	γ_S	5.2	15.7	11.9
Exit rate due to culling (non-mastitis related)	μ_{CU}	0.8	1.0	0.6
Exit rate due to dry-off (non-mastitis related)	μ_{DR}	2.5	2.6	2.6
Remission rate (C to S)	θ	163	61	120
Fraction of new entries that is S	p	0.033	0.020	0.022
Fraction of new entries that is C	q	0.012	0.003	0.000
Fraction of new entries that is R	r	0.049	0.023	0.074
Fraction of new infections from U that goes to S	f_U	0.87	0.87	0.87
Fraction of new infections from R that goes to S	f_R	0.87	0.87	0.87
Change in infectiousness for C relative to S	b	1	1	1
Basic Reproductive Number	R_0	0.53	0.40	0.75

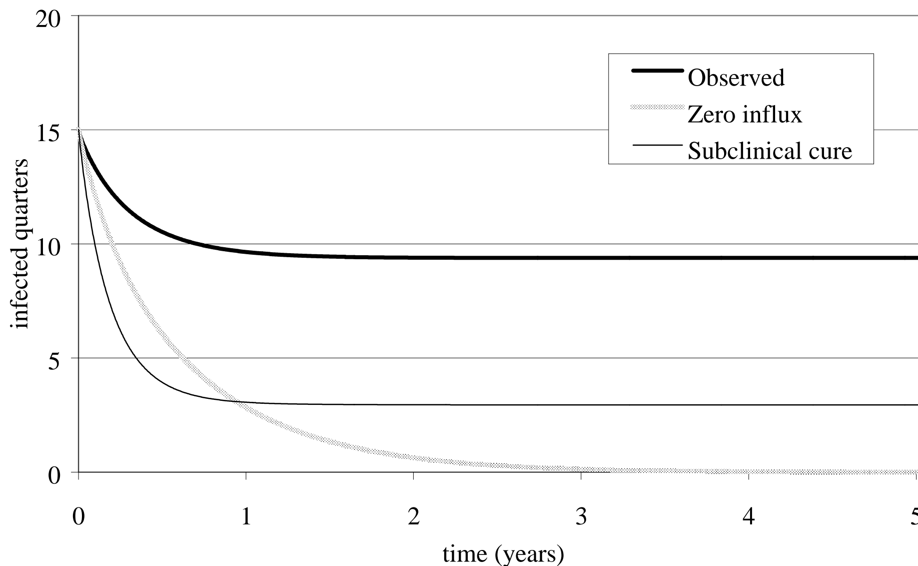
[a)] 95% confidence intervals are (5;10), (8;23), and (9;21) for herds A, B, and C, respectively.
[b)] 95% confidence intervals are (22;80), (12;223), and (21;81) for herds A, B and C, respectively.

Reproductive number and model output

Based on the parameter estimates from Table 2 and equation A2.1 from Appendix 2, values for R_0 were calculated. For each herd, the value of R_0 was below 1 (Table 2). Results for R_t are not tabulated, because R_t changes over time. Using the observed herd composition at onset of the study and the transmission parameters calculated from the data, the value of R_t ranged from 0.53 to 0.67, from 0.40 to 0.44, and from 0.75 to 0.89 for herds A, B and C, respectively, from the start to the end of the first simulated year. R_t was higher than R_0 in each herd, and increased with the proportion of recovered individuals in the simulated population.

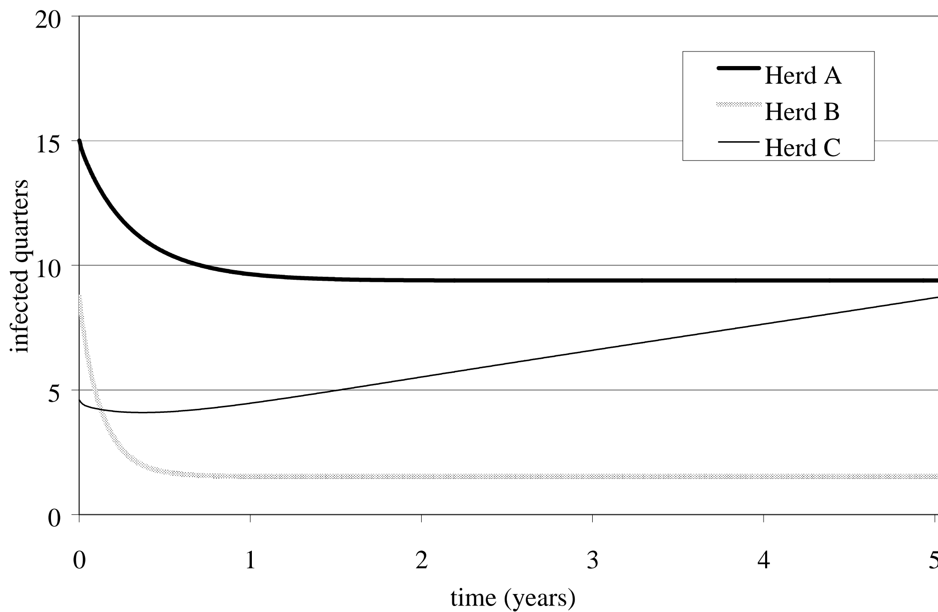
Simulated dynamics for three scenarios (observed situation, higher cure rate of subclinical infections, zero influx of infections) are exemplified for herd A in Figure 5. In the simulation of the observed dynamics, prevalence of subclinical infection levels off at 9.4 infected udder quarters (Figure 5).

Figure 5. Simulated number of quarters with subclinical *Staphylococcus aureus* mastitis in a population of 268 lactating udder quarters (herd A), based on composition of the population at onset of the observational study, and on parameter estimates as calculated from the data (thick black line), or with substitution of a higher cure rate for subclinical infections (thin black line), or assuming zero influx of infected individuals into the population (dotted line). The number of clinically infected quarters is close to zero throughout the simulated period and is not displayed.



During the field study, observed prevalence of infection hovered around a constant level from interval 7 to 21 (Figure 3), and observed average prevalence of subclinical infection in that time period was 191 infected quarterdays per 3-week period. This is equivalent to 9.1 infected udder quarters on any day. Thus, the simulated number of infected quarters reflected the field data. Observed prevalence dropped to the stable level faster than simulated prevalence. Similarly, the prevalence of subclinical infections in herd B leveled off at 1.5 infected udder quarter when observed dynamics were simulated (Figure 6), while field data showed an average prevalence of subclinical infections of 1.3 udder quarter from interval 7 to 21 (Figure 3). Decline of infection prevalence after onset of the study was faster in herd B than in herd A, both for observed and simulated dynamics. For herd C, simulated dynamics showed an increase of infection prevalence over time (Figure 6), in disagreement with field observations. The deterministic model did not simulate changes in infection prevalence that were observed in each herd at the end of the field study.

Figure 6. Simulated number of quarters with subclinical *Staphylococcus aureus* infection based on composition of the population at onset of the observational study, and on parameter estimates as calculated from the data for herds A (thick black line), B (dotted line) and C (thin black line). Population size is 268, 384 and 164 lactating udder quarters, respectively.



Discussion

Staphylococcus aureus is a contagious pathogen that spreads easily in dairy herds unless adequate control measures are taken. Successful control of contagious spread is achieved if R_0 is reduced to a value below 1. One of the aims of this paper was to estimate the value of R_0 for *S. aureus* mastitis in lactating populations, and to use simulation to show how changes in management may contribute to reduction of R_0 to a value below one. Even without simulation of changes, the combined effect of control measures in the study herds was a value of R_0 that was below 1. Therefore, if all transmission of *S. aureus* were the result of cow-to-cow or quarter-to-quarter transmission, it should be possible to eliminate *S. aureus* mastitis from a population. Farmers in the study herds used control measures that are used by many farmers. During our study, the farmers were informed of the infection status of all quarters every three weeks. This led to well-informed treatment, segregation or cull decisions, and may have contributed to the successful control of cow-to-cow spread of *S. aureus* in the participating herds.

Although R_0 was below 1 in each herd, *S. aureus* infections were present throughout the 18-month study period. Prevalence of *S. aureus* infection was highest in herd A, while the probability per time unit that an infected quarter would cause a new infection (β_U) was lowest in herd A. High prevalence and low number of new infections in the lactating population are not necessarily contradictory. It can occur in a population where the pool of susceptible individuals is exhausted, when there is entry of infected individuals into the population, or when infections have long duration. None of those situations explain a low transmission parameter. The low transmission parameter in herd A was unexpected, because herd A was the only herd that did not use a new udder cloth for each cow, PMTD or disinfection of teat cup liners. Those measures were used in the other two herds and reduce transmission of *S. aureus*[23, 30, 46]. Because of the small number of herds involved, it is not possible to determine what caused the observed differences in β_U. Potential contributing factors include production level and breed. At herd level, high production is associated with a higher risk of *S. aureus* mastitis[37]. Production was similar for herds B and C, but much lower for herd A, indicating lower risk in herd A. On the other hand, herd A had the largest proportion of Meuse-Rhine-Yssel cows, and this breed is associated with an increased risk of *S. aureus*[17]. Preliminary evidence suggests that differences between bacterial strains may be associated with differences in spread in a population[26, 50]. In a small scale study, the predominant *S. aureus* strain in lactating quarters in herd A was different from the predominant *S. aureus* strain in lactating quarters from herds B and C[47]. Summarizing, management, cow and pathogen characteristics may have contributed to differences in *S. aureus* spread.

For all three herds, the value of β_R was higher than the value of β_U. Recovered quarters were not immune to reinfection, but, on the contrary, had increased susceptibility to reinfection. This result at population level is in agreement with results at quarter level from a risk factor study in the observed herds[49]. The observed differences in susceptibility between uninfected and recovered-uninfected quarters justified the choice to model uninfected quarters and recovered-uninfected quarters as separate compartments. Farmers may be aware that recovered quarters are at higher risk of infection. Such awareness could explain why culling from compartment R took place at a higher rate than culling from compartment U. Other reasons include decreased milk production and elevated somatic cell counts in quarters that suffered intramammary infection[9, 45]. An interesting consequence of β_R being higher than β_U is that R_t can be higher than R_0, as observed in this study. As a result, measures that are sufficient to prevent spread of *S. aureus* in a wholly susceptible population may not suffice to prevent spread of *S. aureus* in a population with recovered individuals.

Despite the fact that R_0 and R_t were below 1, indicating that contagious transmission of *S. aureus* was controlled in each herd, new infections in lactating individuals were observed. R_0 and R_t indicate the average number of new infections caused by an existing infection. Hence, some new infections should be expected to occur on theoretical grounds, as some existing infections will not cause new infections, while other existing infections cause more than zero new infections. In models, the number of new infections can be fractional. In practice, fractional infections do not occur: an individual does or does not get infected. Over the total observation period, the average number of predicted new infections was equal to the average number of observed new infections. However, for each time interval the observed number of new infections differed from the number of new infections that was predicted based on prevalence. The discrepancy is partly the result of random variability in the number of new infections under field conditions. A deterministic model cannot capture random variability. Similarly, temporary changes in management may affect transmission. Such changes were not reflected in the constant transition rates in the model. Small outbreaks of mastitis that are not predicted based on prevalence, as in herd A during intervals 15 and 16, or in herd C during intervals 7 to 9 (Figure 4), may be the result of temporary “breakdowns” in mastitis control. Such breakdowns can occur at farm level, or at national level. Examples include reduced culling because of Bovine Spongiform Encephalitis in the United Kingdom[22], leading to increased incidence of *S. aureus* mastitis, and Food and Mouth Disease in The Netherlands, which was followed by an increase in national BMSCC in 2001. This increase is partly attributed to reduced culling of infected cows, and to reduced availability of information because herd health visits by veterinarians and routine cow milk SCC testing by the Royal Dutch Cattle Syndicate were suspended (O. C. Sampimon and J. D. Miltenburg, personal communication). Other reasons for lack of agreement between observed and predicted numbers of new infections are discussed below.

In the model, homogeneity of compartments was assumed. This implies homogeneity of susceptibility among individuals in the susceptible compartments and homogeneity of infectiousness among individuals in the infectious compartments. The homogeneity assumption may have been violated, as cows and udder quarters differ with respect to susceptibility to *S. aureus* infection[39, 49], and with respect to numbers of bacteria shed in milk from infected quarters[10, 40]. Similarly, homogeneity of infected compartments with respect to cure rates is assumed, while differences in probability of cure of *S. aureus* infections exist, depending on host factors and pathogen factors[42, 43]. To account for all possible combinations of susceptibility, infectivity, and "curability" levels, the number of compartments in the model would need to be increased dramatically, leading to an intractable model. Different types of models, such as the dynamic discrete event stochastic simulation model developed by Allore and colleagues[3, 4] are better suited to incorporate heterogeneity of susceptible and infected individuals.

Discrepancy between observed and predicted numbers of new infections can be expected if infections in lactating individuals are not the result of cow-to-cow transmission. Evidence that *S. aureus* infections can be of environmental rather than contagious origin is growing. Infections in non-lactating animals are generally considered to be the result of infection from an environmental source[41]. New infections with *S. aureus* in non-lactating animals were observed in this study (Appendix 4) and others[31, 35]. Many environmental sources of *S. aureus* have been identified[34], and environmental factors such as disinfection procedures, bedding replacement and hygienic status of stalls, are associated with the risk of *S. aureus* mastitis in dairy herds[17]. Isolation of different pathogen strains and failure to find a persistent strain may be used as evidence that sustained transmission has been eliminated[12]. So far, strain-typing studies only provide tentative support for an environmental origin of *S. aureus* infections in dry and in lactating animals[47]. A stable incidence of new infections irrespective of prevalence, as observed in herd B, would be in line with a base line infection rate with *S. aureus* from environmental sources. Infections from environmental sources could also explain why *S. aureus* continued to be the third-most occurring cause of clinical mastitis in a British herd with excellent control of contagious transmission of mastitis pathogens[21, 22]. Identification and elimination of sources of *S. aureus*, other than infected quarters, may be crucial for the success of a control program[36].

Despite shortcomings of the deterministic model used in this study, and the failure to describe the dynamics of mastitis in herd C, prevalence of infection in herds A and B was simulated at a realistic level, suggesting that the model could be used to examine the effect of changes in control measures. Changes in cure rate and changes in influx of infections were examined. It must be noted that "cure rate" in this paper is defined as rate, i.e. number of occurrences over time at risk. This is different from cure probabilities that are often reported in literature, i.e. number of cases cured out of number of cases treated. Cure probabilities are commonly called "cure rates" in every day language, which may lead to confusion. In our study, "cure" was defined using bacteriological criteria, and clinically

infected quarters never cured spontaneously. Cure was only achieved after treatment. Cure rate for clinically infected quarters, δ_C, was lower than the rate of cull, α_C, or the rate of remission, θ. This implies that most clinically infected quarters were lost for production, or contributed to the pool of subclinical infections. Cure rate of subclinically infected quarters was lower in treated quarters (δ_S) than in quarters that cured spontaneously (γ_S). This should not be interpreted as delayed cure as a result of treatment. Time-until-treatment was an important component of compartment size and hence, of cure rate. Usually, farmers would not decide to treat subclinically infected quarters unless they were *S. aureus* positive at two or more consecutive samplings, i.e. for at least three weeks. Because farmers were informed of IMI status three weeks after samples had been collected, bacteria were present for six weeks or more before treatment was initiated. As a result, infected episodes that would fall into the category "cured after treatment" had often lasted more than six weeks. Duration of infection does not only affect compartment size, but also the probability of cure[43]. Thus, time-until-treatment affects cure rate through the enumerator and the denominator. In herd B, treatment was usually initiated shortly after diagnosis of subclinical infections, and cure probability after treatment was high (data not shown), resulting in a high cure rate for treated subclinical infections compared to the other two herds. Simulations for herd A showed that increase of the cure rate for subclinical infections resulted in faster decline of the prevalence of infection, and a lower prevalence in the long term compared to the observed situation. Studies on the economic benefit of treatment of subclinical mastitis should take into account effects at population level, in addition to effects at cow level.

Influx of infections, either as a result of infections in non-lactating individuals or after purchase of infected animals, does not contribute to incidence in the lactating population directly, but after entry into the milking herd such infections are a source of *S. aureus* that contributes to the incidence in lactating individuals. In herds B and C, the majority of infections in non-lactating animals were detected in heifers at first calving (Appendix 4), which indicates the importance of mastitis control in young stock. For multiparous animals, the number of infected quarters at calving depends cure of existing infection and prevention of new infections by DCT[30]. In herd A, the success of DCT was limited, and persistent and new infections in dry cows were observed (Appendix 4), indicating a need to re-evaluate treatment and cull strategies in this herd. When animals are introduced into a farm as herd replacements or for herd expansion, *S. aureus* infections may be introduced with them. For newly introduced animals, screening of udders for infection should be an element of mastitis control and biosecurity on dairy farms[28]. The combination of fully successful mastitis control in heifers, DCT and biosecurity is simulated by zero influx of infected individuals. In combination with a reproductive number below 1, zero influx results in prevalence of *S. aureus* infection that approaches zero in the deterministic model, as shown by simulation for herd A.

Can *S. aureus* mastitis be eliminated? If the reproductive ratio is below one, if all infections are the result of contagious transmission, and if there is no influx of infected

individuals, the prevalence of *S. aureus* infection would asymptotically decrease to zero in a deterministic model. In a stochastic model, prevalence would fall to zero as soon as an absorbing state is reached. This would imply elimination of infection, if elimination of infectious disease were understood to mean total absence of cases in the population. A more lenient definition of elimination is a situation in which sustained transmission can not occur[12]. This situation is achieved when $R_0 < 1$. Under this alternative definition, elimination would have been achieved in each herd in this study. To a farmer, this alternative definition of elimination will be of little relevance as new cases of mastitis may continue to occur. Control, or maintaining the prevalence and incidence of mastitis at an acceptable level, would be more meaningful terminology to describe this situation in practice.

Staphylococcus aureus mastitis was not eradicated, but reduced to acceptable levels in all three herds in this study. Well known mastitis control measures, in combination with knowledge of infection status and regular and frequent attention for mastitis control, were sufficient to attain this, as they have been in other studies[19, 21, 36]. Treatment of subclinical infections and prevention of entry of infected individuals in the lactating herd, through DCT, fly control, or screening of replacement animals, contribute to reduction of *S. aureus* prevalence and to reduction of cow-to-cow transmission. Prevention of cow-to-cow transmission does not seem sufficient to reduce *S. aureus* prevalence and incidence to zero, which could be explained by infections from the environment. When control of contagious transmission of *S. aureus* has been achieved, research and management can focus on ways to identify and prevent infections from environmental sources. This can be approached through detection and removal of sources, through limitation of contact between sources and susceptible individuals, or through improved resistance of individuals to mastitis. The economic feasibility of the control measures discussed in this paper remains to be established.

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Appendix 1. Transitions rates describing dynamics of *Staphylococcus aureus* mastitis in a populations of lactating individuals. Identification numbers of equations correspond to identification numbers of transitions in Figure 1.

Elaborating on the differential equation model for the dynamics of contagious mastitis that was developed by Allore and Erb[2], transitions rates were defined to describe the dynamics of S. aureus mastitis in lactating dairy herds:

rate of entry into compartment U = $(1\text{-}p\text{-}q\text{-}r)\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C\}$ [A1.1]

rate of outflow from compartment U = $(\mu_{CU}+\mu_{DR})U$ [A1.2]

rate of subclinical infection from compartment U = $f_U\beta_U U(S+bC)/N$ [A1.3]

rate of clinical infection from compartment U = $(1\text{-}f_U)\ \beta_U U(S+bC)/N$ [A1.4]

rate of entry into compartment S = $p\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C\}$ [A1.5]

rate of clinical flare-up of subclinical infections = χS [A1.6]

rate of entry into compartment C = $q\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_S C\}$ [A1.7]

rate of exit from compartment S = $(\mu_{CU}+\mu_{DR}+\alpha_S)S$ [A1.8]

rate of subclinical infection from compartment R = $f_R\beta_R R(S+bC)/N$ [A1.9]

rate of recovery from compartment S = $(\gamma_S + \delta_S)S$ [A1.10]

rate of remission of clinical infections = θC [A1.11]

rate of recovery from compartment C = $(\gamma_C+\delta_C)C$ [A1.12]

rate of clinical infection from compartment R = $(1\text{-}f_R)\beta_R R(S+bC)/N$ [A1.13]

rate of exit from compartment C = $(\mu_{CU}+\mu_{DR}+\alpha_C)C$ [A1.14]

rate of entry into compartment R = $r\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C\}$ [A1.15]

rate of exit from compartment R = $(\mu_{CU}+\mu_{DR})R$ [A1.16]

where the symbols represent the following (in alphabetical order):

$\alpha_{S,\,C}$ = extra culling rate because of mastitis from S (α_S) or C (α_C)

$\beta_{U,R}$	= transmission parameter for new infections from U (β_U) or R (β_R)
b	= relative change in transmission rate for C compared to S
C	= size of clinically infected compartment
$\gamma_{S,C}$	= spontaneous cure rate from S (γ_S) or C (γ_C)
$\delta_{S,C}$	= cure rate after treatment from S (δ_S) or C (δ_C)
θ	= remission rate (C to S)
$f_{U,R}$	= fraction of subclinicals among infections coming from U (f_U) or R (f_R)
$\mu_{CU,DR}$	= non-mastitis related exit rate due to culling (μ_{CU}) or dry-off (μ_{DR})
N	= total population size
p	= fraction of entries that enters into S
q	= fraction of entries that enters into C
r	= fraction of entries that enters into R
R	= size of recovered uninfected compartment
S	= size of subclinically infected compartment
U	= size of uninfected compartment.
χ	= flare-up rate (S to C)

When an additional culling rate because of recovery from mastitis is added, equations [A1.1], [A1.5], [A1.7], [A1.15] and [A1.16] must be adapted as shown below:

rate of influx into compartment U = $(1-p-q-r)\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C+\alpha_R R\}$ [A1.1a]

rate of influx into compartment S = $p\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C+\alpha_R R\}$ [A1.5a]

rate of influx into compartment C = $q\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C+\alpha_R R\}$ [A1.7a]

rate of influx into compartment R = $r\{(\mu_{CU}+\mu_{DR})N+\alpha_S S+\alpha_C C+\alpha_R R\}$ [A1.15a]

rate of outflow from compartment R = $(\mu_{CU}+\mu_{DR}+\alpha_R)R$ [A1.16a]

where α_R = extra culling rate because of recovery from mastitis.

Appendix 2. The basic reproductive number R_0 for *Staphylococcus aureus* mastitis.

The basic reproductive number is in our model given by: [A2.1]

$$R_0 = \frac{\beta_U \left(f_U \left(\mu + \alpha_C + \gamma_C + \delta_C + \theta + b\chi \right) + \left(1 - f_U\right)\left(\theta + b\left(\mu + \alpha_S + \gamma_S + \delta_S + \chi\right)\right)\right)}{\left(\mu + \alpha_S + \gamma_S + \delta_S + \chi\right)\left(\mu + \alpha_C + \gamma_C + \delta_C + \theta\right) - \chi\theta}$$

For simplicity, μ is used to represent ($\mu_{CU+}\mu_{DR}$).

There are several ways to derive the above expression. Perhaps the most efficient way is by using an elegant scheme introduced by Diekmann and Heesterbeek[14]. Below we present a derivation along a more intuitive line.

Derivation

We start by noting that R_0 is defined as the expected number of secondary infections caused by a single primary infection in an otherwise wholly susceptible population. The primary infection might (initially) be either a subclinical (with probability f_U) or a clinical infection (with probability $1-f_U$), thus giving rise to two contributions (R_S and R_C) to R_0:

$$R_0 = f_U * R_S + \left(1 - f_U\right) * R_C \qquad \text{[A2.2]}$$

R_S is the expected number of secondary infections caused by a single primary infection starting out in compartment *S*. Clearly, this primary infection can cause secondary infections while being in S (we denote these secondary infections with $(R_0)_S$) but it can also move from S to C and cause secondary infections from there ($(R_0)_C$). The probability of moving from S to C is the ratio of the per capita rate χ from S to C to the per capita rate from S to anywhere, so splitting into two contributions again we get:

$$R_S = \left(R_0\right)_S + \frac{\chi}{\mu + \alpha_S + \gamma_S + \delta_S + \chi} * \left(R_0\right)_C \qquad \text{[A2.3]}$$

For R_C we find analogously:

$$R_C = \left(R_0\right)_C + \frac{\theta}{\mu + \alpha_C + \gamma_C + \delta_C + \theta} * \left(R_0\right)_S \qquad \text{[A2.4]}$$

$(R_0)_S$ and $(R_0)_C$ can be obtained by multiplying the expected time of presence in the compartment (denoted with T_S and T_C, respectively) with the respective rates of new infections arising from the primary infection in a totally susceptible population: $(R_0)_S = f_U T_S$ and $(R_0)_C = bf_U T_C$.

This yields the overall expression: [A2.5]

$$R_0 = f_U \beta_U \left(T_S + \frac{\chi}{\mu + \alpha_S + \gamma_S + \delta_S + \chi} bT_C \right) + (1 - f_U)\beta_U \left(bT_C + \frac{\theta}{\mu + \alpha_C + \gamma_C + \delta_C + \theta} T_S \right)$$

To complete the derivation, we need to express T_S and T_C in terms of model parameters. Since infections can make back-and-forth movements between S and C, we can write:

$$T_S = \frac{1}{\mu + \alpha_S + \gamma_S + \delta_S + \chi}(1 + p_r) \quad [A2.6]$$

$$T_C = \frac{1}{\mu + \alpha_C + \gamma_C + \delta_C + \theta}(1 + p_r) \quad [A2.7]$$

Where p_r is the probability of re-entering compartment S or C (by coming back from C or S, summing over all multiple back-and-forth moving processes):

$$p_r = \sum_{n=1} x^n$$

with

$$x = \frac{\chi}{\mu + \alpha_S + \gamma_S + \delta_S + \chi} * \frac{\theta}{\mu + \alpha_C + \gamma_C + \delta_C + \theta} \quad [A2.8]$$

where x is the probability of moving back and forth once.

The sum $1+p_r$ is a geometric series with result [A2.9]

$$1 + p_r = \sum_{n=0} x^n = \frac{1}{1 - x} = \frac{(\mu + \alpha_S + \gamma_S + \delta_S + \chi)(\mu + \alpha_C + \gamma_C + \delta_C + \theta)}{(\mu + \alpha_S + \gamma_S + \delta_S + \chi)(\mu + \alpha_C + \gamma_C + \delta_C + \theta) - \chi\theta}$$

Appendix 3. The effective reproductive number R_t for *Staphylococcus aureus* mastitis.

The effective reproductive number R_t can be derived in a manner comparable to the derivation of the basic reproductive number R_0 that was described in Appendix 2. The resulting expression for R_t has a form similar to the right-hand side of [A2.1], the difference being that β_U is replaced by $(\beta_U U/N+\beta_R R/N)$ and f_U by $(f_U\beta_U U+f_R\beta_R R)/(\beta_U U+\beta_R R)$.

Appendix 4. Observational data on number (and percentage) of events and number (and percentage) of quarterdays at risk in three dairy herds (A, B, C) during an 18-month period. The population of lactating udder quarters in each herd is considered to consist of an uninfected compartment (U), a recovered uninfected compartment (R), a subclinically infected compartment (S), and a clinically infected compartment (C). (Un)infected refers to intramammary infection status with respect to *Staphylococus aureus*.

Event	Compartment				Total
	U	R	S	C	n
	n (%)	n (%)	n (%)	n (%)	
Cull					
herd A	110 (75.3)	16 (11.0)	15 (10.3)	5 (3.4)	146
herd B	199 (90.9)	13 (5.9)	4 (1.8)	3 (1.4)	219
herd C	49 (75.4)	11 (16.9)	4 (6.2)	1 (1.5)	65
Flare-up (S to C)					
herd A	n.a. [a)]	n.a.	10	n.a.	
herd B	n.a.	n.a.	5	n.a.	
herd C	n.a.	n.a.	9	n.a.	
Cure from C after treatment [b)]					
herd A	n.a.	n.a.	n.a.	3	
herd B	n.a.	n.a.	n.a.	3	
herd C	n.a.	n.a.	n.a.	1	
Cure from S after treatment					
herd A	n.a.	n.a.	6	n.a.	
herd B	n.a.	n.a.	11	n.a.	
herd C	n.a.	n.a.	4	n.a.	
Spontaneous cure from S					
herd A	n.a.	n.a.	24	n.a.	
herd B	n.a.	n.a.	17	n.a.	
herd C	n.a.	n.a.	26	n.a.	

Continued overleaf

Appendix 4 (ctd). Observational data on number (and percentage) of events and number (and percentage) of quarterdays at risk in three dairy herds (A, B, C) during an 18-month period. The population of lactating udder quarters in each herd is considered to consist of an uninfected compartment (U), a recovered uninfected compartment (R), a subclinically infected compartment (S), and a clinically infected compartment (C). (Un)infected refers to intramammary infection status with respect to *Staphylococus aureus*.

Event	Compartment				Total
	U	R	S	C	n
	n (%)	n (%)	n (%)	n (%)	
Dry-off					
herd A	322 (89.2)	22 (6.1)	17 (4.7)	0 (0.0)	361
herd B	508 (96.9)	15 (2.9)	3 (0.6)	0 (0.0)	526
herd C	198 (86.1)	20 (8.7)	12 (5.2)	0 (0.0)	230
Remission (C to S)					
herd A	n.a.	n.a.	n.a.	15	
herd B	n.a.	n.a.	n.a.	5	
herd C	n.a.	n.a.	n.a.	9	
Entry					
herd A	467 (90.7)	25 (4.9)	17 (3.3)	6 (1.2)	514 [c]
herd B	654 (95.3)	16 (2.3)	14 (2.0)	2 (0.3)	686 [d]
herd C	253 (90.7)	20 (7.2)	6 (2.2)	0 (0.0)	279 [e]
Quarterdays at risk					
herd A	132,473.5 (91.7)	7,231.0 (5.0)	4,696.5 (3.3)	92.0 (0.1)	144,493.0
herd B	195,563.0 (95.6)	7,915.0 (3.9)	1,080.5 (3.9)	81.5 (0.0)	204,640.0
herd C	76,535.5 (87.9)	8,253.0 (9.5)	2,180.5 (9.5)	75.0 (0.1)	87,044.0

[a] n. a. = not applicable

[b] Spontaneous cure from C was never observed.

[c] Samples were missing at calving for 5 quarters. Ten infections were detected in quarters from heifers, and 13 infections were detected in quarters from multiparous animals.

[d] Samples were missing at calving for 32 quarters. Ten infections were detected in quarters from heifers, and six infections were detected in quarters from multiparous animals.

[e] 271 samples were taken at calving, and 8 samples were taken from quarters that entered the population after purchase of animals. One sample at entry was missing. Six infections were detected in quarters from heifers, and no infections were detected in quarters from multiparous animals.

- Chapter 5 -

Cow and quarter level risk factors for *Streptococcus uberis* and *Staphylococcus aureus* mastitis

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Abstract

This study was designed to identify risk factors for intramammary infections with *Streptococcus uberis* and *Staphylococcus aureus* under field conditions. An 18-month survey with sampling of all quarters of all lactating cows at 3-wk intervals was carried out in three Dutch dairy herds with medium bulk milk somatic cell count (200.000-300.000 cells/ml). Quarter milk samples were used for bacteriology and somatic cell counting. Data on parity, lactation stage and bovine herpesvirus 4-serology were recorded for each animal. During the last year of the study, body condition score and teat end callosity scores were recorded at 3-wk intervals. A total of 93 new infections with *Strep. uberis* were detected in 22665 observations on quarters at risk for *Strep. uberis* infection, and 100 new infections with *Staph. aureus* were detected in 22593 observations on quarters at risk for *Staph. aureus* infection. Multivariable Poisson regression analysis with clustering at herd and cow level was used to identify risk factors for infection. Rate of infection with *Strep. uberis* was lower in first and second parity animals than in older animals, and depended on stage of lactation in one herd. Quarters that were infected with *Arcanobacterium pyogenes* or enterococci, quarters that had recovered from *Strep. uberis* or *Staph. aureus* infection in the past, and quarters that were exposed to another *Strep. uberis* infected quarter in the same cow had a higher rate of *Strep. uberis* infection. Teat end callosity and infection with coagulase negative staphylococci or corynebacteria were not significant as risk factors. Rate of *Staph. aureus* infection was higher in bovine herpesvirus 4-seropositive cows, in right quarters, in quarters that had recovered from *Staph. aureus* or *Strep. uberis* infection, in quarters exposed to other *Staph. aureus* infected quarters in the same cow, and in quarters with extremely callused teat ends. Infection with coagulase negative staphylococci was not significant as a risk factor. Effect of infection with corynebacteria on rate of infection with *Staph. aureus* depended on herd, stage of lactation and teat end roughness. Herd level prevalence of *Strep. uberis* or *Staph. aureus*, and low quarter milk somatic cell count were not associated with an increased rate of infection for *Strep. uberis* or *Staph. aureus*.

Abbreviation key: BCS = body condition score, **BHV4** = bovine herpesvirus-4, **BMSCC** = bulk milk somatic cell count, **CNS** = coagulse negative staphylococci, **pBCS** = previous body condition score, **PMTD** = post-milking teat disinfection, **pSCC** = previous somatic cell count, **TECR** = teat end callosity roughness, **TECT** = teat end callosity thickness.

Introduction

Mastitis is a widely occurring and costly disease in the dairy industry. The major causative agents of mastitis in modern Dutch dairy herds are *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus dysgalactiae*[2, 21]. In addition to the presence of bacteria, dairy herd management and cow or quarter characteristics may contribute to the occurrence of mastitis. Characteristics that increase the risk of infection can be identified in risk factor studies. The ultimate goal of such studies is to develop preventive measures to control spread of disease.

The sampling unit in risk factor studies can be herd, cow or udder quarter and the outcome of interest can be SCC, clinical mastitis[26], subclinical mastitis[6] or intramammary infection (IMI), i.e. the combination of clinical and subclinical mastitis[16, 22]. Some studies do not differentiate between pathogens while others are pathogen specific and demonstrate differences between risk factors for different pathogens[2, 28]. Study designs include experimental and observational studies. The majority are cross-sectional[26, 30], but longitudinal studies have been reported[14, 16]. Cross-sectional studies deal with prevalence data. The time order of occurrence of associated factors is unknown in cross-sectional studies and causal inference is not possible[27]. Longitudinal studies deal with incidence data, and are necessary to support a causal role of risk factors. Despite the vast body of risk factor literature, longitudinal studies are scarce because of their time consuming nature. This is especially true for subclinical mastitis, as detection of new infections requires repeated collection and bacteriological culture of milk samples. To complicate matters, clustering of observations within cows or herds needs to be accounted for in study design or data analysis to avoid invalid statistical inference in risk factor studies[19].

Cows that have no infected quarters or multiple infected quarters occur more frequently than can be expected by chance[4]. This implies differences between cows in susceptibility to mastitis, or within cow-transmission of causative agents. Cow characteristics that influence the susceptibility to mastitis include parity, stage of lactation, and genetic make-up[3, 6, 29]. Recently, bovine herpesvirus 4 (BHV4) isolation from milk was reported in association with mastitis[34]. No effect on susceptibility to mastitis was found for blood vitamin E level, cow conformation, retroviral infections or body condition score (BCS)[11, 29, 32]. Quarter-level factors that affect susceptibility to mastitis include SCC and infections with minor pathogens[14, 16, 29]. Most risk factors at the cow or quarter level have been identified in cross-sectional or experimental studies, or in studies on clinical mastitis.

We present a longitudinal study on the incidence of naturally occurring IMI, based on observations at quarter level at 3-wk intervals. The study was carried out in three Dutch dairy herds that implemented most measures from the five-point mastitis control plan[13]. Risk factors for infection with *Strep. uberis* and *Staph. aureus* were evaluated, while accounting for repeated measures at cow and herd level. Special attention was given to on-farm scoring of body condition and teat end condition because clinical scores may be used as routine tools for monitoring cow and udder health in dairy herds.

Herds, materials and methods

Herd characteristics

Data were obtained from a longitudinal observational study (from May 1997 to December 1998) in three commercial dairy herds (A, B and C) in the Netherlands. Herds were selected based on records that documented a history of at least a year of medium level bulk milk SCC (200,000-300,000 cells/ml) despite reasonably good udder health management (Table 1). Records were supplied by the Dutch Animal Health Service. *Staphylococcus aureus* and non-agalactiae streptococci had been the predominant causes of infection in the herds in the preceding years. The herds were considered to be examples of a relevant level of management and bulk milk SCC (BMSCC) under current farming conditions in the Netherlands.

Table 1. Characteristics of three commercial Dutch dairy herds participating in a longitudinal study on risk factors for intramammary infection with *Streptococcus uberis* and *Staphylococcus aureus*.

Characteristic	Herd A	Herd B	Herd C
herd size (n ± SD)[a]	67 ± 3	95 ± 5	41 ± 2
305-day milk production (kg)[b]	7187 ± 149	8166 ± 459	8508 ± 165
bulk milk somatic cell count[c]	266 ± 76	235 ± 75	205 ± 69
breed[d]	MRY, RH (HF, DF)	HF, DF (MRY)	HF, DF
udder health management			
• teat dipping	no	yes/no[e]	yes
• yearly milk machine check-up	yes	yes	yes
• antibiotic treatment of clinical cases of mastitis	yes	yes	yes
• blanket dry cow treatment	yes	yes	yes
• routine culling of chronically infected cows	no	yes	no
milking parlor	2 x 5 herring bone	2 x 4 open tandem	2 x 5 herring bone

a) Mean number of cows present at each routine sampling during the study ± standard deviation.
b) Mean 305-day milk production (kg) during study.
c) Arithmetic mean of bulk milk somatic cell count (x 1000 cells/ml) in year preceding study.
d) Dominant breed in herd, with minor breeds shown in brackets; HF = Holstein-Friesian, DF = Dutch Friesian, RH = Red Holstein, MRY = Meuse-Rhine-Yssel.
e) Teat dipping was practiced from sampling 1 to 7, and from sampling 16 to 20.

Herd characteristics are summarized in Table 1. Herds were housed in free stall barns with cubicles and concrete slatted floors. Cows mostly grazed on pasture during summer (May through October), but zero-grazing was practiced in herd B during part of the summer in 1998. Animals were milked twice a day. Dry udder preparation was used in all herds. In herd A, cotton towels were used for udder preparation. Cotton towels were replaced when they looked dirty. In herds B and C, single use paper towels were used. At every milking, the first streams of milk from each quarter were checked for signs of clinical mastitis before cluster attachment. During the study, farmers were free to make changes in herd management. Bacteriology results were reported to the farmers three weeks after milk sample collection. In all herds, a number of cows was treated with antibiotics and/or culled because of clinical or subclinical infection with *Strep. uberis* or *Staph. aureus*. In herd B, ten out of fourteen *Strep. uberis* infected animals were housed and milked separately for six weeks in May and June 1998. In herd C, milking clusters were flushed with hot water (90°C) after milking of *Staph. aureus* infected cows to prevent transmission of bacteria via the milking machine.

Sampling and data collection

Single quarter foremilk samples (approximately 15 ml) were collected every three weeks from all lactating animals in each herd. Samples were taken after the first streams of milk were discarded and after teat ends had been disinfected with cotton swabs drenched in methylated spirits[4]. At the start of the study, duplicate samples were taken on two consecutive days to determine the initial infection status of all lactating quarters. Additional quarter milk samples (approximately 5 ml) were collected by farmers at calving (prior to first contact with the milking machine), dry-off, culling and in the case of clinical mastitis (any visual abnormality of milk and/or udder, with or without systemic signs of disease). In herd C, two animals were purchased during the study and sampled before entry into the milking herd. All milk samples that were used for bacteriology were stored at -20°C until processing.

Within three weeks of collection, 0.01 ml of milk was cultured and bacterial species were identified according to National Mastitis Council standards[10]. Colony counts were recorded for each bacterial species. A quarter was considered to have an IMI when $\geq$ 1000 cfu/ml of a pathogen (major or minor) were cultured from a single sample, when $\geq$ 500 cfu/ml of a pathogen were cultured from two out of three consecutive milk samples, when $\geq$ 100 cfu/ml were cultured from three consecutive milk samples, or when $\geq$ 100 cfu/ml were cultured from a clinical sample. Samples containing more than three bacterial species were considered contaminated, and were not informative of IMI status. Samples that were culture negative during antibiotic treatment for udder disease were not considered informative of IMI status either. A previously infected quarter was considered recovered from IMI for a species if none of the above definitions were met and the sample was free of the pathogen[37]. A fraction of the fresh quarter milk samples was preserved with sodium

azide, and used for determination of SCC by a Fossomatic milk cell counter (Foss Electronic, Hillerød, Denmark) within three days of collection.

Data on stage of lactation, parity, clinical mastitis, and dates of calving, dry-off or culling were available from farm records. From October 1997 (sampling 10) until the end of the study, BCS was measured for dry and lactating cows at 3-wk intervals, using a scale from 1 to 5 (where 1 = emaciated and 5 = extremely fat) with quarter intervals[7]. At the same time, teat end condition was scored for lactating cows, using the scale developed by Neijenhuis et al.[23]. Teat end callosity roughness (TECR) was scored as smooth or rough, while teat end callosity thickness (TECT) was scored as absent or thin, moderate, thick, or extreme. Scoring was done by two observers, one of whom was present at every observation.

Blood was collected from the tail vein of periparturient heifers and all lactating and dry animals once in every five samplings (at samplings 1, 6, 11, 16, 21 and 26). Serum was used to detect the presence of antibodies against BHV4 by means of an immunoperoxidase monolayer assay[35]. A BHV4-serostatus was assigned to each observation (milk sample) in the dataset. Serostatus was as determined on the sample date when milk and blood samples were taken on the same date. For other milk sample dates, serostatus was based on interpolation. When a change of serostatus was observed, it was assumed that the change occurred at the midpoint between two serum samplings, unless the animal went through a dry period. In that case, change of serostatus was assumed to have occurred during the dry or transitioning period[33] and one serostatus was assigned to each lactation.

Herd level variables

This study was not designed to identify risk factors at the herd level but rather at the cow and quarter level. However, the risk of infection may depend on herd management factors[3, 26, 30] and on exposure to infected herd mates[15, 37]. Therefore, herd was included as an independent variable in all analyses, and the effect of prevalence of *Strep. uberis* and *Staph. aureus* on the rate of new infections was examined.

The 3-wk period between two routine samplings was called the sampling interval. For each sampling interval, prevalence of *Strep. uberis* and *Staph. aureus,* respectively, were calculated for each herd as described previously[37]. Briefly, quarters were classified as infected or uninfected based on culture results. Duration of infection was calculated from the starting point and end point of the infected episode. The summation of number of infected quarter-days in a sampling interval was considered to be the herd-level prevalence of *Strep. uberis* or *Staph. aureus* for that interval. Because an infected quarter contributes to the prevalence of infection, occurrence of a new infection can be the result or the cause of infection prevalence in that sampling interval. To allow for detection of possible cause-effect relationships, the time order of events must be known. Therefore, the prevalence of infection in the interval preceding the detection of a new infection was used as the

independent variable. For example, for a new *Strep. uberis* infection detected in the fourth sampling interval, prevalence of *Strep. uberis* during the third sampling interval was taken as the value for the independent variable. Because of the definition used, the prevalence in the preceding interval was unknown for new IMI that were detected at the second sampling.

Cow level variables

Variables at the cow level included parity, the occurrence of important events (calving, clinical mastitis, dry-off or culling), DIM, BCS, change in BCS, infection history with respect to *Strep. uberis* and *Staph. aureus*, and BHV4-serostatus. For some variables, the value concurrent with the observation of new infection was used as there could be no confusion about possible cause-effect relationships. For example, IMI does not cause parity. Parity and DIM were initially treated as categorical variables with three and six levels, respectively. For BCS, the value preceding observation of new infection was used. Previous BCS (pBCS) was treated as a categorical variable with four levels (1 = 1.00 to 1.75; 2 = 2.00 to 2.75; 3 = 3.00 to 3.75; 4 = 4.00 to 5.00[32]). Change in BCS was calculated as the difference between the pBCS value at the current observation and the pBCS value at the previous observation, implying that it was the change in BCS over a 3-wk interval. It was treated as a categorical variable with five levels (0 = no change; 1 = 0.25 or 0.50 points increase; 2 = 0.75 or more points increase; 3 = 0.25 or 0.50 points decrease; 4 = 0.75 or more points decrease). Infection history was defined at cow level, because infection of one (or more) quarter(s) may theoretically lead to changes at systemic level, e.g. development of immunity. This could affect susceptibility to future episodes of mastitis in all quarters of the cow. Infection history in the interval preceding the observation of new IMI was used as an independent variable. A cow was considered to have a history of infection when she was infected with the pathogen of interest or when she had recovered from infection with that pathogen.

Quarter level variables

Variables defined at the quarter level were quarter position (right vs. left and front vs. rear), infection status with respect to specified major and minor pathogens, infection history with respect to *Strep. uberis* and *Staph. aureus,* SCC, TECR, TECT, and exposure to other *Strep. uberis* or *Staph. aureus* infected quarters within the same udder. No distinction was made between exposure to one or more than one infected quarter within the udder.

For quarter position, the value concurrent with the observation of new infection was used. For other quarter level variables the value at the observation preceding a new infection was used. In the models, previous SCC (pSCC) was treated as a categorical variable, because a continuous variable assumes a linear cause-effect relation. This assumption of linearity could be evaluated in the categorical response. For routine samples, five levels of quarter milk pSCC (x 1000 cells/ml) were distinguished (1 = < 51; 2 = 51 to

100; 3 = 101 to 250; 4 = 251 to 500; 5 = > 500). For samples taken by farmers (e.g. at calving or clinical mastitis), no SCC was determined. In those situations, pSCC was coded as level 6 for observations following a sample that was taken at calving or within three weeks post calving, and as level 7 for observations following a sample that was taken at more than three weeks in lactation.

Infection history at quarter level was defined, in addition to the infection history at cow level, because infection of a quarter may be associated with local changes that affect susceptibility to future episodes of mastitis in the quarter. A quarter was considered to have an infection history when it was infected with the pathogen of interest, or when it had recovered from infection with that pathogen.

Cow level infection history was strongly correlated with quarter level infection history and with exposure to other infected quarters within the udder. When a quarter had an infection history, or when a quarter was exposed to other infected quarters within the udder, the cow also had an infection history by definition. To be able to look at the effects of cow history (possible systemic immunity) and quarter history (possible local immunity) and within-cow exposure at the same time, a composite variable was created. Five combinations were distinguished: 1 (reference level) = no history of infection in the cow, no history of infection in the quarter, and no exposure to another infected quarter within the cow; 2 = history of infection in the cow, no history of infection in the quarter, and no exposure to another infected quarter within the cow; 3 = history of infection in the cow, no history of infection in the quarter, but exposure to another infected quarter within the cow; 4 = history of infection in the cow, history of infection in the quarter, but no exposure to another infected quarter within the cow; 5 = history of infection in the cow, history of infection in the quarter, and exposure to another infected quarter within the cow.

Statistical analyses

Occurrence or non-occurrence of new infection was the dependent variable in all analyses. Separate analyses were run for new infection with *Strep. uberis* and *Staph. aureus*, respectively. Observations from quarters with existing infections of the pathogen under analysis were excluded from the dataset. Infection status before parturition was unknown in heifers, and management of dry cows was different from management of lactating cows. Therefore, infections that were first detected at calving were also excluded from analysis.

First, univariate analysis of all data was performed to detect extreme values. No observations were excluded for this reason. Next, bivariate analysis for screening of independent variables was done in a Poisson regression model that always included herd as a fixed effect. Mastitis incidence is a person-time rate (number of disease onsets per sum of time at risk for all population members) and use of Poisson regression is conventional for analysis of person-time rates[9]. Finally, all independent variables with $P \leq 0.20$ for at least one level in the bivariate analysis were submitted to a multivariate mixed model, based on general estimation equations[38]. Two-way interactions between herd, parity, DIM, quarter

position, pSCC, previous infection status with respect to CNS, previous infection status with respect to corynebacteria, BHV4-serostatus, TECR and TECT were also tested for statistical significance in the multivariable model, with the exception of interaction between serostatus and quarter position. The latter interation was not considered biologically meaningful.

In the dataset, clustering of observations occurred at multiple levels. Repeated observations over time were clustered within quarters, quarters were spatially clustered within cows and cows were clustered within herds. Modeling of multiple levels of clustering is possible using the GLIMMIX Macro[18]. Due to the small number of new infections relative to the total number of observations in the dataset, computational limitations arose with this method (non-convergence). Using the VARCOMP procedure[1] in SAS version 8.1, it was determined that cow accounted for more variability than quarter. Therefore, analyses were run with herd as fixed effect and cow as repeated effect, accounting for correlation at herd level and cow level, but not at quarter level. Compound symmetry was used as the covariance structure for within-cow correlation[4]. Analyses were run with the GENMOD procedure of SAS version 8.1 (SAS System for Windows. SAS Institute Inc., Cary, CA, USA, 1999) using a regression model with log link and Poisson distributed error. A forward stepwise analysis of main effects and interactions was performed with cut-off for retention set at $P \leq 0.10$ in the likelihood ratio test. The linearized mixed model was as follows:

$$\log P(Y = y) = \beta_0 + \Sigma\beta_i RF_i + R + \varepsilon \qquad [\ 1\ ]$$

where Y is a random variable measuring the number of new infections, y is the actual realization of Y, β_0 = intercept, β_i = regression coefficient for risk factor i, RF_i = value or level of risk factor i, R = random cow effect and ε = residual error. Regression coefficients (β_i) are the natural logarithm of the rate ratio. The rate ratio is the rate of new infections in quarters at a specified level of a risk factor, relative to the rate of new infections in quarters at the reference level for that risk factor.

Model fit of the final model was evaluated using the ratio of the Pearson Chi square statistic to the remaining degrees of freedom[20]. There is no standard method to assess the amount of variability in the dependent variable that is accounted for by the independent variables in a Poisson regression model. Therefore, the ability of the model to differentiate between occurrence and non-occurrence of new infection was assessed semiquantitatively. Predicted values for the probability of new infection were calculated in the GENMOD procedure of SAS[1], and compared between observations of non-occurrence and occurrence of new infection. Sensitivity and specificity of the model as a test for prediction of new infection were calculated after selection of an appropriate cut-off value.
For one independent variable, the proportion of new infections in all observations that could be attributed to the exposure was calculated using equation [2][27]:

$$AP_T = \frac{RR - 1}{RR + 1/P_0 - 1} \qquad [\ 2\ ]$$

with:

AP_T = attributable proportion in all observations
RR = rate ratio
= incidence rate in exposed observations/incidence rate in unexposed observations
P_0 = proportion of all observations that is exposed

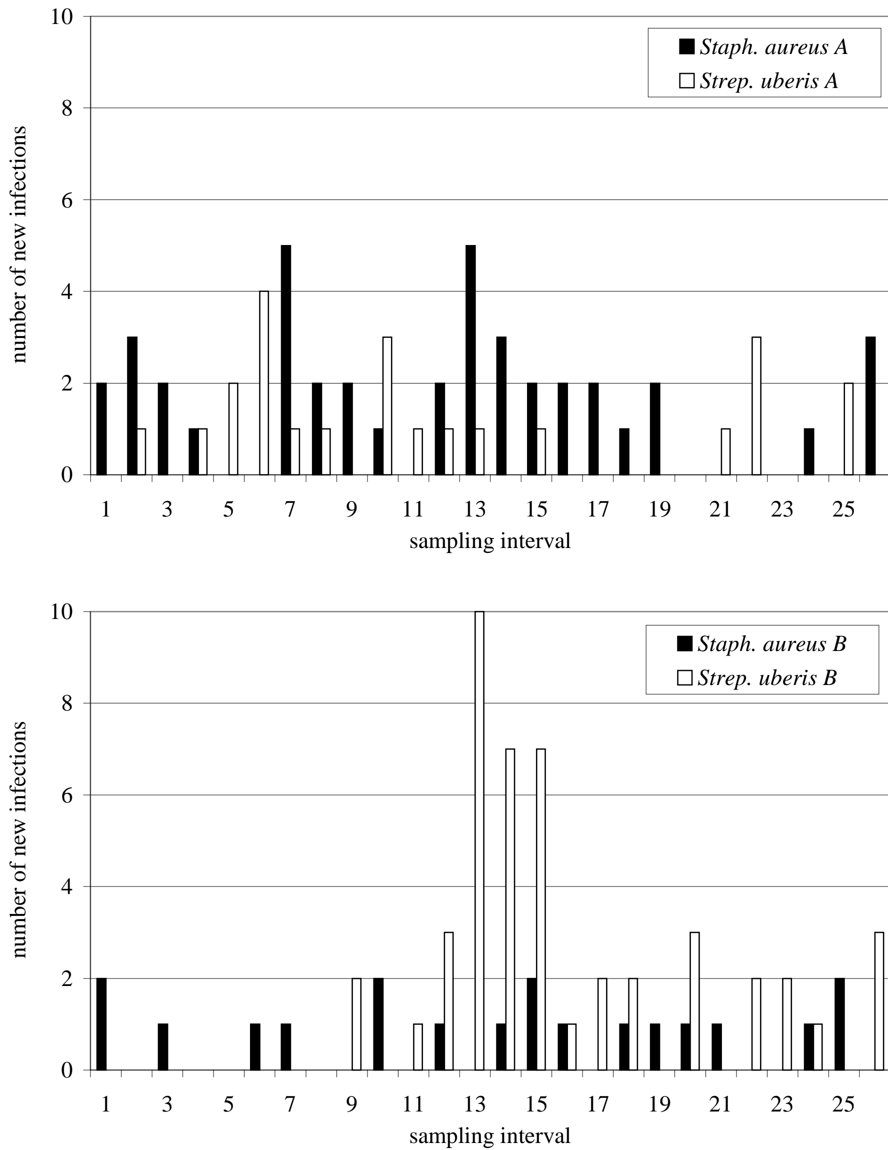
The datasets for quarters at risk of *Strep. uberis* mastitis and *Staph. aureus* mastitis were used to create multiple subsets. The first dataset for either pathogen (FULLset) contained data on herd, important events (clinical mastitis, dry-off, culling), parity, DIM, quarter position, previous infection status with respect to major and minor pathogens, pSCC, previous recovery history at cow and quarter level, and previous exposure to other infected quarters. The second dataset (PREVset) was a subset of FULLset and contained the same variables plus data on the herd level prevalence of *Strep. uberis* or *Staph. aureus* in the previous sampling interval. The third dataset (SEROset) was a subset of FULLset that included BHV4-serostatus and excluded observations that had missing values for BHV4-serostatus. The last dataset (SCOREset) was a subset of FULLset that contained observations with values for pBCS and previous TECR and TECT. Data(sub)sets for analysis of risk factors for *Strep. uberis* mastitis will be referred to as StrepFULLset, StrepPREVset, StrepSEROset, and StrepSCOREset. Data(sub)sets for analysis of risk factors for *Staph. aureus* mastitis will be referred to as StaphFULLset, StaphPREVset, StaphSEROset, and StaphSCOREset.

Results

Descriptive results

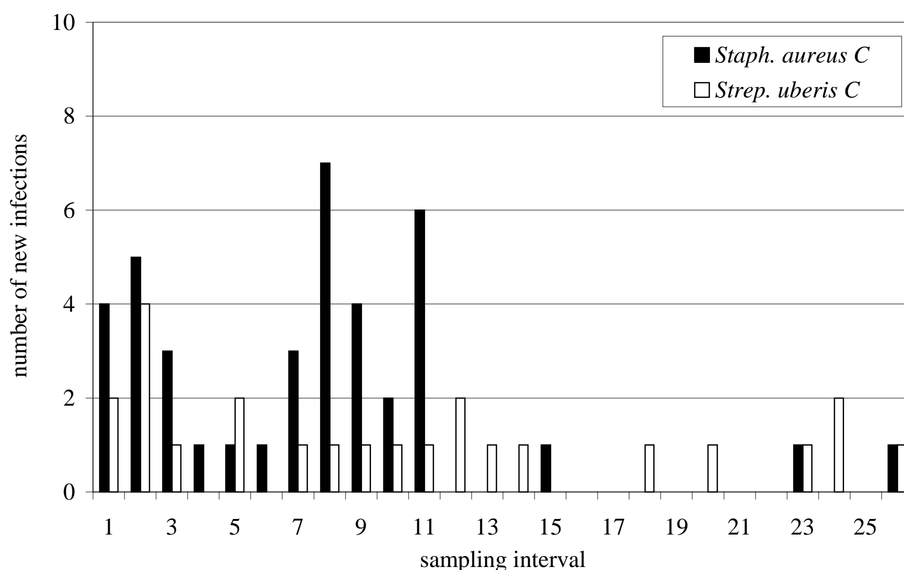
During the 81-wk study period, 93 new infections with *Strep. uberis* and 100 new infections with *Staph. aureus* were detected. Figures 1.a through 1.c show the number of new infections per 3-wk sampling interval for each herd.

Figure 1. Number of new infections with *Streptococcus uberis* (white bars) and *Staphylococcus aureus* (black bars) per 3-week sampling interval in herds A, B, and C. Size of the lactating herd (n ± SD) was 67 ± 3 cows, 95 ± 5 cows, and 41 ± 2 cows, respectively.



Continued overleaf

Figure 1 (ctd). Number of new infections with *Streptococcus uberis* (white bars) and *Staphylococcus aureus* (black bars) per 3-week sampling interval in herds A, B, and C. Size of the lactating herd (n ± SD) was 67 ± 3 cows, 95 ± 5 cows, and 41 ± 2 cows, respectively.



The number of observations on quarters at risk for *Strep. uberis* and *Staph. aureus* infection, respectively, and the total number of new infections are summarized per herd in Table 2. The infections with *Strep. uberis* were detected in 81 quarters of 56 cows. Infections with *Staph. aureus* were detected in 91 quarters of 66 cows. The maximum number of infections per cow and quarter was five and three, respectively, for *Strep. uberis* and six and three for *Staph. aureus*.

Table 2. Number of observations per herd and number of new infections per herd included in analysis of risk factors for *Streptococcus uberis* and *Staphylococcus aureus* infection.

Herd	*Streptococcus uberis*				*Staphylococcus aureus*			
	observations		new infections		observations		new infections	
	n	%	n	%	n	%	n	%
A	7454	32.9	23	24.7	7256	32.1	41	41.0
B	10625	46.9	46	49.5	10752	47.6	19	19.0
C	4586	20.2	24	25.8	48585	20.3	40	40.0
total	22665	100.0	93	100.0	22593	100.0	100	100.0

Risk factors for *Streptococcus uberis* infection

An overview of independent variables and their significance in bivariate and multivariable analysis of risk factors for new infection with *Strep. uberis* is given in Table 3. Of all interactions that were tested, only the interaction between herd and DIM was significant. Parameter estimates for significant variables and interactions in the multivariable model are listed in Table 4. Clinical signs in the quarter were significantly ($P < 0.0001$) associated with new *Strep. uberis* infection but this variable was not included in the multivariable models. The time-order of occurrence of clinical signs and new infection is unknown and therefore causal inference was not possible.

The number of levels was reduced for several categorical variables. For DIM, there was no difference between estimates for 181 to 240 DIM, 241 to 320 DIM, or more than 320 DIM and the levels were collapsed into the reference level. When herd and DIM were included in the model, but the interaction between herd and DIM was not, the remaining levels of DIM were all significantly associated with an increased risk of *Strep. uberis* mastitis. For 1-60 DIM, the relative risk was 2.8 ($P < 0.001$), for 61-120 DIM the relative risk was 1.9 ($P = 0.10$) and for 121-180 DIM the relative risk was 2.0 ($P < 0.05$). When the interaction between herd and DIM was added to the model, overall model fit improved as indicated by the likelihood ratio test. The level of 61-120 DIM in herd 2 remained as the only level with a significantly increased risk of new infection compared to the reference level (>180 DIM in herd 3), with a relative risk of 7.5 ($P < 0.05$).

For quarters that had not recovered from *Strep. uberis* infection in the past, and that were not currently exposed to infected quarters within the same cow, there was no effect of cow-level recovery from *Strep. uberis* infection. The composite variable for cow and quarter level infection history and exposure could thus be reduced to two binary variables, i.e. quarter level infection history and exposure within the udder. Both were significant as independent variables, while their interaction was not significant.

For prediction of *Strep. uberis* infections, the association between pSCC level and new infection was non-linear. Therefore, pSCC could not be treated as a continuous independent variable. In bivariate analysis, level 5 (> 250.000 cells/ml) and 7 (preceding value missing at more than 3 weeks in lactation) of pSCC were significantly associated with increased risk of new infection but pSCC was not significant in the multivariable model.

StrepPREVset, StrepSEROset and StrepSCOREset contained 21791, 22077, and 14493 observations, and 85, 87 and 56 new *Strep. uberis* infections respectively. None of the variables that could only be tested in StrepPREVset, StrepSEROset, or StrepSCOREset were significant.

Model sensitivity (ability to predict occurrence of new infection) was over 50% and model specificity (ability to predict non-occurrence of new infection) was over 90% for the model based on StrepFULLset. The Pearson Chi Square statistic was 0.94 per degree of freedom, indicating good model fit.

Table 3. Significance of independent variables in bivariate and multivariable Poisson regression models for prediction of new IMI with *Streptococcus uberis* or *Staphylococcus aureus*, respectively. + = significant ($P \leq 0.10$); - = non-significant; ... = no estimate (no new infections); x = not tested.

Independent variable	*Strep. uberis* model		*Staph. aureus* model	
	Bivariate	Multivariable	Bivariate	Multivariable
Herd level variables				
herd of origin	-	-	+	+
Strep. uberis prevalence	-	-	x	x
Staph. aureus prevalence	x	x	-	-
Cow level variables				
extra samples				
• clinical mastitis[a]	+	x	+	x
• dry-off	-	-	-	-
• culling	-	-	...	...
parity	+	+	+	+
days in milk	+	+	+	+
recovery from *Strep. uberis*	-	-	-	-
recovery from *Staph. aureus*	-	-	-	-
bovine herpesvirus 4-serostatus	-	-	+	+
previous BCS	-	-	-	-
change in previous BCS[b]	-	-	-	-
Quarter level variables				
right vs. left	-	-	+	+
front vs. rear	-	-	+	-
recovery from *Strep. uberis*	+	+	+	+
recovery from *Strep. dysgalactiae*	+	-	-	-
recovery from *Staph. aureus*	+	+	+	+
previous infection status				
• *Arcanobacterium pyogenes*	+	+	...	...
• *Bacillus species*	-	-	-	-
• coagulase negative staphylococci	-	-	-	-
• corynebacteria	-	-	-	+
• enterobacteria	+	+	-	-
• *Escherichia coli*	-	-	-	-
• *Staph. aureus*	-	-	x	x
• *Strep. dysgalactiae*	+	-	+	-
• *Strep. uberis*	x	x	-	-
• other streptococcus species[c]	+	+	-	-
exposure within udder to quarters with				
• *Staph. aureus*	x	x	+	+
• *Strep. uberis*	+	+	x	x
previous quarter SCC	+	-	+	+
preceding teat end callosity				
• roughness	-	-	-	+
• thickness	-	-	+	+

[a] Significant in bivariate and multivariable models for *Strep. uberis* and *Staph. aureus* ($P <$ 0.0001) but excluded from multivariable analysis because of unknown order of occurrence of new infection and clinical signs.
[b] Estimates for change in previous BCS based on 12187 observations in the *Strep. uberis* dataset, and on 12207 observations in the *Staph. aureus* dataset.
[c] *Streptococcus agalactiae* was never isolated during the study.

The effect of preceding infection with enterococci on other parameter estimates was evaluated, because it was thought that diagnostic interpretation played a role for this variable (see Discussion). When preceding infection with enterococci was omitted from the model for StrepFULLset, estimates for other independent variables changed little in direction, order of magnitude or significance of effect. The proportion of *Strep. uberis* infections that was attributable to exposure to preceding infection with enterococci, i.e. the attributable proportion (AP_T), was 5.1%.

Risk factors for *Staphylococcus aureus* infection

Independent variables and their significance in bivariate and multivariable analysis of risk factors for new infection with *Staph. aureus* are listed in Table 3. Several two-way interactions were significant as predictors for new infection.

Parameter estimates for significant independent variables and interactions for *Staph. aureus* are listed in Table 4, with the exception of the interaction between parity and front or rear quarter. The interaction between parity and front or rear quarter was significant according to the likelihood ratio test. There was no difference between front or rear quarters for parity 2 ($P = 0.36$ in Wald's test) or higher ($P = 0.88$ in Wald's test), but front quarters were significantly associated with a higher rate of infection in first parity animals (17 new infections in front quarters and 3 new infections in hind quarters; $P < 0.01$). Of the interactions terms that were statistically significant in the model for *Staph. aureus*, interaction between parity and quarter position seemed least likely to be biologically meaningful. Addition of this interaction to the model did not change significance, or size or direction of effect for other parameter estimates (results not shown). We decided to omit the interaction between parity and quarter position from the model to facilitate biological interpretation of other interactions that were maintained in the model. Clinical signs in the quarter were significantly associated with new *Staph. aureus* infection ($P < 0.0001$), but were not included in the multivariable models because the cause-effect order of the associated variables was unknown.

As before, the number of levels was reduced for several categorical variables. For DIM there was no difference between estimates for 181 to 240 DIM, 241 to 320 DIM and more than 320 DIM, and the levels were combined into the reference level.

Table 4. Parameter estimates with standard error from multivariable Poisson regression models for prediction of new infections with *Streptococcus uberis* or *Staphylococcus aureus* during lactation. Variables or interactions were significant ($P \leq 0.10$) according to likelihood ratio test. Superscripts indicate significance of levels of categorical variables according to Wald's test.

Independent variable	StrepFULLset[a] n_{obs} = 22665[b] n_{new} = 93[b]	StaphFULLset n_{obs} = 22593 n_{new} = 100	StaphSEROset n_{obs} = 22015 n_{new} = 94	StaphSCOREset n_{obs} = 14386 n_{new} = 53
Intercept	- 5.68 ± 0.40 **[c]	- 5.27 ± 0.37 **	- 5.55 ± 0.44 **	- 5.47 ± 0.55 **
Herd				
• A	- 0.66 ± 0.53 NS	0.06 ± 0.32 NS	- 0.44 ± 0.40 NS	- 0.05 ± 0.40 NS
• B	- 0.66 ± 0.48 NS	- 0.62 ± 0.34 †	- 1.25 ± 0.34 **	- 1.04 ± 0.43 *
• C	reference	reference	reference	reference
Parity				
• first	- 0.55 ± 0.33 †	- 0.43 ± 0.27 NS	- 0.33 ± 0.29 NS	- 1.37 ± 0.44 **
• second	- 0.51 ± 0.30 †	- 0.75 ± 0.29 **	- 0.64 ± 0.30 *	- 1.57 ± 0.52 **
• third and higher	reference	reference	reference	reference
DIM				
• 1-60 days	0.52 ± 0.82 NS	- 0.60 ± 0.50 NS	- 0.46 ± 0.53 NS	- 0.12 ± 0.68 NS
• 61-120 days	- 0.52 ± 0.77 NS	- 0.04 ± 0.41 NS	0.05 ± 0.43 NS	0.79 ± 0.45 †
• 121-180 days	0.14 ± 0.51 NS	0.30 ± 0.46 NS	0.36 ± 0.48 NS	0.22 ± 0.47 NS
• > 180 days	reference	reference	reference	reference
BHV4-seropositive	n. a.[d]	n. a.	0.79 ± 0.34 *	NS
Right quarter	NS	0.38 ± 0.19 *	0.32 ± 0.19 †	NS
Quarter recovered from				
• *Strep. uberis* IMI	1.10 ± 0.38 **	0.85 ± 0.36 *	0.99 ± 0.36 **	1.22 ± 0.42 **
• *Staph. aureus* IMI	0.72 ± 0.36 *	0.88 ± 0.32 **	0.72 ± 0.35 *	NS
Preceded by IMI with				
• *A. pyogenes*	3.06 ± 1.02 **	NS	NS	n. e.
• enterococci	2.01 ± 0.39 **	NS	NS	n. e.
• corynebacteria	NS	0.26 ± 0.43 NS	0.27 ± 0.44 NS	- 2.38 ± 0.94 **
Exposure within udder to				
• *Strep. uberis*	1.44 ± 0.27 **	n. a.	n. a.	n. a.
• *Staph. aureus*	n. a.	1.53 ± 0.31 **	1.47 ± 0.32 **	1.01 ± 0.43 *
Previous SCC	NS			
• < 251,000 cells/ml		reference	reference	reference
• 251,000-500,000		0.74 ± 0.36 *	0.59 ± 0.40 NS	0.53 ± 0.50 NS
• > 500,000		1.06 ± 0.28 **	1.05 ± 0.30 **	1.12 ± 0.40 **
• missing < 21 DIM		1.36 ± 0.57 *	1.41 ± 0.61 *	n. e.
• missing > 20 DIM		- 0.24 ± 0.55 NS	- 0.14 ± 0.56 NS	n. e.

Table 4 (ctd.). Parameter estimates with standard error from multivariable Poisson regression models for prediction of new infections with *Streptococcus uberis* or *Staphylococcus aureus* during lactation. Variables or interactions were significant ($P \leq 0.10$) according to likelihood ratio test. Superscripts indicate significance of levels of categorical variables according to Wald's test.

Independent variable	StrepFULLset[a] $n_{obs} = 22665$[b] $n_{new} = 93$[b]	StaphFULLset $n_{obs} = 22593$ $n_{new} = 100$	StaphSEROset $n_{obs} = 22015$ $n_{new} = 94$	StaphSCOREset $n_{obs} = 14386$ $n_{new} = 53$
DIM * herd		NS	NS	NS
• 1-60 days, herd A	0.80 ± 0.98 [NS]			
• 61-120 days, herd A	0.15 ± 1.06 [NS]			
• 121-180 days, herd A	0.08 ± 0.80 [NS]			
• 1-60 days, herd B	0.55 ± 0.96 [NS]			
• 61-120 days, herd B	2.01 ± 0.91 **			
• 121-180 days, herd B	1.03 ± 0.65 [NS]			
Corynebacteria[e] * herd	NS			NS
• present, herd A		- 1.10 ± 0.52 *	- 1.09 ± 0.55 *	
• present, herd B		- 1.63 ± 0.66 *	- 1.47 ± 0.69 *	
Corynebacteria[e] * DIM	NS			NS
• present, 1-60 days		0.48 ± 0.82 [NS]	- 0.22 ± 1.09 [NS]	
• present, 61-120 days		1.24 ± 0.55 *	1.24 ± 0.56 *	
• present, 121-180 days		- 0.19 ± 0.71 [NS]	- 0.04 ± 0.73 [NS]	
Teat end callosity	n. a.	n. a.	n. a.	
• rough, no corynebacteriac[c]				0.25 ± 0.46 [NS]
• rough, with corynebacteria[e]				2.25 ± 0.99 *
• thickness				
• absent/thin				reference
• moderate/thick				0.24 ± 0.37 [NS]
• extreme				1.62 ± 0.44 **

a) StrepFULLset = full data set on quarters at risk of *Strep. uberis* infection; StaphFULLset = full data set on quarters at risk of *Staph. aureus* infection; StaphSEROset = subset of StaphFULLset including BHV4-serostatus; StaphSCOREset = subset of StaphFULLset including body condition score and teat end callosity scores.

b) n_{obs} = number of observations; n_{new} = number of new infections.

c) NS = not significant (superscript indicates non-significant level of categorical variable; normal script indicates non-significant variable); † $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$.

d) n. a. = not applicable; n. e. = no estimate (model did not converge).

e) Refers to presence or absence of corynebacteria at preceding sampling.

The association between pSCC level and new infection was more or less linear for *Staph. aureus* and a higher pSCC level was associated with a higher risk of new infection compared to the original reference level (<50,000 cells/ml). The differences between estimates for <50,000 cells/ml, 51,000 to 100,000 cells/ml and 101,000 to 250,000 cells/ml were not significant, however, and the three levels were combined into a new reference level. The variable was kept in the model as a categorical variable to facilitate interpretation of parameter estimates, including estimates for observations with missing values. Cow level history of *Staph. aureus* infection was not significant. As for *Strep. uberis*, the composite variable for cow and quarter level history and exposure was reduced to its significant constituent parts, i.e. quarter level history of infection and within-cow exposure.

StaphPREVset, StaphSEROset and StaphSCOREset contained 21729, 22015, and 14386 observations and 92, 94, and 53 new *Staph. aureus* infections, respectively. *Staph. aureus* prevalence was not significant as an independent variable, but BHV4-serostatus was significant in the *Staph. aureus* model. The proportion of BHV4-seropositive observations was 84.1% for herd A, 78.6% for herd B, and 13.8% for herd C. TECT and TECR were both significant as predictors of new *Staph. aureus* infection in the multivariable model (Tabel 4).

Model sensitivity was around 50% and model specificity was around 90% for all models. The Pearson Chi Square statistic per degree of freedom was 1.02, 0.96 and 0.83, respectively, for models based on StaphFULLset, StaphSEROset and StaphSCOREset.

Discussion

Herd level

Longitudinal studies with repeated measurement of IMI status are necessary to identify factors associated with a change of infection status, but they are expensive and rare. The study presented here was specifically designed to identify risk factors for new infections with *Strep. uberis* and *Staph. aureus* under field conditions. During the study, the farmers received reports on the infection status of their animals every three weeks. Using that information and elements from the five-point mastitis control plan, all farmers managed to control the incidence of *Staph. aureus* mastitis in their herds (Figure 1). Similar reduction in incidence of *Staph. aureus* mastitis has been observed by others[13, 22]. Control of *Strep. uberis,* e.g. through disinfection of milking clusters or treatment or culling of infected cows, was less strict in the study herds and an outbreak of *Strep. uberis* mastitis occurred in one herd. Management factors that may have contributed to this outbreak have been described elsewhere[37].

Infected mammary glands are considered to be the main source of *Staph. aureus*, and one of several sources of *Strep. uberis* in dairy herds[17, 22]. In previous studies on *Staph. aureus* mastitis and *Strep. uberis* mastitis, the prevalence of pathogens was a significant predictor for the number of new infections[15, 37]. In the current study, herd level prevalence of pathogens was not significant as a risk factor for new infection. This difference can most likely be attributed to the conscious preventive action taken in the herds involved in the current study, or to the limited number of herds under observation. In addition, the dynamics of infection could differ between outbreaks or epidemic situations where most of the herd is susceptible and has not been exposed to the pathogen, and steady states or endemic situations where most of the population has been in contact with the pathogen. This would be in agreement with the results described by Lam[15] and Zadoks[37] and their co-workers, where transmission differed between outbreak and post-outbreak phases of the studies. In the data presented here, herd level prevalence of *Strep. uberis* was significant as an independent variable for a subset of the data that covered the outbreak in herd B (results not shown).

Observations on cows or quarters within a herd are not independent. The within-herd correlation or dependency of measures must be considered in study design or data analysis to avoid invalid statistical inference[4, 19]. Within-herd correlation is likely to differ between herds, as management differs between herds. When a common within-herd correlation cannot be assumed, mixed models with fixed effects for herd and additional random effects for subgroups within herds, e.g. cows, are needed[20]. In our study, within-herd correlation was not the subject of interest, but rather an effect that had to be corrected for to estimate the effect of cow and quarter level variables correctly. This correction was done as suggested by McDermott et al.[20]. In addition, interactions between herd and other main effects were tested for significance to detect cow or quarter level risk factors that were significant in some herds, but not in others.

Cow level

Clustering of observations also occurred at cow level and at quarter level. When correlation within cow (spatial clustering) and correlation within quarter (temporal clustering) were both modeled, computational limitations arose. Difficulties in fitting of multilevel models with statistical software have been recognized before[4]. We chose to model within-cow correlation, because cow explained more variance than quarter when specified risk factors were not included in the model. Compound symmetry in which the covariance matrix has constant variance and constant correlation was chosen to model within-cow correlation, as had been done by others[4, 29]. Alternatives such as autoregressive or unstructured covariance matrices seemed less justified biologically. Due to the high number of observations per cow (up 108), they would accentuate differences rather than similarities between repeated observations. By treating all observations within a cow as being equally correlated, spatial clustering (quarters within cow) and temporal clustering (consecutive samples within

quarters) were treated as if they were the same. Given the limitations of the dataset, this seemed the best way to model the biology of the repeated measures. Clustering of data commonly causes overdispersion, resulting in a value higher than one for the Pearson Chi Square statistic per degree of freedom[20]. In our models, this statistic had a value close to one, indicating good model fit and suggesting appropriate model selection.

Significant independent variables at cow level included parity and DIM. Incidence of *Strep. uberis* and *Staph. aureus* IMI was lower in first and second parity animals than in third or higher parity animals. This is in line with results for incidence of clinical mastitis[3, 21] and prevalence of subclinical mastitis[6]. Increased prevalence may be the result of increased incidence or increased duration of infection. We showed that increased prevalence is at least partly due to higher incidence in multiparous cows. For *Staph. aureus*, the association between DIM and rate of infection was affected by infection with corynebacteria. Infection with corynebacteria was associated with increased risk of infection at peak lactation (61 to 120 DIM). For *Strep. uberis,* the association between DIM and rate of new infection differed with herd. Peak lactation (61 to 120 DIM) was associated with increased rate of infection in one herd. Several studies document high incidence of clinical mastitis in early stages of lactation[3, 21], but we have not found any reports on incidence of IMI (i.e. clinical as well as subclinical mastitis) in relation to stage of lactation to which we can compare our results.

Increased incidence of mastitis during early or peak lacation may be a result of negative energy balance[31]. In dairy health management, change in BCS is used as an indicator of energy balance. In our study we found no effect of BCS or change in BCS on rate of new infections with *Strep. uberis* or *Staph. aureus*. Similar results have been obtained for increase of SCC[32] and for occurrence of clinical mastitis[11]. The failure of the current and other studies to demonstrate significance of BCS as a risk factor for mastitis in multivariable analyses may be due to differences in definitions of mastitis and energy balance as used by different people, to lack of power in each study, or to inadequacy of body condition scoring as a tool to measure energy balance under field conditions.

BHV4-seroprevalence in study herds A (84% of milk samples from seropositive animals) and B (79% of milk samples from seropositive animals) was high compared to seroprevalence in Dutch cattle in general (18%; Wellenberg et al., 1999). BHV4 is a world wide distributed virus of cattle that is not associated with clearly defined clinical entities[33]. In a study on clinical mastitis, the virus has been isolated from milk that also harboured *Strep. uberis* or *E. coli*. In that study, seroconversion to BHV4 did not differ significantly between case cows and matched controls[34]. In our study, BHV4-serostatus was a significant risk factor for *Staph. aureus* infection. BHV4-positive animals had a higher rate of infection than BHV4-negative animals. The observed statistical association could be a chance effect, the result of increased susceptibility to infections in certain animals (resulting in IMI and in BHV4-infection), or the result of increased susceptibility to mastitis caused by latent BHV4-infection. In latent infections, BHV4 is predominantly situated in nervous ganglia and in mononuclear blood cells. Persistent infection of mononuclear cells with

BHV4 may reduce their phagocytic functions[33]. Theoretically, the phagocytic capacity of udder monocytes and macrophages could be reduced by BHV4-infection which could explain the increased susceptibility to IMI. However, polymorphonuclear cells are thought to be more important than mononuclear cells in the protection of the udder against *Staph. aureus* infection[24]. By contrast, mononuclear cells are considered to be more important than polymorphonuclear cells in *Strep. uberis* IMI[17] but BHV4-serostatus was not significant as a risk factor in the *Strep. uberis* model. Further study into the role of BHV4 in bovine mastitis seems warranted.

Quarter level

Quarter position has been described as a risk factor in studies on incidence of clinical mastitis and prevalence of subclinical mastitis[4, 6, 21]. IMI was found more often in rear quarters than in front quarters. In our study, front vs. rear quarter was significant in univariate analysis for *Staph. aureus*, but not in multivariable analysis. Studies by Barkema[4] and Miltenburg[21] and their coworkers only describe univariate analysis. Busato et al.[6] found more mastitis in rear quarters in late lactation, based on multivariable analysis. Because their study, though presented as a longitudinal study, is in fact a repeated cross-sectional study, it is not possible to distinguish between incidence and duration of infection as the cause of higher prevalence in rear quarters. In our study, right quarters had a higher rate of *Staph. aureus* infection than left quarters in our study. A higher prevalence of infection in right quarters was also found by Barkema et al.[4] and could be associated with lying behavior of cows[8]. For a limited number of herds as in our study, transmission of *Staph. aureus* via teat cup liners[25] may also explain a higher rate in specific quarters, e.g. right quarters.

Quarters that had recovered from *Strep. uberis* or *Staph. aureus* mastitis had a higher rate of infection than quarters that had not experienced infection before. This means that recovery from infection does not confer immunity to reinfection with the same pathogen. Recovery from infection was a risk factor for reinfection with the homologous bacterial species, but also for infection with the heterologous bacterial species. It seems that some quarters are more susceptible to infection than others, irrespective of pathogen. Our field observations were in contrast to reports of immunity to reinfection with *Strep. uberis* that were based on experiments[12]. In the experimental study, the first line of defense was circumvented, which may explain why outcomes differed from those obtained in the observational study with natural exposure of the teat end.

For *Strep. uberis*, preceding infections with enterococci were associated with an increased rate of new infection. No other minor pathogens were associated with increased rate of *Strep. uberis* infection. It is known that identification of streptococci and enterococci based on growth and biochemical characteristics is difficult[17]. Therefore, we think that enterococci and streptococci may have been misclassified in some cases. Because the attributable proportion of new *Strep. uberis* was low for infection with enterococci, and

because other parameter estimates were not affected much by addition or removal of this risk factor, we consider results from the *Strep. uberis* model to be valid despite possible occurrence of misclassification. We recommend that in studies of *Strep. uberis* mastitis all enterococcal and streptococcal isolates be tested with more sensitive and specific techniques than standard NMC-recommended methods alone.

Preceding infection with coagulase negative staphylococci (CNS) or corynebacteria was not associated with an increased or decreased rate of *Strep. uberis* infection. Hogan et al.[14] found an increased rate of infections with environmental streptococci in quarters infected with *Corynebacterium bovis* or *Staphylococcus* species. In their study, within-cow correlation and quarter level risk factors were not taken into account, and no distinction was made between *Strep. uberis* and *Strep. dysgalactiae*. Lam et al.[16] found a protective effect of corynebacteria but not CNS on subsequent infection with *Strep. uberis*. By performing a within-cow comparison of case and control quarters, cow effects were corrected for. Quarter characteristics such as pSCC or teat end condition were not accounted for in their study. For *Staph. aureus* infection, CNS infections were not significant as a risk factor in our study, in agreement with reports by other authors[16, 29]. The situation for corynebacteria is more complex. Corynebacteria were not significant as a risk factor for *Staph. aureus* IMI in late lactation animals in herd C (reference level). However, infection with corynebacteria was associated with a lower rate of *Staph. aureus* IMI in herds A and B, and with a higher rate of *Staph. aureus* IMI at peak lactation (61 to 120 days) and in rough teat ends. Schukken et al.[29] found a protective effect of corynebacteria after experimental infections. They used intracisternal challenge with *Staph. aureus*, thus surpassing the teat end. Similar results after intracisternal challenge were obtained by Brooks and Barnum[5]. When experimental challenge consisted of exposure of the teat orifice to *Staph. aureus,* no protective effect of corynebacteria was found[5]. This underscores the importance of the teat end in (non-)occurrence of new infections. Results from Lam et al.[16] are ambiguous with respect to the effect of infection with corynebacteria on subsequent infection with *Staph. aureus*. Our study on naturally occurring infections showed that the role of minor pathogens differs with minor pathogen species, and with herd, cow and quarter level factors, including teat end callosity. Together with the many different study types, challenge methods and definitions of infection that have been used, this may explain why conflicting results have emerged from numerous studies on the role of minor pathogens. Knowing that post-milking teat disinfection (PMTD) affects the prevalence of minor pathogens[16], it is interesting to note that herds A and B did not use PMTD throughout the entire study or parts of the study, as opposed to herd C. This difference in management may be associated with the difference in the significance of corynebacteria as risk factor for IMI. Unfortunately, with only three herds enrolled in this study, a role of management factors cannot be proven.

As mentioned earlier, multiple infections within a cow occur at a higher rate than would be expected based on independence of quarters and this may be due to increased susceptibility of certain cows or to within-cow transmission of pathogens[4]. In our study, exposure to other

quarters infected with *Strep. uberis* within a cow was associated with increased rate of *Strep. uberis* infection. Exposure to other quarters infected with *Staph. aureus* within a cow was associated with increased rate of *Staph. aureus* infection. Because clustering at cow level and several cow and quarter level risk factors associated with susceptibility were accounted for in the model, this suggests that within-cow transmission does occur for both pathogens.

Bulk milk SCC is decreasing in several countries, but the decline in BMSCC has not been accompanied by a decrease in incidence of clinical mastitis[21, 26]. On the contrary, studies on clinical mastitis in herds with low BMSCC have led to the suggestion that low SCC may be associated with increased risk or severity of mastitis[3, 32]. In our study on naturally occurring infections, low quarter SCC was not associated with an increased rate of subsequent infection. This is in contrast to results obtained by others. In an experimental study, low pre-challenge SCC at quarter level was associated with an increased risk of *Staph. aureus* mastitis[29]. Quarters were challenged by intracisternal infusion of *Staph. aureus*, thus eliminating the role of the first line of defense that is important in natural infections. This may explain the difference in results between the experimental study and our field study. In a case-control study on low SCC as risk factor for mastitis, IMI with clinical signs was associated with lower pre-infection SCC than IMI without clinical signs[32]. The case-control study was carried out as a field study in a low bulk milk SCC herd. The majority of clinical mastitis cases yielded no growth or growth of E. *coli* while les than 15% of clinical cases yield *Strep. uberis* or *Staph. aureus*. The disagreement between studies with respect to effect of low SCC on subsequent mastitis is probably the result of differences in study design, herd type, pathogen, and definitions of mastitis. It is a reminder that conclusion based on one pathogen in one type of study or one herd cannot automatically be generalized to other pathogens, other categories of animals at risk, or other types of herds.

Missing values for SCC were not missing at random. Therefore, they were coded as specific categories. Values were mostly missing when the preceding sample was a farmer-collected sample. Level 6 generally indicated that the preceding sample was taken at calving (data not shown). Significance of pSCC level 6 for *Staph. aureus* could be interpreted as evidence that the rate of new infection with *Staph. aureus* was high when animals were introduced to the milking parlor after calving, although early lactation (1-60 DIM) was not associated with an increased rate of infection. The difference between presence of infection at calving and onset of infection in early lactation is relevant because management strategies should be targeted at the non-lactating or the lactating period, depending on the time of occurrence of new infections. More data, or a different approach such as the use of strain typing techniques[36], would be needed to differentiate between the two scenarios.

The system that was used to classify teat end callosity was recently developed by Neijenhuis et al.[23]. The study reported here is the first to examine the role of teat end callosity as a risk factor for IMI, including subclinical mastitis. An increased rate of *Staph.*

aureus infection was observed in quarters with extreme thickness of teat end callus and in quarters with rough teat ends that were infected with corynebacteria. An effect of TECR or TECT on the rate of *Strep. uberis* infection was not found. The mechanism through which teat end callosity and *Staph. aureus* IMI are associated is unknown. Poor milking machine function could lead to teat end callosity and to increased risk of *Staph. aureus* IMI. In that case, both factors are the result of a common cause, even though teat end callosity preceded IMI in time. Alternatively, rough and extremely callused teat ends themselves could harbor *Staph. aureus*. Further research is needed to explain the difference between effects of teat end callosity on rate of *Staph. aureus* IMI and *Strep. uberis* IMI, and to unravel the biological relation between teat end callosity and presence of minor and major pathogens.

Conclusions

Rate of infection with *Strep. uberis* and *Staph. aureus* was lower in first and second parity animals than in older animals. There was no association of infection rate with body condition score, or change in body condition score, while association of lactation stage with infection rate differed with herd and with pathogen. BHV4-infection may play a role in susceptibility to mastitis, especially for *Staph. aureus*. Quarters that recovered from infection with *Strep. uberis* or *Staph. aureus* showed an increased rate of new infection with either pathogen. Quarters that were exposed to *Strep. uberis* or *Staph. aureus* within the same cow show an increased rate of new infection with the homologous pathogen. There was no effect of minor pathogens on subsequent infection with *Strep. uberis*, while the effect on rate of *Staph. aureus* infection depended on presence of minor pathogen species, herd, stage of lactation, and teat end callosity. Low quarter SCC (<50,000 cells/ml) was not associated with increased rate of IMI with *Strep. uberis* or *Staph. aureus*. Teat end roughness and extreme teat end callosity increased the rate of *Staph. aureus* mastitis but not *Strep. uberis* mastitis.

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- Chapter 6 -

Analysis of an outbreak of *Streptococcus uberis* mastitis

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Abstract

An outbreak of *Streptococcus uberis* mastitis was described to gain insight into the dynamics of *Strep. uberis* infections at a herd level. Data were obtained from a longitudinal observational study on a commercial Dutch dairy farm with good udder health management. Quarter milk samples for bacteriological culture were routinely collected at 3-week intervals from all lactating animals (n = 95 ± 5). Additional samples were collected at calving, clinical mastitis, dry-off and culling. During the 78-week observation period, 54 *Strep. uberis* infections were observed. The majority of infections occurred during a 21-week period that constituted the disease outbreak. Incidence rate was higher in quarters that had recovered from prior *Strep. uberis* infection than in quarters that had not experienced *Strep. uberis* infection before. Incidence rate of *Strep. uberis* infection did not differ between quarters that were infected with other pathogens compared to quarters that were not infected with other pathogens. The expected number of new *Strep. uberis* infections per 3-week interval was described by means of a Poisson logistic regression model. Significant predictor variables in the model were the number of existing *Strep. uberis* infections in the preceding time interval (shedders), phase of the study (early phase vs. post-outbreak phase), and prior infection status of quarters with respect to *Strep. uberis*, but not infection status with respect to other pathogens. Results suggest that contagious transmission may have played a role in this outbreak of *Strep. uberis* mastitis.

Abbreviation key: **β** = transmission parameter, the probability per unit of time that an infectious quarter will infect a non-infected quarter, **IMI** = intramammary infection, **IR** = incidence rate, **OP** = other pathogens, **PMTD** = postmilking teat disinfection, **R** = recovered from infection with *S. uberis* (irrespective of presence of other pathogens), $\mathbf{U_1}$ = uninfected (never infected with *S. uberis*, irrespective of presence of other pathogens), $\mathbf{U_2}$ = uninfected (not infected with other pathogens, irrespective of recovery from *S. uberis*).

Introduction

Streptococcus uberis is a widely occurring causative agent of mastitis in modern dairy herds. It is responsible for the majority of clinical and subclinical cases of mastitis in New Zealand[23] and the UK[14], and ranks among the most prevalent causes of mastitis in the USA[15] and the Netherlands[1]. Progress has been made in the control of *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Staphylococcus aureus* mastitis, but there has been little reduction in the incidence of *Strep. uberis* mastitis over the past 30 years[22].

Streptococcus uberis, like *Escherichia coli*, is considered to be an environmental pathogen[28]. The primary reservoir of environmental pathogens is the dairy cow's environment, and exposure of uninfected quarters to environmental pathogens can occur at any time during the life of a cow [31]. By contrast, the primary reservoir for contagious mastitis is the cow, and exposure of uninfected mammary quarters to contagious pathogens is restricted to the milking process[28, 31].

Diseases that are transmitted through individual-to-individual contact, such as contagious mastitis, can be described by a mathematical model called the Reed-Frost model. This model assumes that the probability of infection for a susceptible individual depends on the number of infectious individuals to which it is exposed[4]. For other disease agents the probability of infection depends on characteristics of the susceptible individual and its environment, as is assumed to be the case for environmental mastitis pathogens. In this situation, the probability of exposure does not depend on the number of infectious individuals. A Greenwood model describes this in mathematical terms[4]. Comparison of the output of mathematical models with observational data can be used to understand the mode of transmission of infectious agents[7].

Mathematical models can also be used to compare susceptibility of groups with specific characteristics, such as absence or presence of other pathogens or a history of prior infection[10, 20]. Minor pathogens were shown to reduce the risk of intramammary infection (IMI) with *Strep. uberis*[21]. However, other studies indicated an increased risk of infection with environmental pathogens in quarters infected with minor pathogens[17]. Recovery from *Strep. uberis* IMI conferred protection against subsequent reinfection in experimental studies[13].

The purpose of this paper is to describe the dynamics of an outbreak of *Strep. uberis* mastitis with mathematical models. Susceptibility of quarters without a history of *Strep. uberis* infection is compared to susceptibility of quarters that recovered from *Strep. uberis* infection, and susceptibility of uninfected quarters is compared to susceptibility of quarters infected with other pathogens. Finally, fit of a model that assumes the number of new infections to be a function of the number of existing infections is compared to a model that assumes the number of new infections to be independent of the number of existing infections.

Materials and Methods

Data collection

Data were obtained from a longitudinal observational study (from June 1997 to December 1998) in a commercial Dutch dairy herd. Cows were mainly Holsteins, partly cross-bred with Dutch Friesians or Meuse-Rhine-Yssel cows. In winter, animals were housed in a free stall barn with a concrete slatted floor and cubicles with wood shavings as bedding material. Lactating cows were on pasture during summer. Cows were milked twice daily in a two times four open tandem parlor. Milking hygiene included regular monitoring of milking machine function, use of individual paper towels and, at the start of the study, post milking teat disinfection. Predipping was not practiced, as it is illegal in the Netherlands. Blanket dry cow treatment was used, as had been herd practice for a number of years.
Quarter milk samples were collected every three weeks from all lactating animals ($n = 95 \pm 5$), using aseptic technique. Additional quarter milk samples were collected by the farmer at calving (prior to first contact with the milking machine), dry-off, culling and in the case of clinical mastitis (any visual abnormality of milk and/or udder, with or without systemic signs of disease). Samples were stored at -20°C until processing. Within three weeks of collection, 0.01 ml of milk was cultured and bacterial species were identified according to National Mastitis Council standards[12]. Colony counts were recorded for each bacterial species. Up to 10 colony forming units (cfu) per plate all colonies were counted, while higher counts were categorized as 11-50 cfu/plate, 51-200 cfu/plate, or >200 cfu/plate. Preliminary identification of *Strep. uberis* was based on colony morphology and aesculin hydrolysis on Edward's medium. Cultures were confirmed as *Strep. uberis* using the API 20 Strep system[19]. Shedding levels of *Strep. uberis* during the pre-outbreak and outbreak part of the observation period (early phase) were compared to shedding levels during the post-outbreak period (late phase) by means of a Chi-square test (Statistix for Windows, Version 2.0, Analytical Software Co., La Jolla, CA, USA, 1998).

Definition of infection and reinfection

A quarter was considered to have an IMI when ≥ 1000 cfu/ml of a pathogen (major or minor) were cultured from a single sample, when ≥ 500 cfu/ml of a pathogen were cultured from two out of three consecutive milk samples, when ≥ 100 cfu/ml were cultured from three consecutive milk samples, or when ≥ 100 cfu/ml were cultured from a clinical sample. Samples containing more than three bacterial species were considered contaminated, and were not informative of IMI status. A previously infected quarter was considered recovered from IMI for a species if none of the above definitions were met and the sample was free of the pathogen[1, 30] with slight modifications).

Based on IMI status, quarters were classified as belonging to one of six compartments in a compartmental model (Figure 1). Categorization was repeated each time samples were obtained. Quarters that were not infected with any pathogen and that had not been infected with *Strep. uberis* at any stage during the study were classified as "uninfected". Quarters infected with *Strep. uberis* only were called "infected". Quarters that were not currently infected with any pathogen but had been infected with *Strep. uberis* earlier in the study were considered "recovered-uninfected". Quarters infected with any pathogen other than *Strep. uberis* were called an "other pathogen" infection if infection with *Strep. uberis* had not been observed at any point during the study. Quarters with mixed infection of *Strep. uberis* and other intramammary pathogens were termed "infected, including other pathogens". Quarters that were infected with pathogens other than *Strep. uberis*, but had recovered from *Strep. uberis* IMI, were considered "recovered and infected with other pathogens". The upper level of Figure 1 represents quarters that were never infected with *Strep. uberis*, the middle level represents quarters that are infected with *Strep. uberis,* and the lower level represents quarters that recovered from infection with *Strep. uberis*. The left side of Figure 1 represents quarters that are not infected with other pathogens, while the right side of the diagram shows quarters that are infected with other pathogens. It is impossible to return to the upper level of the diagram from either the lower or the middle level.

Quarter-days at risk in a susceptible compartment (any compartment that represents quarters that are not infected with *Strep. uberis*) or quarter-days infected with *Strep. uberis* were calculated based on starting point and endpoint of uninfected and infected periods. For IMI that were first detected at calving or as clinical mastitis, sample date was assumed to be the date of onset. For IMI that were last detected at dry-off or at culling, sample date was taken as endpoint of infection. For all other combinations, e.g. IMI starting during lactation or ending between a clinical sample and the consecutive routine survey sample, the midpoint of the last negative and first positive sample was taken as starting point, and the midpoint between last positive and first negative sample was taken as endpoint of IMI. The terms "positive" and "negative" sample refer to IMI status of the sample.

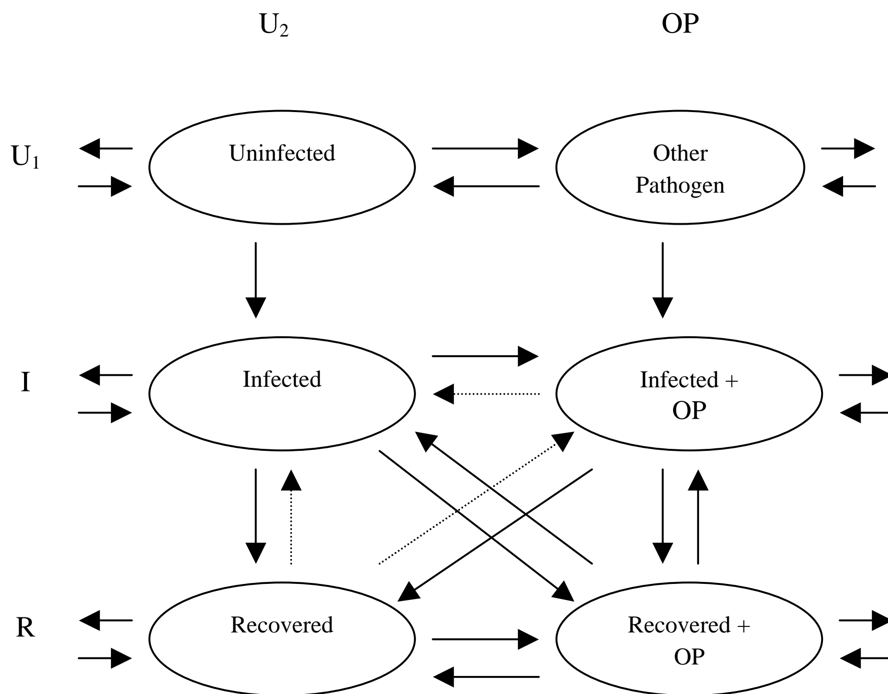
Comparison of incidence rates

Incidence rates (IR) were calculated as number of new IMI per quarter-day at risk[1, 17]. When no IMI were observed in a group of quarters, the highest incidence rate that is compatible with this observation was calculated as[11]:

$$1 - \text{maximum risk} = \alpha^{1/n} \qquad [1]$$

with α = probability of type 1 error
n = number of observations

Figure 1. Six compartment model describing *Strep. uberis* dynamics in a dairy herd. “Infected” represents infection with *Strep. uberis.* “Other pathogen” represents infection with any other intramammary pathogen. U_1 denotes the compartments that were never infected with *Strep. uberis* (Uninfected, Other pathogen) and is contrasted to R, which denotes the compartments that have recovered from infection with *Strep. uberis* (Recovered-Uninfected, Recovered + Other pathogen). U_2 denotes the compartments that are not infected with *Strep. uberis* or any other pathogen (Uninfected, Recovered-Uninfected) and is contrasted to OP, which denotes the compartments that are infected with other pathogens, but not with *Strep. uberis* (Other pathogen, Recovered + Other pathogen). I denotes the compartments that are infected with *Strep. uberis* with or without other pathogens (Infected, Infected + Other pathogens). Arrows indicate possible transitions between compartments. Dotted arrows indicate transitions with zero observations during an 18-month observation period in a 95-cow herd.



To compare all quarters never infected with *Strep. uberis* to all recovered quarters, incidence rate in compartments denoted as U_1 (top row in Figure 1), was contrasted to incidence rate in compartments denoted as R (bottom row in Figure 1). To compare all quarters infected with other pathogens to all quarters without other pathogens, incidence rate in compartments denoted as OP (right hand column in Figure 1) was contrasted to incidence rate in compartments denoted as U_2 (left hand column in Figure 1).

Incidence rates were compared between compartments as described by Greenland and Rothman[9], using a two-sided test. This statistical analysis of incidence rates assumes an underlying Poisson probability model. To assure validity of the Poisson model, distribution of incidence data (number of new IMI) was examined with BestFit (BestFit for Windows, Version 2.0d, Palisade Corporation, Newfields, NY, USA, 1993). When the distribution of number of new IMI was overdispersed, statistics for significance testing were deflated by the square root of the overdispersion factor[5].

Regression analysis

Probability of transmission of a disease can be expressed in transmission parameter β, the probability per unit of time that an infectious quarter will infect a non-infected quarter. Values for β can be estimated from De Jong's modification of a model originally described by Becker[7]:

$$\text{number of new } IMI_i \text{ per time interval} = \beta_i * (I/N) * S_i \qquad [2]$$

with
- IMI_i = intramammary infection in susceptible quarter from compartment i
- I = number of infected quarters
- S_i = number of susceptible quarters in compartment i
- N = total number of quarters

Because incidence data (number of new IMI) are count data, a Poisson regression model was used for analysis[5]. Values for β_i were estimated using a modification of the generalized linear model with log-link and Poisson error described for *Staph. aureus* by Lam et al.[20]:

$$\varepsilon\,[\ln(IMI)] = \ln(\beta') + \ln(S/N) + \theta_1 * \ln(I) + \theta_2 * y + \theta_3 * U_m + \theta_4 * y * U_m \qquad [3]$$

with
- ε = expected value
- IMI = number of new infections in susceptible compartent
- β' = transmission parameter for model with ln(S/N) as offset
- S = size of susceptible compartment expressed in quarterdays at risk
- N = size of total population expressed in quarterdays lactating
- θ_i = regression parameters
- I = exposure expressed in quarterdays infected in preceding time interval
- y = dummy for phase (y = 0 for early phase, y = 1 for late phase)
- U_m = dummy for compartment U_1 vs. R (m = 1) or U_2 vs. OP (m = 2)
- $\theta_4 * y * U_m$ = interaction term for compartment and phase

At first, ln(S/N) was used as model offset. If ln(I) was found to be a significant predictor variable, ln(S*I/N) was used as model offset, replacing ln(S/N) + θ_1 * ln(I), and ln(β') was replaced by ln(β_i) in model [3]. In this model, infected quarters were assumed to be infectious, as the definition of infection was based on shedding of the bacteria of interest. The mastitis outbreak reported by Lam et al.[20] was best described by a model that stratified the analysis by start ("outbreak") versus remainder ("steady state") of the observation period. Therefore, a variable for phase of study was included in model [3]. To determine the starting point of the late phase for the *Strep. uberis* outbreak described here, models were fitted with each possible sampling interval as start of the post-outbreak period. Models with lowest deviance were considered the best models.

The model was run to compare compartments U_1 to R and U_2 to OP, respectively. Compartment sizes were expressed in number of quarter-days at risk. Significance of predictor variables in the regression analysis was declared at $\alpha = 0.01$. A conservative level was chosen because correlation between repeated observations (quarters within cow) could not be corrected for. Analysis was performed using statistical software (Statistix, 1998).

Results

Descriptive results

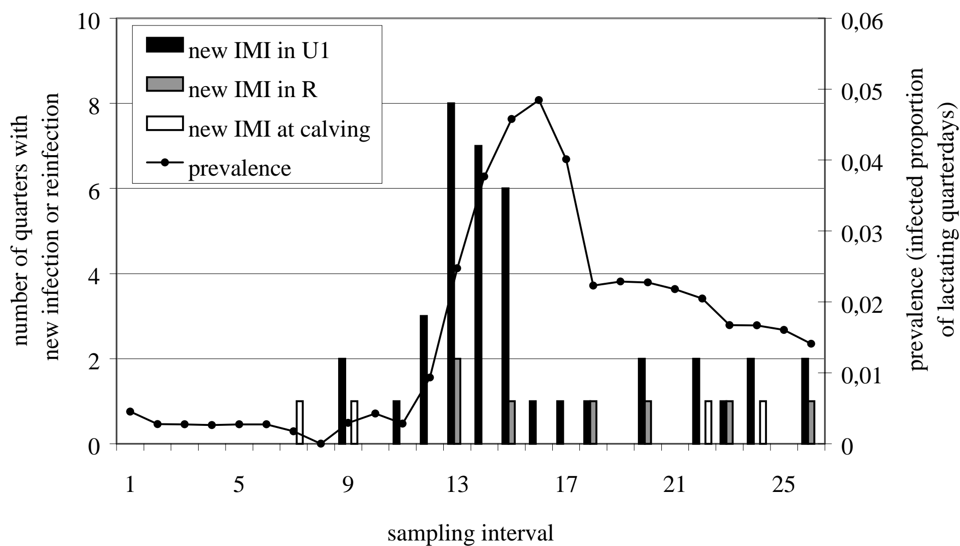
A total of 11,932 quarter milk samples were obtained, out of which 686 were collected at calving, 263 from cows with clinical mastitis, 526 at dry-off, 146 at culling, and 91 as extra samples prior to treatment of subclinical IMI with antibiotics. Samples were missing for eight cows at calving, and for 17 cows at culling. Cows with a blind quarter did occur (n=10). Eighty-eight samples were excluded from the analysis because of contamination (0.2% of all samples).

During the 18-month observation period, 39 new *Strep. uberis* infections and seven reinfections were observed in lactating animals, and 4 infections were observed in quarters at calving (Figure 2, Appendix 1). The majority of cases occurred in a limited time interval, covering sampling intervals 9 through 15 (from November 1997 to April 1998). This 21-week period is called the outbreak period. Six weeks after the outbreak had ended, at sampling 18, most infected animals (10 out of 14) were separated from uninfected animals, and infected animals were treated with antibiotics. Treated animals were added to the main herd again at sampling number 20, irrespective of bacteriological cure.

Infections with *Strep. uberis* were observed in uninfected quarters, in quarters infected with other pathogens and in recovered quarters infected with other pathogens (seven reinfections in six quarters from five cows), but not in recovered-uninfected quarters (Appendix 1). With 1315 recovered-uninfected quarter-days at risk, the 99% confidence interval for the maximum risk of infection per recovered-uninfected quarter-day at risk

(maximum incidence rate) is $0 \leq IR \leq 0.0035$. Incidence rates for compartments U_1, R, U_2 and OP are shown in Table 1.

Figure 2. Prevalence (infected proportion of lactating quarter-days, right vertical axis) and incidence (number of new infections and reinfections, left vertical axis) of *Strep. uberis* IMI per 3-week sampling interval in a 95-cow herd, showing new infections at quarter level in compartments U_1, R and at calving. U_1 = never infected with *Strep. uberis*, with or without presence of other pathogens; R = recovered from prior *Strep. uberis* infection, with or without presence of other pathogens.



Infected quarters that were present at the start of the study (n = 4) did not contribute to incidence, but did contribute to prevalence of infection. Prevalence of infection, expressed in number of infected quarter-days (with or without other pathogens) as proportion of the total number of lactating quarter-days per sampling interval, is included in Figure 2.

Shedding levels did not differ between early (interval 1-15) and late phase (interval 16-26) of the observation period (shedding levels ≤10, 11-50, 51-200 and >200 cfu/ml; $\chi^2 = 2.72$, $P = 0.44$).

Table 1. Probability distribution of number of new *Streptococcus uberis* IMI within susceptible compartments per 3-week interval, based on 26 observed intervals. U_1 = never infected with *Strep. uberis*, with or without other pathogens at sampling prior to detection of *Strep. uberis* IMI; R = recovered from infection with *Strep. uberis*, with or without other pathogens at sampling prior to detection of *Strep. uberis* IMI; U_2 = not infected with *Strep. uberis* or other pathogens at sampling prior to detection of *Strep. uberis* IMI, irrespective of recovery history; OP = infected with other pathogens at sampling prior to detection of *Strep. uberis* IMI, irrespective of recovery history.

Compartment	Distribution	Mean	Variance	Overdispersion factor
U_1	NegBin[a] (1.00,0.40)	1.50	3.75	2.5
R	NegBin(5.00,0.95)	0.27	0.28	1.1
	Poisson(0.27)	0.27	0.27	
U_2	NegBin(1.00,0.68)	0.46	0.67	1.5
	Poisson(0.46)	0.46	0.46	
OP	NegBin(1.00,0.43)	1.31	3.00	2.3

[a)] NegBin = negative binomial distribution with shape parameters

Other pathogens

Infections with other pathogens were predominantly caused by corynebacteria (81.9% of IMI with other pathogens) and coagulase negative staphylococci (26.0%). Other minor pathogen infections (Enterococci, *Bacillus spp.*, non-*dysgalactiae* and non-*uberis* Streptococci, Micrococci) and major pathogen infections (*Streptococcus dysgalactiae*, *Escherichia coli*, *Staphylococcus aureus*, *Arcanobacterium pyogenes*) accounted for 1.7% and 3.1% of other pathogen infected samples, respectively. Total of percentages adds up to more than 100% because mixed infections occurred in 13.1% of samples from quarters infected with other pathogens. *Streptococcus agalactiae* was never isolated.

Comparison of incidence rates

Incidence data (number of new IMI in each time interval) for compartments U_1 and OP were best described by a Negative Binomial distribution or a Geometric distribution. Incidence data for compartments R and U_2 were adequately described by a Negative Binomial, Geometric or Poisson distribution. Distribution parameters and overdispersion factors are shown in Table 1. Overdispersion factors for U_1 and OP were used to deflate statistics for significance testing.

Incidence rate (number of new IMI per quarter-day at risk) in quarters that had recovered from *Strep. uberis* infection was 7.5 times as high as incidence rate in quarters

that had never experienced *Strep. uberis* infection (*P* < 0.001, Table 2). Incidence rate in quarters infected with other pathogens was 1.3 times as high as incidence rate in quarters that were not infected with other pathogens. The difference was not significant (*P* > 0.2, Table 2).

Table 2. Number and rate of *Streptococcus uberis* IMI per susceptible compartment of origin. U_1 = never infected with *Strep. uberis*, with or without other pathogens at sampling prior to detection of *Strep. uberis* IMI; R = recovered from infection with *Strep. uberis*, with or without other pathogens at sampling prior to detection of *Strep. uberis* IMI; U_2 = not infected with *Strep. uberis* or other pathogens at sampling prior to detection of *Strep. uberis* IMI, irrespective of recovery history; OP = infected with other pathogens at sampling prior to detection of *Strep. uberis* IMI, irrespective of recovery history.

Susceptible compartment	Number of new infections[a]	Rate of new infections[b]
U_1	39	0.20
R	7	1.50
U_2	12	0.19
OP	34	0.25
non-lactating	4	-[c]

a) Total number of new infections detected during an 18-month observation period

b) (Number of new *Streptococcus uberis* infections : susceptible quarterdays at risk) * 1000

c) Three out of four infections in non-lactating animals were detected in heifers at calving. Number of quarter-days at risk for heifers was unknown. Hence, infection rate was not calculated. Infections in non-lactating animals were not included in any of the mathematical models described in this paper.

Regression analysis

Model deviance was calculated with a range of starting points for the post-outbreak period in model [3]. Model fit was best when the late phase started at sampling interval 16, both when contrasting U_1 to R and when contrasting U_2 to OP.

For comparison of compartments U_1 and R, phase of study was a significant predictor variable. Other significant predictor variables were compartment of origin and number of quarter-days infected in the preceding 3-week interval (Table 3). The interaction between compartment and phase of study was not significant, and was omitted from the model. Although raw data were overdispersed, modeled data could be described by a Poisson distribution (deviance 61.46, *P* = 0.06, df = 46).

Table 3. Estimates, standard errors and *P*-values for ln(β') and regression coefficients of explanatory variables in Poisson logistic regression model ε [ln(IMI)] = ln(β') + ln (S/N) + θ_1 * ln(I) + θ_2 * y + θ_3 * U_m where ε = expected value, IMI = number of new intramammary infections with *Streptococcus uberis* in current time interval, β' = transmission parameter for model with ln(S/N) as offset, S = number of quarter-days susceptible in current time interval, N = total number of quarter-days in current time interval, I = number of quarter-days infected in preceding time interval, y = dummy variable for phase (y = 0 for early phase, y = 1 for late phase), U_m = dummy variable for compartment (U_m = 0 for R or OP, U_m = 1 for U_1 or U_2) and θ_i = regression coefficient

Model	Parameter[a]	Estimate	Standard error	*P*-value
U_1 vs. R[b]	ln(β')	0.11	0.78	0.8793
	ln(I)	0.68	0.15	< 0.0001
	study phase	-1.55	0.35	< 0.0001
	compartment	-2.06	0.42	< 0.0001
U_2 vs. OP[c]	ln(β')	-1.84	0.67	0.0067
	ln(I)	0.71	0.15	< 0.0001
	study phase	-1.47	0.35	< 0.0001
	compartment	-0.36	0.34	0.2846

a) For ln(β') the estimated value is shown, for other parameters estimated values of the regression coefficients are shown. No value is given for ln(S/N) because this was used as the model offset.

b) U_1 = never infected with *Strep. uberis*, with or without other pathogens at sampling prior to detection of *Strep. uberis* IMI; R = recovered from infection with *Strep. uberis*, with or without other pathogens at sampling prior to detection of *Strep. uberis* IMI.

c) U_2 = not infected with *Strep. uberis* or other pathogens at sampling prior to detection of *Strep. uberis* IMI, irrespective of recovery history; OP = infected with other pathogens at sampling prior to detection of *Strep. uberis* IMI, irrespective of recovery history.

For comparison of U_2 and OP, phase of study and number of quarter-days infected during the preceding 3-week interval were significant predictor variables (Table 3). The transmission parameter estimate for compartment U_2 was not significantly different from the transmission parameter estimate for compartment OP. Again, the interaction between compartment and phase of study was not significant, and was omitted from the model. Modeled data were slightly overdispersed (deviance 68.93, $P = 0.02$, df = 46).

Estimates for transmission parameters, based on model [3] with ln(S*I/N) as offset, are shown in Table 4.

Table 4. Estimates of transmission parameters β (probability per unit of time that an infectious quarter will infect a non-infected quarter; based on linear regression model with ln(S*I/N) as offset) for uninfected quarters without infection history (U_1), for quarters recovered from *Streptococcus uberis* IMI (R), for quarters without other pathogens (U_2) and for quarters with other pathogens (OP) during early (intervals 1-15) and late phase (intervals 16-26) of the study. Within a model, values with different superscript are different ($P < 0.001$).

Model	Compartment	$\beta_{early\ phase}$	$\beta_{late\ phase}$
U_1 vs. R	U_1	0.033^a	0.005^b
	R	0.246^c	0.040^d
U_2 vs. OP	U_2	0.029^e	0.005^f
	OP	0.041^e	0.007^f

Discussion

In this paper we describe an outbreak of *Strep. uberis* mastitis. Susceptibility of groups or compartments of quarters at risk is compared with the use of mathematical models. In addition, the hypothesis is tested that a model that includes number of infected quarters as predictor variable (Reed-Frost model) describes the outbreak better than a model that does not include this factor (Greenwood model).

Quarters of a cow were treated as independent units. Exposure to infected quarters within the same cow can be considered of minor importance, because proportions of new IMI resulting from cross-infections between quarters of one cow are small compared to new IMI resulting from transmission between cows[3, 23]. Quarters within a cow are clustered with respect to exposure to sources outside the cow itself, and with respect to susceptibility to infection. Barkema *et al.*[2] provide pathogen specific estimates for correlation of quarters within cows, and give examples of how to correct for this correlation in clinical trials and cross-sectional prevalence studies. This correction does not apply to regression analysis of incidence data in longitudinal studies. As an alternative to analysis of data at quarter level, analysis at cow level has been considered. This alternative would not allow for classification of cows as belonging in one compartment only, because infection status may differ per quarter. Therefore, we preferred analysis at quarter level.

Number of new IMI with *Strep. uberis* varied over time, with zero cases during the first months of the study period, and high numbers during an outbreak that lasted 21 weeks. Although no IMI with *Strep. uberis* were observed in recovered quarters that were not infected with other pathogens, the 99% confidence interval for incidence rate in this group covers the observed incidence rates for compartments U_1, R, U_2 and OP. Hence, it cannot

be concluded that recovered uninfected quarters are fully resistant to reinfection. Incidence rates of *Strep. uberis* IMI in our study (number of new IMI per quarter-day at risk) were in the same range as incidence rates reported by Todhunter et al.[32] and Hogan et al.[17] for environmental streptococci, and by Barkema et al.[1] for clinical *Strep. uberis* mastitis.

Based on comparison of incidence rates, and on transmission parameters for transmission to uninfected (U_1) and recovered quarters (R), it is concluded that susceptibility of quarters that recovered from *Strep. uberis* infection is higher than susceptibility of quarters that never experienced *Strep. uberis* infection. Hill[13] described reduced susceptibility to infection after previous exposure to *Strep. uberis* in an experimental situation. The same strain of *Strep. uberis* was used for primary and subsequent infections. Finch et al.[8] demonstrated that vaccination was less effective against strains other than the immunizing strain. The difference between Hill's findings and our study may be a result of differences in bacterial strains, in cow susceptibility or in study type. Use of quarter-days at risk as denominator in incidence calculations does not take into account that repeated observations within a quarter over time are correlated. Some cows or quarters may be more susceptible to IMI than others. This effect can be more pronounced in compartment R than in compartment U_1, because the number of quarter-days at risk in compartment R is smaller. However, use of person-time at risk is routine practice in epidemiology[9] and comparison of incidence rates for mastitis pathogens is usually based on time at risk expressed in cow-days or quarter-days[17, 20, 32]. Despite its shortcomings, we therefore consider the calculation of incidence rates justified. Poor sensitivity of bacterial culture is an issue when dealing with detection of *Stap. aureus*. For isolation of *Strep. uberis* poor culture sensitivity is hardly ever reported as a problem. Because of that, and because all quarters yielded at least two *Strep.uberis* negative samples before reinfection was observed, it is unlikely that apparent reinfections were a result of persistent infections that went undetected in the time period between *Strep. uberis* isolations.

The majority of infections in the study herd that were not caused by *Strep. uberis* were attributable to corynebacteria and coagulase negative staphylococci. Those species are commonly grouped under the collective denominator "minor pathogens"[28]. In our analysis, minor pathogens and the small proportion of major pathogens other than *Strep. uberis* were all treated as one group. When comparing quarters that were not infected with other pathogens (U_2) to quarters infected with other pathogens (OP), no difference in incidence rate or transmission parameters was detected *for Strep. uberis*. Lam[21] found the same result for quarters infected with *Staphylococcus* species, but observed a decreased risk of *Strep. uberis* IMI in quarters infected with *Corynebacterium bovis*. Hogan et al.[17] found the rate of environmental streptococcal mastitis to be significantly higher in quarters infected with *Corynebacterium bovis* or *Staph. spp.* compared to uninfected quarters. An explanation for the discrepancy between results obtained from different studies could be the fact that Lam used a matched case-control analysis, comparing quarters within cows to correct for possible confounding by cow effect. Results presented here and results obtained by Hogan et al.[17] are based on observational studies without correction for cow or quarter effects.

Phase of study is a significant predictor variable for the number of new IMI with *Strep. uberis*, with lower transmission parameters during the late phase of the observation period. Observational data are best described by the model when a dummy for post-outbreak period is included, starting with interval 16 as the first post-outbreak interval. A similar result has been found by Lam et al.[20] for an outbreak of *Staph. aureus* mastitis. Significance of study phase can be interpreted as a change in transmission parameter β at the end of the disease outbreak. This change may be due to a change in infectiousness of infected quarters or a change in susceptibility of exposed individuals[10]. Theoretically, a change in infectiousness could be caused by a change in shedding levels, or a change in pathogen population at the subspecies level. In our study, shedding levels did not differ between early and late phase of the observation period. Lam et al.[20] postulated that a change in susceptibility of exposed individuals may be the result of immunity developed after short duration IMI. In their study, short duration IMI could go unnoticed as a result of the sampling scheme. In the present study, sampling was more frequent and samples that contained >1000 cfu/ml only once were considered to be infected to improve detection of short duration IMI. Still, short duration IMI that occurred between samplings may have gone unnoticed. Development of immunity in response to short duration IMI would be in disagreement with the increased susceptibility in recovered quarters observed in this study, unless short duration IMI and long term IMI have different pathogenesis and cure mechanisms.

A change in transmission rates could also be the result of a change in herd contact structure, or a change in probability of transmission upon contact[10]. Segregation of animals is an example of a management strategy that reduces contact between individuals. Post milking teat disinfection (PMTD) is an example of a control procedure that reduces the probability of infection after contact. In the study herd, segregation of animals was used temporarily. Most *Strep. uberis* infected cows were housed separately and milked last for six weeks (10 out of 14 animals). Separation of animals violates the assumption of random mixing underlying the Reed-Frost and Greenwood model. However, this change in management was temporary, and took place several weeks after the end of the outbreak period. Hence, it does not explain or affect a change in transmission at the end of the outbreak itself. PMTD was used during intervals 1 to 6 and 16 to 19, but discontinued during intervals 7 to 15 and 20 to 26 (exact dates unknown). When added to model [3], PMTD was a significant predictor variable ($P < 0.01$) and did not affect significance or direction of effect of other predictor variables. Use of PMTD reduced transmission parameter β. PMTD may have contributed to the end of the outbreak.

In all models for calculation of transmission parameters, the number of infected quarter-days in the preceding 3-week interval was significant, with higher exposure to shedders predicting higher numbers of new IMI. This implies that the Reed-Frost assumption is more appropriate than the Greenwood assumption, and suggests that *Strep. uberis* can behave as contagious pathogen. Though this may seem contrary to the prevailing classification of *Strep. uberis* as an environmental pathogen, several authors include the option of contagious transmission when describing environmental streptococci[22, 24].

The characteristic setting environmental pathogens apart from contagious pathogens is that in addition to the possibility of contagious transmission through exposure during milking time, there can be non-contagious transmission at other times and through exposure to other sources than the milking process. The present study and other studies provide several arguments in favor of contagious transmission, in addition to environmental transmission. Arguments in favor of contagious transmission from this study include the significance of infection prevalence as predictor for the number of new IMI, and the decrease in predicted number of new IMI during periods that PMTD was used. PMTD is thought to kill bacteria that are transmitted during the milking process, e.g. via teat cup liners, and thus reduce the incidence of IMI. For *Staph. aureus*, transmission via contaminated teat cup liners has been described[25]. Using liner swabs, we detected *Strep. uberis* in teat cup liners after milking of *Strep. uberis* shedding cows, and after milking of up to two non-shedding cows following a shedding cow (data not shown). This indicates that transmission of *Strep. uberis* via teat cup liners is possible, which may explain part of the effect of PMTD. An outbreak of *Strep. uberis* mastitis described by Cattell[6] also illustrates the role of infected quarters as source of infection.

Other observations in our study are in agreement with an environmental mode of transmission. Four *Strep. uberis* infections were observed in non-lactating animals, out of which three were heifers. Heifer mastitis is a well-known phenomenon[26]. Because heifers have not been exposed to the milking machine, contagious transmission during the milking process cannot explain infections in preparturient mammary glands. One possible source of exposure to mastitis pathogens in the environment is bedding material. Cows in this study were housed in a free stall with wood shavings for bedding. In wood-based materials gram-negative bacteria tend to predominate over gram-positive bacteria[29], but numbers of *Strep. uberis* in bedding can increase with increased organic contamination[16]. Bedding management may have played a role in this outbreak of *Strep. uberis* mastitis, but attempts to culture *Strep. uberis* from wood shavings were unsuccessful (results not shown) and the role of bedding material cannot be proven.

Middle ground between the so-called contagious and environmental modes of transmission exists. The fact that a model including the number of infected individuals describes incidence data better than a model without the number of existing IMI does not prove a causative role of infected individuals, nor a specific mechanism of transmission. This is an inherent limitation of mathematical models[7]. It is possible that the number of infected quarters merely reflects unobserved changes in environmental conditions, or exposure to an increasing and then decreasing environmental *Strep. uberis* load. It is also possible that infected quarters themselves are indeed the main source of exposure, but that transmission takes place via the environment, e.g. when infected cows contaminate stalls through milk leaking. Another transmission mechanism could be via flies, as shown for *Staph. aureus* by Owens *et al.*[27]. If cows are the major source of exposure, but transmission is not via the milking process, the dynamics of *Strep. uberis* infections at herd level can not be classified in the traditional "contagious" vs. "environmental" dichotomy.

In principle, strain typing of *Strep. uberis* isolates could support or contradict the notion of contagiousness. Thus, mathematical and molecular techniques could supplement each other in the approach of an epidemiological problem. Many techniques have been used for typing of strains within the species *Strep. uberis*[18, 22] and a wide variety of strains has been shown to cause intramammary infections. In the case of contagious transmission, all IMI that are part of an outbreak should be attributable to a limited number of strains. Not all isolates from this outbreak were available for strain typing. However, if strain typing in future outbreaks would show that isolates belong predominantly to one or a few strains, we can agree with Cattell's suggestion[6] that *Strep. uberis*, like *Strep. dysgalactiae*[31], can be characterized as intermediate between contagious and environmental.

Conclusion

Udder quarters that recovered from prior *Strep. uberis* IMI had a higher incidence rate of Strep. uberis IMI than quarters that had not experienced *Strep. uberis* IMI before. Incidence rate of *Strep. uberis* IMI did not differ between quarters that were infected with other pathogens and quarters that were not infected with other pathogens. A prediction model for number of new *Strep. uberis* infections that included the number of existing infections fit the observed data better than a model that did not include exposure to existing infections. Contagious spread of the pathogen may have played a role in this outbreak of *Strep. uberis* mastitis.

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Appendix 1. Data used for analysis in this paper. For each 3-week sampling interval the number of quarter-days at risk or infected is shown for each compartment, as well as the number of new *Streptococcus uberis* infections observed in quarters originating from each of the four susceptible compartments is shown. U = uinfected; I = infected with *S. uberis*, OP = infected with other pathogens; R = recovered from *S. uberis*.

Interval	Quarter-days at risk or infected with *Strep. uberis* per compartment							new *Strep. uberis* infections per susceptible compartment				
	U	OP	I	I + OP	R	R + OP	Total	U	OP	R	R + OP	Total
1	3103.5	4804.5	34.5	1.5	1.5	10.5	7956	0	0	0	0	0
2	2440.0	5110.0	21.0	0.0	0.0	21.0	7592	0	0	0	0	0
3	2376.5	5229.5	21.0	0.0	0.0	21.0	7648	0	0	0	0	0
4	2524.0	5412.0	21.0	0.0	0.0	21.0	7978	0	0	0	0	0
5	2565.5	5044.5	21.0	0.0	9.0	30.0	7670	0	0	0	0	0
6	2563.0	5069.0	21.0	0.0	31.5	31.5	7716	0	0	0	0	0
7	2528.5	4859.5	6.0	7.0	49.0	6.0	7456	0	0	0	0	0
8	2750.5	4570.5	0.0	0.0	63.0	0.0	7384	0	0	0	0	0
9	2661.5	4577.5	0.5	21.0	63.0	20.5	7344	0	2	0	0	2
10	2883.5	4333.5	0.0	31.5	84.0	52.5	7385	0	0	0	0	0
11	3027.5	4224.5	10.5	10.5	93.0	54.0	7420	0	1	0	0	1
12	3142.0	4517.5	52.5	21.0	70.5	76.5	7880	1	2	0	0	3
13	3277.5	4976.5	158.5	54.0	42.0	89.5	8598	2	6	0	2	10
14	3099.0	4777.0	239.5	74.0	67.5	63.0	8320	3	4	0	0	7
15	2661.0	4905.0	284.0	87.5	94.5	84.0	8116	2	4	0	1	7
16	2609.0	5067.5	327.5	73.5	66.0	136.5	8280	0	1	0	0	1
17	2467.5	5333.0	239.5	97.5	72.5	194.0	8404	0	1	0	0	1
18	2172.0	5680.5	138.5	50.5	149.0	281.5	8472	0	1	0	1	2
19	1886.0	6000.0	155.0	39.5	68.5	352.0	8501	0	0	0	0	0
20	1719.0	5713.0	127.0	52.5	11.5	277.0	7900	1	1	0	1	3
21	1737.5	5951.5	104.5	73.5	24.5	272.5	8164	0	0	0	0	0
22	1868.5	6048.5	111.5	60.5	42.0	277.0	8408	0	2	0	0	2
23	1790.5	5947.0	94.5	42.0	10.5	283.5	8168	0	1	0	1	2
24	1833.0	5528.0	60.5	69.0	28.5	237.0	7756	2	0	0	0	2
25	2307.0	5523.0	59.5	73.5	83.5	241.5	8288	0	0	0	0	0
26	2504.5	5194.5	51.5	63.0	90.0	232.5	8136	1	1	0	1	3
Total	64498.0	134397.5	2360.5	1003.0	1315.0	3366.0	206940	12	27	0	7	46

- Chapter 7 -

Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds

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Submitted

Abstract

A longitudinal observational study was carried out in two Dutch dairy herds to determine clinical, epidemiological and molecular characteristics of intramammary *S. uberis* infections in dairy cattle. Intramammary infections (n = 84) were detected in 70 quarters of 46 cows. The majority of infections were subclinical. Chronic infections and infections with non-elevated somatic cell count were common. A selection of isolates was RAPD-fingerprinted. In each herd, multiple strains were identified but one RAPD-type predominated. Persistent infections within a quarter were usually caused by one strain, while recurrent infections could be caused by the same or different strains. When multiple quarters of a cow were infected, infections were mostly caused by the same strain. Associations between strains and disease characteristics in vivo are discussed. Epidemiological and molecular data support within-cow and between-cow transmission of *S. uberis* mastitis, with possible transfer of bacteria via the milking machine, as well as infection from environmental sources.

Abbreviation key: IMI = intramammary infection, **RAPD** = random amplified polymorphic DNA, **QMSCC** = quarter milk somatic cell count.

Introduction

Streptococcus uberis is an important udder pathogen in the modern dairy industry. It ranks among the main causes of mastitis in countries around the world, including Australia[28], Brazil[6], Canada[31], The Netherlands[1, 29], New Zealand[8], the United Kingdom[20], and the United States[14]. The bacterium is a major barrier to the control of bovine mastitis[4, 20], which is partly due to the fact that the epidemiology of the disease is incompletely understood[27, 28].

Streptococcus uberis is usually classified as "environmental pathogen"[20, 32]. The primary reservoir of environmental pathogens is the dairy cow's environment, and exposure is not limited to the milking process[32]. Sources of *S. uberis* in the environment of the bovine udder include body sites, manure, pasture, and bedding material[3, 7, 11]. New infections with *S. uberis* may occur in dry cows and in heifers before calving[24, 30, 34]. Dry cows and preparturient heifers are not milked, and infections in those animals cannot be the result of cow-to-cow transmission in the milking parlor. Therefore, the environment is most likely the source of infection. In recent years, molecular techniques have contributed evidence for the environmental origin of *S. uberis.* Many strains of *S. uberis* can cause bovine mastitis, implying that mastitis does not result from contagious transmission of a limited number of strains within a herd[8, 20, 28, 36].

The emphasis on the environmental nature of *S. uberis* is a result of the failure to eradicate *S. uberis* mastitis through management that controls transmission of contagious mastitis[4]. However, indications for cow-to-cow transmission of *S. uberis* are emerging from molecular and mathematical approaches to epidemiology. While multiple genotypes are commonly found within dairy herds, strain typing studies have also yielded evidence for direct transmission and predominance of particular strains in some herds[2, 28]. Zadoks et al.[37] showed that the prevalence of *S. uberis* was a predictor for incidence of new infections, which suggests that infected cows act as a source of infection for uninfected herd-mates.
Intramammary infections (IMI) caused by *S. uberis* may be clinical or subclinical, and may vary in duration. Subclinical infections may hamper the control of mastitis because they often go unnoticed, resulting in a long duration and the possibility of spread. Persistent infections with *S. uberis* are known to occur[26, 36] and a role for chronic subclinical infections in the epidemiology of *S. uberis*, possibly through transmission at milking time, has been suggested[13, 27].

The paper presented here describes *S. uberis* infections that were observed in a longitudinal field study in two commercial dairy herds in The Netherlands. The objective of the paper is to outline clinical characteristics of infections and strain typing results, and to examine the relevance of clinical and molecular findings for the epidemiology of *S. uberis* infections in dairy herds. Strains were identified by randomly amplified polymorphic DNA (RAPD) fingerprinting. Results from a pilot experiment on the role of the milking machine in spread of *S. uberis* are included.

Herds, Material and Methods

Herds and animals

Data and isolates were obtained from two commercial dairy herds in The Netherlands. The herds participated in a longitudinal observational study (June 1997 to December 1998) on the population dynamics of mastitis. Cows were mainly Holstein-Friesians, partly cross-bred with Dutch Friesian or Meuse-Rhine-Yssel cows. In winter, animals were housed in a free-stall barn with a concrete slatted floor, and cubicles with wood shavings as bedding material. Lactating cows were mostly on pasture during the summer. Herd 1 consisted of 95 ± 5 lactating cows with an average 305-day milk production of 8166 ± 459 kg, and arithmetic mean bulk milk somatic cell count of 235 ± 75 ($*10^3$) cells/ml in the year preceding the study. Herd 2 consisted of 41 ± 2 lactating cows, with average 305-day milk production of 8508 ± 165 kg, and arithmetic mean bulk milk somatic cell count of 205 ± 69 ($*10^3$) cells/ml.

In herd 1, cows were milked twice daily in a two by four open tandem parlor. Mastitis management included regular monitoring of milking machine function, use of individual paper towels, antibiotic treatment of (sub)clinical cases of mastitis, and antibiotic treatment of all cows at dry-off. Post-milking teat disinfection was practiced from June through September 1997, and again from April through June 1998. An outbreak of *S. uberis* mastitis occurred in this herd between November 1997 and April 1998 and has been described in detail elsewhere[37]. In herd 2, cows were milked twice daily in a two by five herringbone parlor. Mastitis management was similar to herd 1, but post-milking teat disinfection was practiced throughout the study.

Collection and processing of samples

Quarter milk samples were collected every three weeks from all lactating animals, using aseptic technique[12]. Farmers collected additional quarter milk samples at calving (prior to first contact with the milking machine), dry-off, culling, and when clinical mastitis was detected (any visual abnormality of milk or udder, with or without systemic signs of disease). A fraction of fresh quarter milk samples was used for determination of quarter milk somatic cell count (QMSCC) by a Fossomatic milk cell counter (Foss Electronic, Hillerød, Denmark), with the exception of farmer collected samples.

All milk samples for microbiological analysis were stored at -20°C until processing. Within three weeks of collection, 0.01 ml of milk were cultured and bacterial species were identified according to National Mastitis Council standards[12]. Preliminary identification of *S. uberis* was based on colony morphology and aesculin hydrolysis on Edward's medium. The number of colony forming units (cfu) was recorded for all samples. When ≤ 10 cfu of *S. uberis* were identified, individual colonies were counted. Higher cfu-

counts were categorized as 10 to 49 cfu/plate, 50 to 199 cfu/plate or ≥200 cfu/plate. Isolates from herd 1 were confirmed as *S. uberis* using the API 20 Strep system[18]. For herd 2, only a convenience selection of isolates was confirmed as *S. uberis* using the API 20 Strep system. Milk samples from herd 2 had been stored frozen for over a year before repeated isolation of bacteria for API 20 Strep confirmation of species identity was attempted, and re-isolation of bacteria was not always successful.

Definition of infection

A quarter was considered infected when ≥ 1000 cfu/ml of *S. uberis* were cultured from a single sample, when ≥ 500 cfu/ml were cultured from two of three consecutive milk samples, when ≥ 100 cfu/ml were cultured from three consecutive milk samples, or when ≥ 100 cfu/ml were cultured from a sample obtained from a quarter with clinical mastitis. Samples containing more than three bacterial species were considered contaminated, and were not informative of IMI status. Samples that were culture negative during antibiotic treatment for udder disease were not considered informative of IMI status either. A previously infected quarter was considered recovered from IMI if none of the above definitions were met and the sample was free of the pathogen[37].

For IMI that were first detected at calving or as clinical mastitis, the sample date was assumed to be the date of onset of infection. For IMI that were last detected at dry-off or at culling, sample date was taken as endpoint of infection. For all other combinations, e.g. IMI that started during lactation or ended between a clinical sample and the consecutive routine survey sample, the midpoint of the last negative and first positive sample was taken as starting point, and the midpoint between last positive and first negative sample was taken as endpoint of IMI. The terms "positive" and "negative" sample refer to IMI status of the sample[37].

Liner swabs

In herd 1, swabs were taken from milking machine unit liner s on two occasions (swab experiment 1 and swab experiment 2) to determine presence of *S. uberis* on liners before and after milking of *S. uberis* infected cows. Five cows that were chronically infected with *S. uberis* in one quarter were selected. Cows were led into the milking parlor so that the milking unit and teat cup that would be used for the infected quarters were known. Before milking started, designated teat cup liners were swabbed to check cleanliness. Next, cows with infected quarters were milked, using standard milking routine. In swab experiment 1, liners were swabbed in duplicate after infected quarters had been milked. In swab experiment 2, milking of cows with infected quarters was followed by milking of two cows that had no *S. uberis* infection in any quarter. Swabs were not taken after milking of the infected cow, but after milking of the first non-infected cow following the infected cow, and again after milking of the second non-infected cow. Infection status of infected and

non-infected cows was determined based on prior milk culture results, and checked with bacteriological culture from milk samples taken on days when swabs were obtained.

Swabs consisted of sterile cotton wool, mounted on plastic rods and stored in a sterile container. Swabs were inserted into the teat cup up to the point were the liner joined the short milking tube, and withdrawn in a spiraling motion while rotating the swab. Swabs were reinserted into containers together with 5 ml of cooled sterile peptone-saline solution (0.85% w/v sodium chloride and 0.1% w/v peptone water (Oxoid, CM9, Haarlem, The Netherlands) in sterile water). Swabs were cooled and transported to the laboratory within a few hours. In the laboratory, swabs were inserted into 5 ml of serum broth (0.5% w/v Lab Lemco (Oxoid, Haarlem, The Netherlands), 1.0% w/v Bacto peptone (Oxoid, Haarlem, The Netherlands), 0.5% w/v sodium chloride and 0.1% glucose in sterile water, pH 7.4) and broth was shaken vigorously. Inocula were diluted 1:10 in serum broth and 0.1 ml was plated on Edward's medium (experiment 1). In experiment 2, dilutions (1:10) were incubated overnight at 37 ± 1°C before 0.1 ml was plated on Edward's medium. All plates were incubated for 21 ± 3 hrs at 37 ± 1°C. Isolates were confirmed as *S. uberis* using the API 20 Strep system. Results were expressed as absence or presence of *S. uberis*.

RAPD fingerprinting

DNA isolation was performed as described by Gillespie et al.[10]. Amplification of bacterial DNA using primer OPE-04 (5'-GTGACATGCC-3'; Operon Technologies, Alameda, CA, USA) was done as described by Jayarao et al.[17]. Amplified products were electrophoresed in 2% agarose with TBE buffer (0.9 *M* Tris bse, 0.09 *M* boric acid, 2.5 m*M* EDTA; pH 8.3). Gels were run at 150 V for 3.5 h and were stained with ethidium bromide (1.0 µg/ml; Sigma Chemical Co., St. Louis, MO, USA). The DNA was visualized by transillumination (Fotodyne Inc., New Berlin, WI, USA) and photographed with type 55 Polaroid® film (Polaroid Corp., Cambridge, MA, USA). Each DNA amplification pattern was examined for number and size of DNA fragments. Isolates with the same number and size of DNA fragments were considered to belong to the same strain, irrespective of band intensity of fragments. Strains were designated an arbitrary letter to identify a genotype.

Statistical analysis

For between-group comparison of categorical outcomes, Fisher's exact test was used when both categorical variables had two levels, and the Mantel-Haenszel Chi-square statistic was used when categorical variables had more levels. Analyses were performed in SAS version 8.01 (SAS Institute Inc., Cary, NC, USA, 1999). For between-group comparison of continuous outcomes that were not normally distributed, a Kruskal-Wallis one-way nonparametric AOV was used. Association between median QMSCC and number of observations was examined using Pearson's correlation coefficient. Analyses were done in Statistix for Windows version 1.0 (Analytical Software Co., La Jolla, CA, USA, 1996).

Significance was declared at $P < 0.05$. For statistical analysis, bacterial content of milk samples was indicated by a categorical variable with five levels (level 0 = 0 cfu/plate, level 1 = 1-9 cfu/plate, level 2 = 10-49 cfu/plate, level 3 = 50-199 cfu/plate, and level 4 = ≥200 cfu/plate).

Results

Clinical and bacteriological observations

In herd 1, 54 infected episodes were observed in 47 quarters of 31 cows. Four infected quarters were present at the start of the study and 50 new infections were detected during the 18-month observation period. Four infections were detected in heifers at calving, one infection was detected in a multiparous cow at calving, one infection was detected as severe clinical mastitis in the early dry period, and 44 infections were first detected during lactation. In herd 2, 30 infected episodes were observed in 23 quarters of 15 cows. Seven infected quarters were present at the start of the study, and 23 new infections were detected during the study. One infection was detected in a multiparous animal at calving, and all other infections were detected during lactation.

Table 1. Number of clinical, subclinical and combined clinical/subclinical intramammary infections with *Streptococcus uberis* observed over an 18-month period in two Dutch dairy herds. Herd 1 consisted of 95 ± 5 lactating animals. Herd 2 consisted of 41 ± 2 lactating animals.

Course of infected episode	Herd 1	Herd 2
Clinical onset		
• clinical only	3	3
• followed by subclinical only	2	2
• followed by subclinical with clinical flare-ups	0	1
Σ Subtotal clinical onset	5	6
Subclinical onset		
• followed by subclinical only	40	16
• followed by subclinical with clinical flare-ups	6	3
Σ Subtotal subclinical onset	46	19
Unknown onset		
• followed by subclinical only	3	4
• followed by subclinical with clinical flare-ups	0	1
Σ Subtotal unknown onset	3	5
Total number of episodes	54	30

Out of 54 observed infected episodes in herd 1, eleven showed clinical signs and 43 (80%) were subclinical throughout the observation period. Infections that showed clinical signs began as clinical mastitis in five cases and as subclinical infections in six cases. The number of recorded clinical episodes per infection ranged from one to five. In herd 2, 20 of 30 episodes were fully subclinical (67%). Infections that showed clinical signs started as clinical mastitis in six of ten cases. Per infection one or two clinical episodes were recorded (Table 1).

Estimated duration of infections ranged from 1 to 309 days in herd 1 (median = 46 days), and from 1 to 280 days in herd 2 (median = 28 days). The true duration of infections may have been underestimated, because duration was truncated for infections that were present at the start or the end of the study. Of course, estimates also depend on the definitions of onset and end of infected episodes. Dry-off or culling was considered as the end of an infected episode. All quarters that were infected at dry-off and that were examined again at the next calving, were cured during the dry period. To avoid loss of information and biased selection of data, only infections that were first detected at dry-off or at the last sampling of the study were excluded from calculations of duration. For those two categories there was no follow-up after onset of infection and calculation of duration was not considered meaningful. In herd 1, the duration of infection was estimated for 47 infected episodes and in herd 2 the duration of infection was estimated for 27 infected episodes. Reasons for truncation of infections are summarized in Table 2, which also shows the number of infections that cured with or without treatment. Infections that were treated but not cured are included as truncated infections.

Table 2. Reasons for truncation or end of infected episodes in *Streptococcus uberis* infected quarters in two Dutch dairy herds observed over an 18-month period.

Event	Episodes in herd 1	Episodes in herd 2
Treatment and cure[a]	14[b]	6[b]
Spontaneous cure	14	10
Start of study	1	1
End of study	7	3
Dry-off	4	5
Cull	11	1[c]
Start of study and dry-off	3	3
Start of study and cull	0	1[c]
Total	54	30

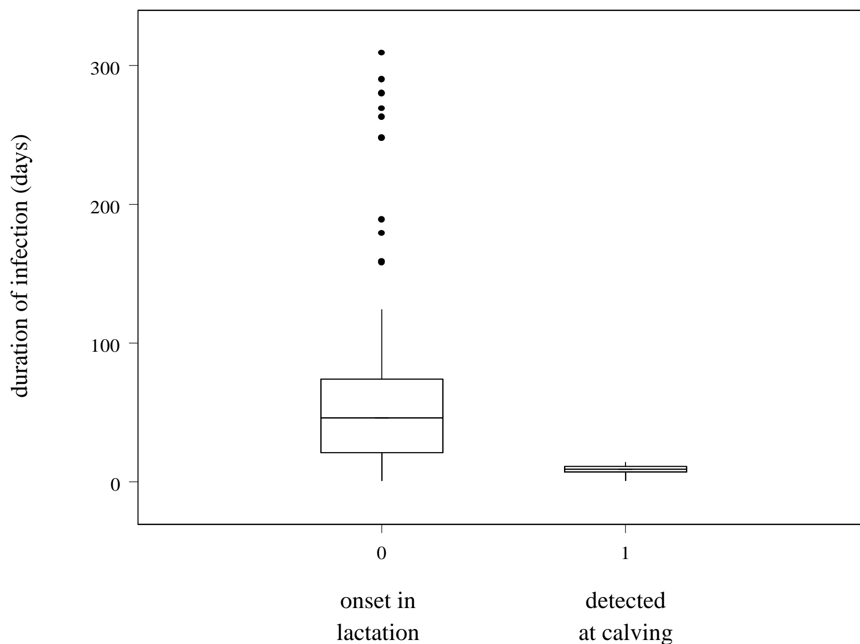
a) It is not claimed that treatment was the cause of cure.

b) Treatments that were not followed by cure are not reported here.

c) Milking of this quarter was terminated and the quarter was "blind" for the remainder of the study period.

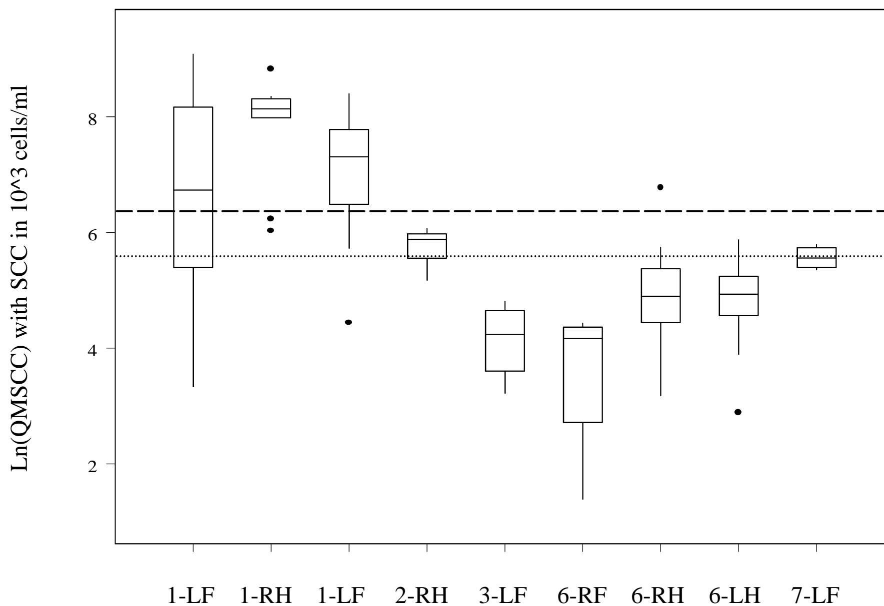
Infections that were detected at calving (n = 5, median duration = 9 days) were significantly shorter than infections that were first detected at a week or more in lactation (n = 65, median = 46 days; Kruskal-Wallis AOV, $P < 0.01$; Fig. 1). Two infections for which duration of infection was estimated had unknown time of onset because they were already present at start of the study. Two other infections were detected within a week post calving and were thought to be infections that could originate in the dry period or in the lactating period. They were not included in either category. Duration of infection did not differ between the two herds or between positions of udder quarters (left or right, front or rear) (Kruskal-Wallis AOV, $P = 0.47$, 0.22, and 0.67, respectively). Infections that started as clinical mastitis were significantly shorter (n = 9, median = 13 days) than infections that started subclinically (n = 56, median = 45 days) (Kruskal-Wallis AOV, $P < 0.001$). Infections with subclinical onset and clinical flare-ups were longer (n = 9, median = 90 days) than infections that were fully subclinical (n = 47, median = 42 days) (Kruskal-Wallis AOV, $P = 0.01$).

Figure 1. Duration of *Streptococcus uberis* infections for quarters that became infected during lactation (n = 65; infection detected at >7 days after calving) and for quarters that were infected at calving (n = 5).



Quarter milk somatic cell count in infected quarters ranged from 5,000 cells/ml to 9,999,000 cells/ml (upper limit of detection system). For 21 quarters, at least four observations of QMSCC during infection were available. In herd 1, the lowest median QMSCC in such a chronically infected quarter was 215,000 cells/ml and the highest median QMSCC was 4,719,000 cells/ml. In herd 2, lowest median QMSCC was 65,000 cells/ml and the highest median QMSCC was 3,412,000 cells/ml. Examples of high and low QMSCC in quarters with chronic *S. uberis* infection are shown in Figure 2.

Figure 2. Natural logarithm of somatic cell count of quarters with chronic *Streptococcus uberis* infection. Numbers on horizontal axis indicate cows. Abbreviations indicate quarter position. RF = right front, LF = left front, RH = right hind, LH = left hind. Number of observations per quarter ranged from 4 through 16. Dashed line indicates quarter milk somatic cell count (QMSCC) of 500,000 cells/ml. Dotted line indicates quarter milk somatic cell count of 250,000 cells/ml.



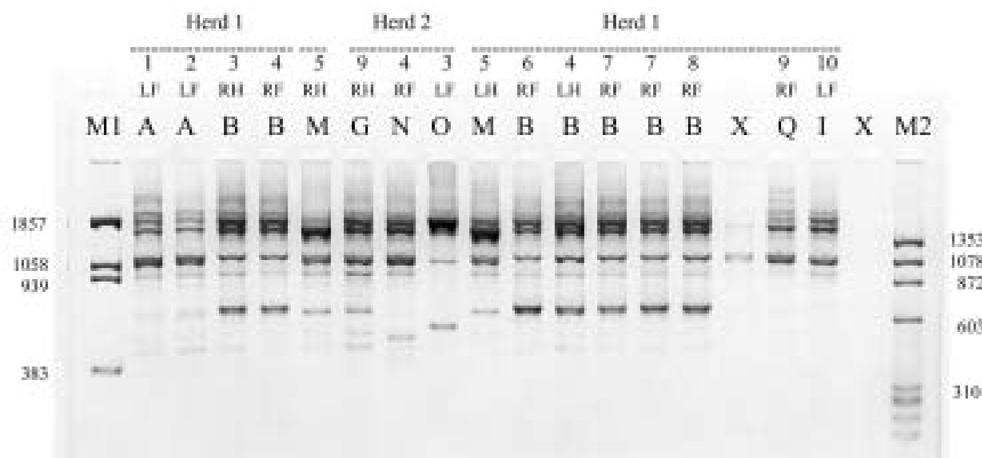
Two cut-off values for somatic cell count that are used commonly as indicators of infection are included in the figure. Ten of 21 quarters had median QMSCC ≤ 500,000 cells/ml and five had median QMSCC ≤ 250,000 cells/ml. Median QMSCC was not significantly associated with herd of origin, quarter position (front or rear, right or left), or duration of infection (expressed in number of observations) (Kruskal-Wallis AOV, $P = 0.09$, 0.12, and 0.59, and Pearson correlation, $P = 0.47$, respectively).

The number of bacteria in milk from infected quarters was generally high, with infected quarters shedding > 1000 cfu/ml in 84.9% of 230 samples in herd 1, and in 88.3% of 139 samples in herd 2. Samples from infected quarters were culture negative for *S. uberis* in 5.7% of samples in herd 1, and in 5.8% of samples in herd 2.

Strain typing

In herd 1, twelve strains were identified among 111 mammary isolates that were RAPD fingerprinted. In herd 2, seven strains were identified among 41 mammary isolates that were RAPD fingerprinted. An example of strain typing results is shown in Figure 3.

Figure 3. Example of *Streptococcus uberis* fingerprints, obtained through RAPD analysis. Lane M1 is pBR322 DNA digested with *Bst*NI (New England BioLabs, Beverly, MA, USA); Lane M2 is OX174 DNA digested with *Hae* III (New England BioLabs, Beverly, MA, USA). X indicates empty lane. Herd, cow, and quarter are indicated for each sample. RF = right front, LF = left front, RH = right hind, LH = left hind. Letters represent *S. uberis* strains. Molecular size standards (kb) are indicated on either side of the figure.



The number of isolates, quarters and cow per strain is summarized in Table 3. A dominant strain was identified in each herd. The dominant *S. uberis* strain from herd 2, strain A, was also detected in herd 1. The dominant strain from herd 1, strain B, was not detected in herd 2. Of 17 strains that were identified, two occurred in both herds.

Table 3. Number of isolates, infected episodes, infected quarters, and infected cows per *Streptococcus uberis* strain in two dairy herds. In herd 1, 111 out of 217 *S. uberis* isolates were RAPD fingerprinted. In herd 2, 41 out of 131 *S. uberis* isolates were RAPD fingerprinted. Only infections with known strain types are included.

Strain	Herd 1				Herd 2			
	Isolates	Episodes	Quarters	Cows	Isolates	Episodes	Quarters	Cows
A	12[a]	4	4	4	30	9	8	5
B	83	29	29	22	-	-	-	-
C	1[b]	1	1	1	-	-	-	-
D	2	1	1	1	-	-	-	-
E	-	-	-	-	3	2	2	1
F	1	1	1	1	-	-	-	-
G	1	1	1	1	3	3	3	2
H	-	-	-	-	1	1	1	1
I	5	1	1	1	-	-	-	-
J	1	1	1	1	-	-	-	-
K	-	-	-	-	2	1	1	1
L	1	1	1	1	-	-	-	-
M	2	1	1	1	-	-	-	-
N	-	-	-	-	1	1	1	1
O	-	-	-	-	1	1	1	1
P	1	1	1	1	-	-	-	-
Q	1	1	1	1	-	-	-	-
Total	111	39[c]	37[c]	28[c]	41	18	15	10

a) In one quarter, a mixed infection was detected (strain A and strain F were present in the same sample; colonies differed in aspect on culture plate). In one quarter, two different strains (A and B) were detected on two consecutive occasions. In one quarter, three different strains (A, B and Q) were detected on three consecutive occasions).

b) Superinfection with strain C in a quarter that was chronically infected with strain B. Strain C was isolated from an extra sample taken during a clinical episode.

c) Column does not add up to total, due to detection of multiple strains per infected episode in some quarters.

When multiple isolates were typed per infected episode, isolates usually belonged to one RAPD type (Table 4). For 40 infected episodes, more than one isolate was typed (range = 2 to 8 isolates; median = 3 isolates). For the majority of infections (87.5%), the same strain was found upon every isolation.

Table 4. Strains of *Streptococcus uberis* isolated from selected quarters of dairy cows in herd 2 over an 18-month period with 3-weekly sampling. Infection with *S. uberis* is represented by strain type for isolates that were RAPD-fingerprinted; S = sampling; + = *S. uberis* infection present, but isolate not fingerprinted; - = no *S. uberis* infection present; dp = dry period; * = clinical mastitis. Empty cells indicate absence of cows due to culling.

S	1[a]			2	3	4	5	6		7	8			9	10
	LF	RH	LH	RH	LF	RF	RF	RH	LH	LF	LF	RH	LH	RH	LH
1	-	-	-	A	-	-	-	+	+	+	-	E	-	-	-
2	-	-	-	+	-	-	+	A	A	+	E	E	-	-	-
3	A	A	A	+	-	dp	-	A	A	A	dp	dp	dp	-	-
4	A	A	A	A	-	-	dp	+	A	A	-	-	-	-	A*
5	A	A*	A	A	-	-	dp	+	+	-	-	-	-	-	-
6	+	+	+	+	-	-	dp	+	+	-	-	-	-	-	-
7	+	+	+	+	-	-	dp	+	+	-	-	-	-	-	-
8	A	A	+	+	-	-	dp	+	+	-	-	-	+	-	-
9	+	+	+	+*	-	-	-	+	+	-	G	-	+	-	-
10	+	A	A	-	-	-	-	+	+	-	-	G	-	-	-
11	+*	+	+	-	-	-	-	+	+	-	-	+	H	-	dp
12	+	+	A	-	-	-	-	A	+	dp	-	-	-	-	dp
13	+	+	+	-	dp	-	-	+	+	-	-	-	-	-	-
14	+	A	+	-	dp	-	-	+	A	-	dp	dp	dp	-	-
15	A	+	+	-	-	-	-	dp	dp	-				G	-
16	dp	dp	dp	-	-	-	-	dp	dp	-					-
17				-	-	-	-	dp	dp	-					-
18				-	-	-	-	-	-	-					-
19				dp	-	N	-	-	-	-					-
20					-	-	-	-	-	-					-
21					-	-	-	-	A*	-					-
22					-	-	-	-	+*	-					
23					-	-	-	-	-	-					
24					+	dp	-	-	-	-					
25					O	dp	K	-	+	-					
26					+	-	K	-	+	-					
27					+	-	+	+	+	-					

[a)] Numbers identify cows. Letters identify quarters. RF = right front, LF = left front, RH = right hind, LH = left hind.

Multiple quarters of a cow could be infected. Within an udder quarter infection could occur in multiple lactations. Per lactation, one or more infected episodes could occur. Infected episodes in a quarter that were separated by periods of cure could be caused by the same strain or by different strains. Examples are shown in Table 4. Cow 6 in herd 2 was infected with strain A in the left hindquarter in two lactations, separated by the dry period and a non-infected period after calving. In the same herd, the right hindquarter of cow 8 was infected with strain E in one lactation, followed by a dry period and months without infection, before a new infection occurred with strain G in the next lactation. When multiple quarters within a cow were infected, the same strain was isolated from all quarters on most occasions. Onset of infection could be detected simultaneously for multiple quarters (e.g. Table 4, cow 1). Alternatively, infection with a specific strain in one quarter could be followed by infection with the same strain in other quarters of the cow (e.g. Table 4, cow 8; and five cows in herd 1 (data not shown)). When multiple quarters within a cow were infected, quarters were usually adjacent. Concurrent infection in diagonally opposed quarters was observed twice, once with identical strains and once with unidentified strains.

Associations between strains and clinical characteristics

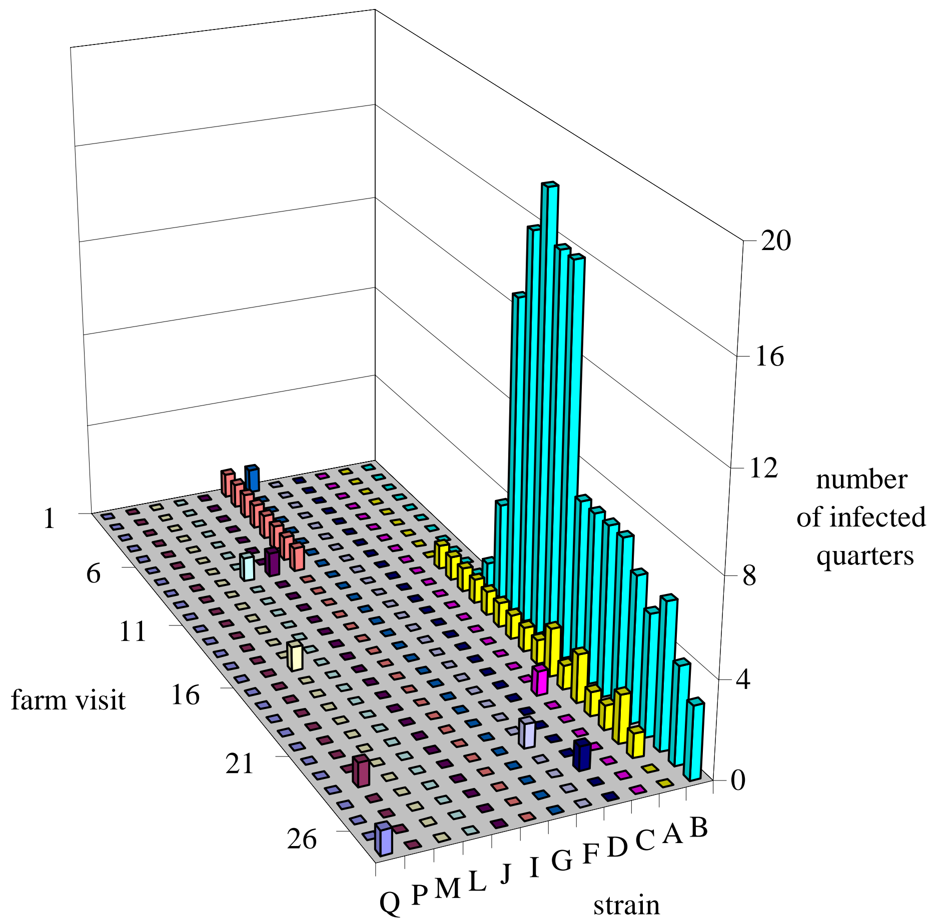
In herd 1, eight of ten infections (80%) with clinical signs and known strain type were associated with the dominant strain (strain B), and two infections were associated with other strains (J and P). For infections without clinical signs and with known strain type, 21 out of 27 (78%) were associated with the dominant strain, and 11 were associated with other strains (A, D, F, I, L, M and Q; due to infections with multiple strains total does not add up to 27). There was no significant association between observed duration of infection and strain (dominant or non-dominant; Kruskal-Wallis AOV, $P = 0.10$). In herd 2, infections with clinical signs were associated with strain A or undetermined strain types. There was more heterogeneity among isolates that caused subclinical infections (strains A, E, G, H, K, N, O, and undetermined strains). Because of the limited number of observations per strain, statistical analysis was not attempted. In herd 2, infected episodes caused by the dominant strain were significantly longer than episodes caused by other strains (Kruskal-Wallis AOV, $P < 0.01$).

No significant associations were identified between median QMSCC and strain of *S. uberis* (strain A, B or other) for quarters that had at least four QMSCC measurements and known strain type (n=19; Kruskal-Wallis AOV, $P = 0.46$). In herd 1, bacterial content of milk samples was lower for quarters infected with the predominant strains (strain B, 166 observations) than for quarters infected with other strains (39 observations) (Mantel-Haenszel Chi-square = 12.6 for 1 df, $P < 0.01$).). In herd 2, shedding levels did not differ between quarters infected with the predominant strains (strain A, 103 observations) or other strains (18 observations) (Mantel-Haenszel Chi-square = 0.02 for 1 df, $P = 0.89$). No correction was used for correlation of repeated observations within infected quarters.

Association between strains and epidemiological characteristics

In herd 1, an outbreak of *S. uberis* mastitis occurred[37]. A convenience selection of isolates from the pre-outbreak, outbreak and post-outbreak periods was RAPD fingerprinted. For 39 of 54 observed infected episodes, *S. uberis* strains were identified. For each of the 27 consecutive herd visits, the number of quarters that was infected with a specified strain was determined. Numbers are depicted in Figure 4.

Figure 4. Number of quarters that was infected with a specified strain of *Streptococcus uberis* during an 18-month observation period with 27 farm visits at 3-week intervals in a dairy herd with 95 ± 5 lactating animals. Strains were specified for 39 out of 54 observed infected periods.



The majority of infections during the outbreak of *S. uberis* mastitis was attributable to strain B. Strain B had not been identified in the pre-outbreak period. Other strains were identified throughout the pre-outbreak, outbreak and post-outbreak period, but never in more than two quarters at any one time. Infections with strain B always started during lactation, while six out of nine infections with other strains started during the non-lactating period (two-sided Fisher's Exact test, $P < 0.0001$).

Liner swabs

No *S. uberis* was cultured from any of the liner swabs taken from milking machine unit liners before milking. In swab experiment 1, *S. uberis* was isolated from five of five liner swabs immediately after milking of *S. uberis* infected cows. Of five *S. uberis* infected cows, four shed strain B in their milk, and one shed strain A. For each cow-liner combination, the same strain was obtained from the infected quarter and from the teat cup liner. In swab experiment 2, milking of an infected cow was followed by milking of two uninfected cows, and swabs were taken after milking of the uninfected cows. Three out of five liner swabs taken after one uninfected cow had been milked harbored *S. uberis*, and one out of five liner swabs taken after two uninfected cows had been milked was positive for *S. uberis* (Table 5).

Table 5. Culture results from *Streptococcus uberis* infected quarters, from non-infected quarters and from swabs of milking machine unit liners.

Experiment[a]	Clean liner	First[b]		Second		Third	
		Cow	Swab	Cow	Swab	Cow	Swab
1	- [c]	A	A				
	-	B	B				
	-	B	B				
	-	B	B				
	-	B	B				
2	-	B		-	-	-	-
	-	B		-	B	-	-
	-	B		-	B	-	B
	-	B		-	B	-	-

a) Swabs were taken before milking, and after milking of infected quarters (experiment 1) or after milking of subsequent uninfected quarters (experiment 2).

b) First second and third indicate order of milking and collection of unit liner swabs.

c) Letter indicates strain of *S. uberis* that was isolated, - indicates that no *S. uberis* was isolated, and an empty cell indicates that no sample was taken.

Discussion

Control of mastitis pathogens such as *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Staphylococcus aureus* has reduced the number of clinical mastitis cases caused by those bacteria. As a result, there has been a relative (and possibly absolute) increase in the number of cases of clinical mastitis caused by *S. uberis*[20]. Together with studies that report the majority of *S. uberis* infections to be clinical[34], this has led to emphasis on *S. uberis* as a cause of clinical mastitis, which makes the suggestion that subclinical mastitis may also be important seem new[27]. Our study proves that *S. uberis* can be a major cause of subclinical mastitis in dairy herds, and that clinical cases constitute a small proportion of infections caused by *S. uberis*. This is in agreement with observations by Jayarao et al.[16] who report as many as 95% of cases within a herd to be subclinical. In The Netherlands, *S. uberis* is the second most frequently isolated pathogen from milk samples from cows with subclinical mastitis[29].

The range of duration of *S. uberis* infections observed in our study (1 to 309 days) is similar to the range reported elsewhere (1 to 370 days[34]). Mean duration in the study by Todhunter et al.[34] was 12 days. This is close to the median duration of infections with clinical onset in our study, but much shorter than the overall mean duration of 72 days. Because duration was not normally distributed, the median duration was thought to be a more appropriate measure for the central value. Half of the observed infections lasted more than an estimated 42 days, and approximately one in four infected episodes lasted more than 72 days, emphasizing that chronic infections are no exception.

In both herds, clinical infections in dry cows were rare. This is in contrast to reports from the United States[24, 32, 34] and may be a result of successful dry cow treatment in the study herds, and of differences between herds and countries in dry cow management or bacterial flora. Several new infections were detected at calving, most of them subclinical. Such infections probably originated in the non-lactating period. Infections that were present at calving were of shorter duration than infections that occurred during lactation and had limited impact on the incidence of clinical mastitis or the prevalence of subclinical mastitis in the study herds. The effect of infections at calving on production in the subsequent lactation was not examined.

For diagnosis of subclinical infections, bacteriological culture of milk samples is necessary. Whole herd bacteriological surveys of quarter milk samples are too expensive to be performed on a routine basis. Therefore, milk somatic cell count is used commonly as an indirect indicator of infection. In The Netherlands, 250,000 cells/ml is used as the cut-off value to discriminate between non-infected quarters and quarters that are suspect of infection[29]. This value is used at cow level, i.e. for pooled samples in which milk from infected quarters is mixed with milk from uninfected quarters that normally have lower QMSCC. At a quarter level, a cow-side test such as the California Mastitis Test (CMT) can be used. This screening test is positive for quarters with QMSCC 400,000 to 500,000

cells/ml or higher. In our study, a considerable proportion of infected quarters had median QMSCC below those detection limits so that they would go unnoticed with standard screening strategies. Infected quarters may be a source of infection for other animals in the herd, as will be discussed in the context of epidemiological findings. Furthermore, even at cell-counts below 250,000 cells/ml, milk quantity and milk quality are affected by subclinical mastitis[15].

Previous studies found that isolates from one milk sample usually belong to the same strain[26, 28]. Therefore, only one isolate per milk sample was RAPD fingerprinted in the present study. One exception was a milk sample from a heifer at calving, which yielded two colony types and two strains. For several infected episodes, multiple consecutive isolates were typed. Within an episode in a quarter, isolates mostly belonged to the same strain. This has been reported by others, and supports the idea that infections are persistent rather than recurrent with different strains[26, 28]. When multiple episodes in a quarter occurred, episodes could be associated with the same strain or different strains, as has been shown by Oliver et al.[26] for isolates from different lactations within a quarter. When multiple quarters in a cow were infected, infections were usually caused by the same strain. This confirms results from Phuektes et al.[28] and suggests within-cow transmission of bacteria, although exposure of multiple udder quarters to the same environmental source cannot be ruled out. However, infection in one quarter often preceded infection of other quarters within the cow and it is not likely that different quarters would be exposed to the same environmental source at different points in time.

Within each herd, multiple *S. uberis* strains were identified. This is in agreement with reports from Australia[36], New Zealand[8], and the United States[26]. The variety of strains is consistent with an environmental source of *S. uberis*[20, 28]. In addition, a predominant strain was identified in each herd. The predominance of a single strain may be the result of infection of multiple quarters from a common environmental source. Alternatively, it could be the result of cow-to-cow transmission of bacteria, as suggested in other strain typing studies[2, 28].

Predominance of particular strains in a herd could be the result of differences in pathogen virulence. The outcome measure of infection that is most easy to determine is the occurrence of clinical signs in infected quarters or animals. Hill[13] and Phuektes et al.[28] report that some strains are more likely to cause clinical mastitis than others. Jayarao et al.[19] found less heterogeneity among isolates from clinical mastitis than among isolates from subclinical infections. Results for herd 2 are consistent with results reported by Jayarao, but within herd 1, an association between strain and clinical disease was not observed. Many studies have addressed virulence of *S. uberis*[20, 25] but most studies are based on in vitro work, and the relevance of different virulence factors in vivo or at herd level is unknown. The in vivo study by Hill[13] is the only one to have been linked to in vitro virulence characteristics[21], and more work needs to be done to determine the in-vivo relevance of in-vitro traits.

No association was found between strain and QMSCC. Cow-factors may be more important than bacterial virulence factors in evoking influx of somatic cells to the infected mammary gland. In herd 1, strain type was not associated with duration of infection. Cow factors and management measures, such as treatment or culling, are likely to be important determinants of duration. Lack of statistical significance of associations between specified strains and clinical characteristics may also be a result of the limited number of observations for most bacterial strains. Infections with clinical onset were shorter than infections with subclinical onset. If it is true that some strains are more likely to cause clinical onset of infection, such "clinical strains" could be associated with shorter duration of infection. Short duration of clinical infections could well be the result of early detection. Early detection and treatment of infection may result in higher probability of cure, as described for *Staphylococcus aureus*[33]. It would be of interest for farmers to determine cow and quarter factors (e.g. parity, quarter position, duration of infection, QMSCC) and bacterial characteristics (e.g. antibiotic susceptibility, strain type) that influence the probability of cure of *S. uberis* infections with or without treatment. If risk factors were known, the probability of cure for a specific individual could be determined and well-informed treatment decisions could be made. In the long term, this approach would contribute to prudent use of antibiotics.

The number of *S. uberis* found in milk was lower on average in quarters infected with strain B than in quarters infected with others strains. Phuektes et al.[28] observed no association between strains and shedding levels. This may be caused by limited statistical power due to small sample size (38 samples were included in this part of their study), or to differences between strains in their study as compared to our study. Differences in shedding levels may be of limited clinical importance, because even in quarters that were infected with strain B, 86% of samples contained >1000 cfu/ml.

One of the most striking results from this study is the predominance of one strain in each herd. The outbreak of *S. uberis* mastitis that was observed in one herd was almost entirely attributable to one *S. uberis* strain. Outbreaks of *S. uberis* mastitis have been described before, and it was suggested that cases of *S. uberis* mastitis that were left untreated may have resulted in spread of the pathogen to other individuals in the dairy herd[5]. This cow-to-cow transmission or contagious spread seems very likely, because infections with strain B were only observed in lactating animals, and never in non-lactating animals. Furthermore, *S. uberis* could be isolated from teat cup liners, not only after milking of infected quarters, but also after subsequent milking of uninfected quarters. This shows that transmission via the milking machine, which has long been accepted to play an important role in spread of *S. aureus* infections[23], may also play a role in transmission of *S. uberis*. In an earlier report, mathematical analysis was used to determine the statistical significance of existing infections as predictors for new infections[37]. Findings from the current paper support the mathematical conclusion that cow-to-cow transmission played a role in an outbreak of *S. uberis* mastitis. It is the contagious spread of *S. uberis* that can be controlled through implementation of management measures that reduce cow-to-cow

transmission of bacteria. The success that can be achieved with such programs has been known for many decades[22, 30] and is still important today.

In addition to contagious spread with infected animals as source of infection, there is non-contagious spread of *S. uberis* with the environment as source of infection. A vast body of literature supports this notion[20, 32, 34]. The wide variety of strains isolated from cases of *S. uberis* mastitis, and the occurrence of new infections in animals at calving observed in our study is in line with an environmental origin of the bacteria. So far, molecular epidemiology has been used primarily to determine variability in *S. uberis* strains isolated from the bovine mammary gland. As a next step in *S. uberis* research, strains isolated from cattle should be compared to strains isolated from the environment, including skin, mucosa, rumen, manure, bedding, and pasture, to determine the relative importance of different environmental sources in the dynamics of *S. uberis* mastitis.

Finally, to allow for comparison of result obtained by different research groups world wide, it would be desirable to have a reproducible typing method with enough discriminatory power and yet sufficient simplicity to allow easy storage and comparison of data. The need to develop such a method has been recognized before[2], but most reports are based on pulsed-field gel electrophoresis[2, 28, 36] or RAPD-fingerprinting[17, 26, this study]. Both methods require interpretation of complex banding patterns on gels that are often difficult to standardize, interpret, store and compare. It would be a great asset if a library typing system, such as the binary typing system for *S. aureus*[35] or multilocus sequence typing as used for *S. aureus* and *Streptococcus species*[9] could be developed for typing of *S. uberis*.

Conclusion

Chronic subclinical intramammary infections with *S. uberis* occur frequently and may serve as a source of infection for other cows in a herd. A contagious route of spread, possibly via transmission during milking, plays a role in the dynamics of *S. uberis* mastitis in some dairy herds. When dealing with *S. uberis* problems, both environmental and contagious sources and routes of transmission should be given due consideration. To facilitate comparison of results from different studies, it would be desirable to have an internationally standardized typing system for *S. uberis*.

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- Chapter 8 -

General discussion

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Introduction

The aims of this study were to improve our understanding of the epidemiology of *Staph. aureus* and *Strep. uberis* mastitis in dairy herds that implement the standard mastitis prevention program, and to identify host and pathogen characteristics that affect the occurrence and the outcome of intramammary infections. To this end, mathematical models that describe the spread of *Staph. aureus* and *Strep. uberis* were developed (Chapter 4 and Chapter 6) and host factors that influence the risk of intramammary infection were examined (Chapter 5). Pathogens were identified at the subspecies level with a variety of techniques, and associations between strains and the spread and clinical manifestation of infections were explored (Chapters 2, 3 and 7). In the current chapter, results from the preceding chapters are discussed with a focus on herd level, host level, and pathogen level. Separate paragraphs are dedicated to discussions of mathematical modeling and molecular epidemiology of mastitis. Finally, implications for udder health management and directions for future research are considered.

Contagious and environmental mastitis: from dichotomy to sliding scale

"Contagious mastitis" and "environmental mastitis" are labels used to classify the epidemiology of pathogens that cause intramammary infections in dairy cows[25, 132]. The primary reservoir of contagious pathogens consists of infected udders, while the primary reservoir of environmental pathogens is the dairy cow's environment. Contagious mastitis is transmitted from cow to cow during milking, whereas exposure to environmental pathogens can occur at any time during an animal's lactating or non-lactating life[50, 132]. *Staphylococcus aureus* is categorized as a contagious pathogen[50], and *Strep. uberis* is categorized as an environmental pathogen[86, 132]. Results from our studies contradict this dichotomous paradigm. We provide evidence for environmental *Staph. aureus* mastitis and for contagious *Strep. uberis* mastitis. For both pathogens, arguments that support contagious epidemiology as well as arguments that support environmental epidemiology are presented below.

For *Staph. aureus*, there is general consensus that infected mammary glands are the main reservoir of infection in dairy herds[25, 50]. The pathogen is spread during milking, via udder cloths, milkers' hands, or milking machine unit liners[35, 50, 51, 109, Chapter 3]. Post-milking teat disinfection limits the spread of *Staph. aureus* mastitis[78]. Together with other measures aimed at control of cow-to-cow transmission of udder pathogens, post-milking teat disinfection has been successful in reducing the incidence and prevalence of *Staph. aureus* mastitis[59, 107]. In many herds, one or a few strains of *Staph. aureus* predominate[71, 79, 83, 120]. Predominance of a limited number of strains is likely to be the result of contagious

spread[79, 86]. Alternatively, it may indicate that some strains of *Staph. aureus* in the environment are better adapted to survival in the bovine udder or more virulent than others[79, 105].

Interest in environmental sources of *Staph. aureus* was originally sparked by the awareness that preparturient heifers were infected with the pathogen[19, 153, Chapter 4]. Because preparturient heifers have not been exposed to the milking machine, infections are thought to originate from environmental sources rather than from contagious transmission. Infections resulting from cross-suckling by calves have been postulated, but this mechanism is not supported by data[121, 122]. Transmission by flies does occur[95, 115, 121], but the question as to what the source of *Staph. aureus* remains unanswered. New infections in dry cows[Chapter 4] are also unlikely to result from contagious transmission. Matos and co-workers[95] were the first to use the term "environmental staphylococci" in a paper on isolation of *Staph. aureus* from sites other than the lactating mammary gland. Paradoxically, they proposed to use the designation "environmental staphylococci" for *Staphylococcus* species other than *Staph. aureus*. Elaborating on existing nomenclature, the name "environmental *Staph. aureus*" will be used in the current discussion to mean *Staph. aureus* isolated from sites other than the mammary gland. *Staphylococcus aureus* has been isolated from many sources, including body sites, skin lesions, non-bovine animals and humans[51, 82, 95, 121, 122, Chapter 3], bedding material, feedstuffs, air and water[95, 121]. The existence of extra-mammary reservoirs could explain why control of contagious transmission alone is not sufficient to eliminate *Staph. aureus* mastitis[61, 124, Chapter 4]. Especially in low SCC herds where contagious transmission of pathogens has been controlled, clinical *Staph. aureus* mastitis continues to be a problem[14, 42, 127]. Environmental factors (no regular disinfection of stall, no regular replacement of bedding) have been associated with an increased risk of clinical *Staph. aureus* mastitis[42]. Ancillary support for environmental *Staph. aureus* comes from strain typing studies on isolates from farms with intensive longitudinal monitoring of mastitis dynamics[Chapter 2]. In addition to the predominant *Staph. aureus* strains, a variety of strains is isolated from milk samples at low frequency in many studies[11, 71, 83, 98]. Heterogeneity and a low frequency of isolation within herds could be explained by an environmental origin of the strains.

For *Strep. uberis*, an environmental origin is dictated by the paradigm. Arguments for this classification include the failure of the five-point mastitis program to control *Strep. uberis* mastitis[59, 116, 132], and the importance of *Strep. uberis* as cause of mastitis in dry cows[110, 123, 134]. *Streptococcus uberis* mastitis is also found in preparturient heifers[113, 117, Chapter 6]. As for *Staph. aureus*, many sources of *Strep. uberis* have been identified outside of the infected mammary gland. They include bovine skin and nares, the rumen, feces, pasture, soil, and bedding[23, 33, 54, 63, 77]. A multitude of *Strep. uberis* strains occurs within dairy herds, as can be expected for infections of environmental origin[40, 86, 118, 164, Chapter 7].

The success of the five-point control plan in reducing *Strep. agalactiae*, *Staph. aureus*, and *Strep. dysgalactiae* mastitis and the failure to reduce *Strep. uberis* mastitis to the same extent has led to the misconception that control of contagious transmission does

not prevent the spread of *Strep. uberis*. In fact, implementation of the five-point control plan leads to a decrease in incidence and prevalence of *Strep. uberis* mastitis[107, 123]. When measures from the mastitis prevention program are omitted, outbreaks of *Strep. uberis* mastitis may occur[29, Chapter 6]. Like *Staph. aureus*, *Strep. uberis* can be isolated from milking machine unit liners[Chapter 7]. This supports the idea that cow-to-cow transmission at milking time may occur [57], and is in accordance with results from strain typing studies[16, 118]. Strain typing studies and biometrical analyses implicated contagious transmission via the milking machine as most plausible explanation for the outbreak of *Strep. uberis* mastitis that we observed[Chapter 6, Chapter 7].

In summary, the arguments that are used to claim contagious transmission of *Staph. aureus* also apply to *Strep. uberis*, and arguments that are used to claim the environmental origin of *Strep. uberis* also apply to *Staph. aureus*. To which extent the arguments pertain, differs between the species. In addition, different strains within the same pathogen may differ in their speed and mode of spread, as indicated by recent studies on *Staph. aureus*[101, Chapter 4] and on *Strep. uberis*[Chapter 7]. Thus, the concepts of “contagious mastitis” and “environmental mastitis” need to be interpreted at the level of the pathogen strain rather than at the level of the pathogen species. Classifying all *Staph. aureus* as contagious and all *Strep. uberis* as environmental is an oversimplification of mastitis epidemiology. The epidemiology of mastitis pathogens is better represented by a sliding scale where the balance of contagious and environmental transmission shifts gradually, than by a species-based dichotomy (Figure 1).

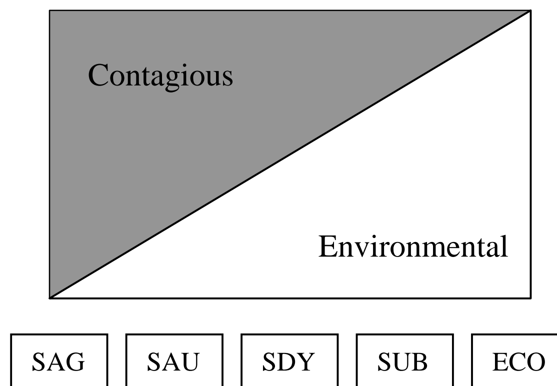

Figure 1. Sliding scale from contagious to environmental epidemiology of mastitis pathogens. SAG = *Streptococcus agalactiae*. SAU = *Staphylococcus aureus*. SDY = *Streptococcus dysgalactiae*. SUB = *Streptococcus uberis*. ECO = *Escherichia coli*.

The contagious end of the scale is represented by *Streptococcus agalactiae*, which is generally considered to be strictly udder-bound and to spread through cow-to-cow-transmission[73]. So far, *Strep. agalactiae* has been the only mammary pathogen that can be eliminated from dairy herds[89]. *Staphylococcus aureus* spreads predominantly as a contagious pathogen, but infected udders are not the only reservoirs, and control of cow-to-cow transmission leaves the problem of environmental *Staph. aureus* unsolved[121, Chapter 2, Chapter 4]. *Streptococcus dysgalactiae* epidemiology is intermediate between contagious and environmental[25, 50, 132]. *Streptococcus uberis* epidemiology is predominantly environmental, but the bacterium can also behave as a contagious pathogen[29, 107, Chapter 6, Chapter 7]. *Escherichia coli* is the prime environmental pathogen[132].

With the continued implementation of mastitis control programs, the epidemiology of mastitis may change. When contagious *Strep. agalactiae* mastitis is controlled, sporadic cases in low SCC herds remain[44]. Those cases are likely to be of environmental or human origin, and have different clinical and epidemiological characteristics than infections caused by bovine strains[68, 70]. Mirroring this, evidence is emerging that *E. coli* may not behave as a purely environmental pathogen[21]. As stated by Jensen[69] and reiterated by others[133], the majority of control efforts should not focus on exceptions. However, as time, mastitis control and consumer demands progress and change, the situation may alter and pathogens, strains or routes of spread that were of minor importance before, may become more relevant to bovine health and milk quality. This shift is occurring for coagulase negative staphylococci[106, 133] and it may also occur for other bacterial species and strains.

Mathematical modeling in mastitis epidemiology

Nearly all mastitis pathogens can be considered to be contagious to some extent[50, 131]. For accurate advice on mastitis control, quantification of the degree of contagiousness is necessary. Contagiousness can be quantified by the basic reproductive number, R_0, and the success of control measures can be quantified by changes in R_0[78]. For microparasites, represented by a compartmental model, R_0 is the average number of secondary infections produced by one infected individual in a fully susceptible population[10]. The concept has mostly been used to quantify transmission of viral diseases and malaria, but it also applies to bacterial diseases. Examples from veterinary medicine include dynamics of infections with *Mycobacterium paratuberculosis*[159], *Staph. aureus*[78] and *Strep. uberis*[Chapter 6] in dairy cattle, and *Actinobacillus pleuropneumoniae*[162] in pigs.

If R_0 for a mastitis pathogen is reduced to a value below one, sustained cow-to-cow transmission of mastitis cannot occur. Some authors consider a situation where $R_0 < 1$ to qualify as elimination[36]. Most farmers would interpret "elimination" to mean total absence of cases, as can be achieved for *Strep. agalactiae*. A situation where infections occur but spread of the pathogen is limited, as described for *Staph. aureus* in Chapter 4,

would be described as a situation where mastitis is not eliminated, but "under control". When the prevalence of contagious pathogens is low, it is important that farmers use management procedures that ensure that $R_0 < 1$, to prevent mastitis outbreaks. Such outbreaks could be sparked by an infection from an environmental source, or by introduction of an infected animal into the lactating herd after calving or purchase. Changes in management that cause an increase in R_0 may result in mastitis outbreaks, as described for *Staph. aureus*[78] and *Strep. uberis*[29]. Recovered quarters are more susceptible to mastitis than quarters that have not experienced intramammary infection[Chapters 4, 5 and 6]. As a result, R can be higher in a non-naive population than in a fully susceptible population and control measures that are adequate in a naive population may not be sufficient to prevent spread of mastitis in a population with a number of recovered individuals.

The concept of R_0 is very useful, but calculation of R_0 is based on a number of assumptions[Chapter 4]. The validity of the use of R_0 depends on the validity of the underlying assumptions, such as homogeneity of susceptibility and infectiousness, and assumptions with respect to contacts between individuals[37, 99]. Velthuis and De Jong[162] quantified transmission of *A. pleuropneumoniae* using experimental infections in pigs in isolations units. In a situation like that, it is probably justified to assume that the population was more or less homogeneous, that one strain of the infectious agent was used and that the contact structure for pigs within different isolation units was largely identical. Those assumptions are not always met for bacterial diseases in field studies.

All major species of mastitis pathogens are comprised of a number of strains[2, 39, 69, Chapter 1]. Some strains have a higher propensity to spread than others. In the Washington State University dairy herd, one strain of *Staph. aureus* caused an outbreak despite the use of control measures that were sufficient to prevent the spread of other strains[101, 135]. In Chapter 4, transmission of *Staph. aureus* in three dairy herds is quantified, and the transmission parameter β was found to be lowest in the herd where management was most conducive to spread of mastitis. Strain typing of a limited number of isolates identified a different predominant strain in the herd with low transmission, than in the other two herds[Chapter 2]. For *Strep. uberis*, a large number of strains has been identified[40, 86, 164]. Only a fraction of strains is identified in multiple quarters or cows within a herd, suggesting that contagiousness is a characteristic of a limited number of strains[16, 118]. In Chapter 7, we present evidence for the spread of one strain of *Strep. uberis* in a herd. Other strains of *Strep. uberis* were present, but did not cause outbreaks. For *Strep. agalactiae,* spread differs with serotype, as evidenced by the average number of infected cows and quarters in a large number of infected herds[68, 68]. All in all, there is ample evidence that mathematical modeling of the dynamics of bacterial mastitis should not be done at level of bacterial species, but at the level of bacterial strains.

R_0 is composed of the cure rate, α, and the transmission parameter, β, which depends on the contact rate between individuals in a population and the probability that a contact between a susceptible and an infectious individual will result in transmission of infection[10, 37, 99]. Different strains of a pathogen may differ in rate of spontaneous clearance,

as demonstrated for *Strep. agalactiae*[69], or in probability of cure after treatment, as is the case for penicillin-resistant *Staph. aureus* compared to penicillin-susceptible isolates[136]. Strain specific differences in cure rate and transmission are accounted for when modeling is done at strain level. However, cure rate, contact rate and the probability of infection upon contact also depend on dairy herd management and on host factors. Cure rate, measured as number of cures per total time at risk of cure, depends on diagnostic procedures, the decision to use treatment, and treatment protocols. Early detection of mastitis is necessary for early treatment decisions, and can result in higher probability of cure upon treatment[138] and higher cure rates[Chapter 4]. Segregation of infected cows or disinfection of milking machine unit liners after infected cows are milked, and use of post-milking teat disinfection are examples of management measures that affect contact between infected and susceptible cows and the probability of infection after contact. The effect of host factors will be discussed in the next section. The number of possible combinations of host factors that affect cure rate and susceptibility is large. As a result, it is difficult to account for all known host factors in compartmental models. Other models, such as discrete event simulation models, are better suited for this purpose[7]. Because R_0 depends on herd management and on strains within bacterial species, and because management and strains differ between herds, it is not meaningful to average R_0 over multiple herds. Despite its limitations, R_0 can be compared between herds or between cows or quarters within herds[78] to identify management or strain characteristics that promote or reduce contagious transmission of mastitis pathogens.

Although mathematical models can be used to simulate herd dynamics of mastitis[5, 6] and to quantify transmission of pathogens when contagiousness is assumed[78, Chapter 4, Chapter 6], it is difficult to provide full proof for a contagious route of mastitis transmission. An environmental origin of mastitis can be shown by means of mathematical, bacteriological and molecular methods. When the number of new infections is independent of the number of existing infections, contagious transmission is unlikely. When the bacteria can be isolated from the environment, and isolates from different cows belong to unrelated strains, it is certain that infections do not originate from cow-to-cow transmission. For contagious transmission to be plausible, the number of new infections has to be a function of the number of existing infections and the composition and contact structure of the population. Furthermore, isolates from different cow must belong to the same strain[Chapter 6, Chapter 7]. However, those prerequisites can also be met following other scenarios, as discussed below.

The mathematical and molecular criteria for contagiousness are met when the number of infected individuals is a proxy that reflects a changing environmental exposure. For example, *Strep. uberis* is known to multiply in straw[63], and increasing numbers of new infections over time could be the result of an increasing pathogen population in straw bedding. When transmission takes place via the environment, infected quarters may still be the original reservoir, e.g. when cows that leak milk contaminate bedding material. If cows are the major source of pathogens, but transmission takes place via the environment, the

infection dynamics cannot be classified in the traditional dichotomy of contagious or environmental spread. Similarly, it is not clear how spread of mastitis pathogens by insect vectors should be classified. Insects can transmit *Staph. aureus* and *Strep. dysgaclactiae*[115, 170]. Mathematically, a model that assumes contagious transmission would represent vector-borne transmission adequately if infected cows are the source of the pathogen, if insects visit infected and susceptible cows at random, and if the number of secondary infections transmitted by flies after contact with a primary infection is limited. When insects derive the pathogen from environmental sources, or when contact with one infected cow is sufficient for transmission of infection to an unlimited number of susceptible cows, the number of new infections may seem independent of the number of existing infections. The best a mathematical model and strain typing can do is to not reject the hypothesis of contagious transmission, which is different than proving it[37]. Studies into mechanisms of transmission, e.g. through isolation and strain identification of pathogens from flies[115], the environment[122], or fomites that explain contagious transmission[Chapter 3, Chapter 7] are necessary to justify the use of specific mathematical models and to support their conclusions.

The mathematical models of mastitis dynamics that are presented in Chapter 4 and Chapter 6 are deterministic models. Random components are not included. When random or probabilistic elements are incorporated in a model, the model is stochastic[84]. Many factors play a role in the dynamics of mastitis, including management[13], host characteristics[Chapters 4, 5 and 6], pathogen characteristics[Chapters 2, 3, 4 and 7], and a wide range of unknown or unmeasured factors, including social behavior of animals, and climate. Point estimates for transition rates or relative risks are not necessarily adequate representations of underlying distributions[84]. Furthermore, any of these characteristics or factors may change over time, and factors may not be independent. Like most real-world systems, the dynamics of mastitis are in fact too complex to be represented by a deterministic model[5]. Even when all risk factors and underlying distributions were known, the best approach is currently to calculate the probability of an event, e.g. the probability of infection or cure. In reality, either the event happens, or it does not. Unmeasured or chance effects make it is impossible to predict what the exact outcome for any specified situation will be. This argument alone is sufficient to explain that in some situations an outbreak occurs, while in other situations, where the same pathogen may be involved and where R_0 may have the same value, a major outbreak does not occur[37]. Using stochastic simulation, a number of outcomes can be generated for any given combination of input parameters[6]. This approach allows for the quantification of the expected average outcome, e.g. average pathogen prevalence or BMSCC, which may be similar to the outcome of a deterministic model. Moreover, with the use of stochastic models, it is possible to estimate a range of possible outcomes, and the likelihood of exceeding a threshold value, such as the BMSCC limit[6].

Host factors in mastitis susceptibility and response to infection

As discussed above, knowledge of host factors does not make it possible to predict with certainty which cow or quarter will develop mastitis or become cured from mastitis. Knowledge of host factors is useful because it may help to predict and influence the probability of infection or cure. At the host level, risk factors for infection, risk factors for severity of disease, and risk factors for cure can be identified. Cow and quarter level risk factors for infection with *Staph. aureus* and *Strep. uberis* mastitis are discussed in Chapter 5. The severity of disease that results from infection depends on host factors, and on diagnostics methods and nomenclature. Sensitivity of bacteriological culture of milk varies with inoculum size and with use of single or multiple samples. Differences in nomenclature can be a source of much confusion. For example, repeated isolation of *Staph. aureus* from human nares is considered to constitute "carriage"[161], repeated isolation of *Staph. aureus* from nares and other human body sites has been called "colonization"[93], and this term that is also used for bovine teat skin[121], but repeated isolation of *Staph. aureus* from milk samples is interpreted as "infection". Throughout this dissertation, isolation of bacteria has been the criterion to define infection, as is customary in bovine mastitis research.

In experimental studies of mastitis, particularly *E. coli* mastitis, severity is commonly measured as the area under the curve of a plot of bacterial concentrations over time[76, 160]. Such detailed information is not available in field studies. Under field conditions, SCC, absence or presence of visual abnormalities of the milk or udder, and systemic signs of disease (rectal temperature, hydration status, rumen activity, attitude) are used to measure disease severity[102, 145, 165]. Data on the association between cow characteristics and severity of *Staph. aureus* are scarce. Within one strain of *Staph. aureus*, marked differences in severity of disease were observed between preparturient heifers and multiparous lactating cows. Overall, our pilot study suggested that pathogen effects, rather than host effects were associated with disease severity[Chapter 2]. Pathogen effects will be discussed in the section on strain characteristics.

For *S. uberis*, the majority of infections is subclinical, i.e. not accompanied by visible abnormalities of milk, udder or habitus[66, 104, Chapter 7]. When cases are clinical, they are usually not accompanied by systemic signs[102, 103]. The proportion of infections that is accompanied by clinical signs decreases with increasing stage of lactation, and increases with parity[66], suggesting a cow effect on outcome of infection. The outcome of infection (subclinical or clinical) in early lactation may theoretically be affected by energy balance and/or ketosis [144], as has been shown for *E. coli* under experimental conditions[76]. In studies of clinical mastitis, early lactation is often identified as a period of increased risk of "mastitis"[102]. It should be noted that for *Strep. uberis*, early lactation is a period of increased risk of showing clinical signs[66], rather than of increased risk of infection[Chapter 5].

Severity of subclinical infections can be quantified based on SCC elevation[Chapter 2, Chapter 7]. This is relevant, because SCC elevation is indicative of damage to the udder tissue and reduced milk production[28, 65]. For *Staph. aureus*, SCC response seemed strain

dependent[Chapter 2]. For *Strep. uberis*, SCC response differed between cows, and seemed independent of bacterial strains[Chapter 7]. This is a preliminary conclusion, based on observation of a limited number of cows in two dairy herds. No other reports on SCC as cow- or strain-dependent response to infection were found. On average, *Strep. uberis* and *Staph. aureus* infected quarters have an increase in ln(SCC) of 2.21 and 2.32, respectively, compared to uninfected quarters[125]. In contrast to what could be expected based on SCC, Morin et al.[103] found production levels in cows with streptococcal infection to be higher than in non-infected cows. They suggest that this could indicate that high producing cows are more susceptible to mastitis, and/or that streptococcal infection has no detrimental effect on production. The second explanation may well apply to infections without elevation of SCC and for infections of short duration. Although *S. uberis* infections may last for more than a year, many infections are of short duration[104, 149, Chapter 7]. A third explanation for high 305-day milk production in cows with streptococcal infections could be that infected cows with high production are less likely to be culled than infected cows with low production, and that 305-day production data for infected cows are biased towards high producing cows. However, preliminary analyses indicated that *Streptococcus* infected cows were less likely to be culled than non-infected cows[103]. To assess economic losses due to mastitis, the impact of infection on milk production needs to be established, at species specific and preferably even at strain specific level[103, Chapter 4, Chapter 7].

The probability of infection and the severity of disease can both be affected by vaccination, as shown for *E. coli* mastitis[64]. Attempts to develop vaccines that protect dairy cows against *Staph. aureus* or *Strep. uberis* mastitis have been under way for decades and still continue today[27,53, 62, 87]. A recent review by Yancey[169] ends with the expectation that efficacious vaccines for the most common mastitis pathogens will become available in the next 10 years. Vaccination is also tested as part of treatment strategies for *Staph. aureus* infections[148]. In the long term, improved resistance to infection may be achieved through selective breeding. Genetic traits of cows are known to contribute to susceptibility to *Staph. aureus* mastitis[128], but genetic progress through selection of cows is a slow process. Recently, testing of bulls showed that there is an association between in vitro response of lymphocytes to *Staph. aureus* and SCC in bull progeny[49]. The possibility to test bulls directly, rather than through calculation of predicted transmitting abilities based on daughter records, would open the way to faster progress in breeding for mastitis resistance.

Once a cow is infected, risk factors for cure, i.e. factors that affect the probability of cure, become more important than risk factors for infection. Risk factors for cure have been described for subclinical and clinical *Staph. aureus* mastitis after treatment in lactation or the dry period[114, 136-138]. They include parity, mean SCC before treatment, number of infected quarters, and occurrence of clinical episodes in the preceding lactation. *Strep. uberis* specific risk factors for cure of mastitis after therapy will need to be identified in future research. It can be anticipated that known risk factors, together with cow characteristics that determine an animal's economic value, will be incorporated in decision support software for treatment and culling decisions on dairy farms.

Strain characteristics associated with mastitis epidemiology

In addition to host characteristics, pathogen characteristics affect the probability of infection, the severity of disease, and the chance of cure. *Staph. aureus* lineages that cause intramammary infections in cows are largely different from human *Staph. aureus* lineages[72, 82, 90, Chapter 2]. For *Strep. agalactiae* and *Strep. dysgalactiae*, differences between bovine and human strains exist, too[18, 70, 92]. *Staph. aureus* isolates from healthy teat skin differ from isolates from milk[Chapter 3]. All in all, some strains within the species are more likely to cause intramammary infections than others. This is also apparent from the fact that some strains of *Staph. aureus*[101, 135], *Strep. uberis*[120, Chapter 7], and *Strep. agalactiae*[68, 69] spread more rapidly than others, as discussed in the section on mathematical modeling. While there is a large variety of *Staph. aureus* strains, the majority of intramammary *Staph. aureus* infections in multiple regions and countries is caused by a limited number of predominant strains[4, 26, 47, 72, 83, 105, 120].

Staph. aureus strains with high prevalence show more resistance to phagocytosis by neutrophils in vitro than strains with low prevalence[3, 105, 143]. Resistance to host immune responses enhances the probability of establishment of infection and of survival in the mammary gland for prolonged periods of time. Other factors that potentially contribute to evasion of mechanical (milking) as well as immunological clearance are adhesion to and invasion into udder epithelial cells[8, 9, 80, 86, 146]. Most studies on invasion are carried out in vitro[17, 41, 166], and the role of *Staph. aureus* invasion in vivo is debated[91]. Presence of intracellular *Staph. aureus* in udder epithelial cells from naturally infected cows has been demonstrated[55]. This supports the idea that invasion plays a role in vivo, but quantitative evidence for the importance of this phenomenon remains to be provided. In vitro, adhesion and invasion of *Staph. aureus, Strep. uberis* and *E. coli* is strain dependent[38, 56, 97]. Intracellular bacteria are protected from the immune system, as well as from the effect of most antibacterial treatments. Particularly for *Staph. aureus*, intracellular survival is thought to play a role in the probability of cure[9, 55, 80]. Strain specific abilities to survive and multiply intracellularly may lead to strain specfic probabilties of cure. Another virulence factor that can tip the balance between infection and clearance is the growth potential of bacterial strains in milk[75, 86, 119]

Pathogen characteristics can be associated with the likelihood of inducing infection, and with the severity of the response to infection. In a pilot study, associations between strain of *Staph. aureus* and severity of disease were observed, including differences in presence or absence of SCC elevation and clinical signs[Chapter 2]. An association between *Staph. aureus* strain and milk production of infected quarters has also been observed[100]. *Staph. aureus*[50, 146] and *Strep. uberis*[86, 111] produce many virulence factors, but the importance of those factors in vivo is largely unknown. For staphylococci, a role of enterotoxins in severity of disease has been suggested[74, 96]. In vitro, leukotoxins and superantigens induce killing of polymorphonuclear neutrophils and proliferation of mononuclear cells[126]. *Staph. aureus* strains that produce enterotoxin A are better at evading

host defense mechanisms than strains without enterotoxin, and this trait is associated with high prevalence of enterotoxin producing strains[105]. However, no association was found between toxin production and clinical outcome of infection in vivo[46, 126, 150]. In some regions, hardly any *Staph. aureus* produce enterotoxins, while staphylococcal mastitis exists in subclinical and clinical manifestations[1, 82, 85]. Thus, the role of enterotoxins in clinical manifestation of *Staph. aureus* infections remains poorly understood. Similarly, the significance of capsule for virulence is unknown[151]. For a limited number of *Strep. uberis* strains, the ability to resist phagocytosis and killing by neutrophils in vitro has been associated with severity of experimentally induced disease in vivo[57, 88]. In field studies, clinical disease is sometimes associated with one out of a number of *Strep. uberis* strains, but in other herds such an association is not found[67, 118, Chapter 7].

Response of *Staph. aureus* infections to treatment is often poor[50]. Strain-specific intracellular survival and resistance to host immune responses, as discussed above, are likely to contribute to differences in cure rates. A more direct link between strain characteristics and chances of cure is found in the penicillin resistance of *Staph. aureus*. Compared to infections by penicillin-sensitive strains, infections by penicillin resistant strains were numerically and statistically less likely to be cured upon treatment of subclinical and clinical mastitis, respectively, even when antibiotics were used to which the staphylococcal isolate was susceptible in vitro[136-138]. The pathobiology underlying this association is unknown. For *Strep. uberis*, cure rates after treatment are often high, although differences between herds are observed[24]. Such differences could be associated with management, cow or strain characteristics, but no research has been done in this area.

Molecular epidemiology of bovine mastitis

The main concepts and techniques used in molecular epidemiology have been introduced before[Chapter 1], and are discussed in detail in a number of reviews[52, 94, 142, 155]. Molecular techniques are used to identify bacterial species and strains, and to examine their relatedness. Phenotypic techniques are less reliable as indicators of relatedness than genotypic techniques, because expression of genes may vary with (experimental) circumstances[81, 141]. Non-identity of isolates can be proven by many genotypic techniques. Identity of isolates can only be proven by DNA-sequencing, but genetic relatedness of isolates can be made plausible when multiple techniques are combined and fail to discriminate between isolates. This is especially true when isolates also have a common epidemiological background[141]. In the absence of a gold standard for strain designation, epidemiological information is often used to validate a typing technique. Once the typing technique has been validated, it can subsequently be implemented to enhance our understanding of epidemiology[52].

In mastitis research, strain-typing techniques have been used to determine whether indistinguishable or different strains are found when typing multiple isolates

1. from a single sample (differentiation between infection with a single strain and simultaneous infection with multiple strains[118, 171])
2. from consecutive samples from a single udder quarter (differentiation between re-infection and non-cure[39, 112]; susceptibility to reinfection with homologous or heterologous strains[45, 58])
3. from samples from multiple quarters within a cow (differentiation between possible cross-infection and infection from multiple sources[22, 118, Chapter 7])
4. from multiple cows within a herd (differentiation between contagious and environmental epidemiology[16, 118, Chapter 7])
5. from multiple herds (identification of targets for vaccination[53, 139])
6. from milk, body sites and surroundings of dairy cows (identification of fomites and reservoirs[51, 124, Chapter 3])
7. from animals, dairy products and humans (identification of sources of antibiotic resistant bacteria[30]; identification of sources of food poisoning[167]; role of humans as carriers of animal pathogens[82, 147, Chapter 3])
8. from cows and food processing equipment (quality control of foods of animal origin[34, 152]).

As safety standards for food of animal origin become more strict, it is to be expected that retailers or processing plants will start to implement typing techniques on a routine basis as part of CCPM (critical control point management) or HACCP (Hazard Analysis Critical Control Point) procedures. Typing techniques can be used to trace the origin of foods that are contaminated with pathogenic bacteria that are possibly of animal origin. Routine use of strain typing, other than antibiotic susceptibility testing, is not feasible yet in veterinary practice and dairy herd management. This may change, if associations between strains and characteristics such as cure rates, severity of disease, or speed of spread can be shown to hold true across animals, herds and countries. For strains that occur in multiple herds and regions, investments into identification of strain specific cure probabilities could pay off, if such knowledge is used to produce diagnostic tests that are coupled with strain specific prognosis. This approach could contribute to rational decision-making about the choice of treatment or culling, and could contribute to selective use of antibiotic therapy. Similarly, if virulence and speed of spread within a herd is strain dependent[135] and consistent across herds, strain specific advice on mastitis prevention and control could be formulated. To prevent damage from a highly virulent, fast-spreading strain, immediate action and strict control measures are of paramount importance. For less virulent and slow spreading strains, control is less urgent, and allocation of farm resources to other areas than udder health management may be rational.

Applications of strain typing techniques in herd management and food production are most likely to be used for *Staph. aureus* in the foreseeable future. For *Strep. uberis*, information on distribution of strains in intramammary infections is limited, and information on strains from extra-mammary sources is virtually non-existent. As a consequence, hardly anything is known about associations between specific strains and characteristics that play a role in pathogenesis or epidemiology. In situations where the number of strains is almost as large as the number of isolates[40] studies into strain specific characteristics may not be relevant. In herds or regions with predominance of a limited number of strains, knowledge of strain-specific cure rates or virulence, and identification of fomites and reservoirs of *Strep. uberis* may contribute to mastitis control.

One of the major challenges in future strain typing studies will be the choice of appropriate techniques[141, 154]. If researchers around the world want to compare results, use of library typing techniques[43, 142] or highly standardized experimental protocols[163] is necessary. Currently, PFGE is the most popular technique for typing of *Staph. aureus*[154] and *Strep .uberis*[40, 118, 164]. Unfortunately, inter-center reproducibility of PFGE is still limited[31, 32, 156]. Binary typing, which is a RAPD derived technique, is suitable for typing of human *Staph. aureus*[157, 158] but would need to be developed for bovine *Staph. aureus* and *Strep. uberis* to be applicable in the veterinary field. Sequence based typing of single[129, 130] or multiple genes[43, 140] and the use of DNA microarrays[48] is too expensive for routine diagnostic laboratories, but will be used increasingly in research laboratories. With the advent of ever more sophisticated typing techniques, the real challenge will be to interpret typing results in terms of genetic relatedness, and to understand their relevance in terms of pathobiology and epidemiology.

Udder health management in dairy herds

Ideally, mastitis research should result in recommendations for herd management that help farmers to reduce the incidence and prevalence of mastitis in a cost-effective manner. The standard mastitis prevention program or five-point plan is one of the most successful products of mastitis research. Since its development in the 1960s[107], it has been applied in many dairy countries around the world with considerable success[20]. Meanwhile, additional measures have been developed, as discussed in Chapter 1, and summarized in the National Mastitis Council's recommended mastitis control program or ten-point plan (http://www.nmconline.org/docs/NMC10steps.pdf). In The Netherlands, a number of programs related to udder health and milk quality have been started, including herd specific planning of control strategies, routine sampling of milk from cows with subclinical mastitis, and combined analysis of milk recording and mastitis data[12].

Herd specific management should be based on the farm situation (high or low BMSCC, high or low incidence rate of clinical mastitis), the farmer's goals, and the

predominant pathogen[12, 15]. The word "pathogen" needs to be interpreted at species level, but also at strain level. The traditional classification of pathogen species as either contagious or environmental is an oversimplification of reality. Within staphylococcal and streptococcal species, strains with contagious epidemiology and strains with environmental epidemiology occur. In herds with *Staph. aureus* mastitis, control of contagious transmission is essential, but not sufficient to eradicate the bacteria. The risk of new infections is always present, as *Staph. aureus* occurs in the environment of the cow. In addition, introduction of the pathogen into the milking herd may occur with herd additions or with animals that enter the lactating population after calving. To avoid spread of *Staph. aureus*, a control program that limits contagious transmission needs to be in place at all times. Herd additions should be screened for mastitis before they enter the milking herd. For *Strep. uberis*, the environment is relatively more important as a source of infection. Implementation of the five-point control plan may seem unrewarding because it doesn't prevent infections of environmental origin. However, if cow-to-cow transmission is not prevented, e.g. when infected animals are not treated, segregated from the herd, or culled, outbreaks of *Strep. uberis* mastitis may occur. Thus, prevention of contagious transmission is part of *Strep. uberis* prevention too.

Segregation of infected animals can be an effective way to prevent spread of mastitis pathogens via the milking machine[168]. Segregation can be physical, i.e. by means of separate housing and milking of a group of infected animals, or functional, as achieved through disinfection of milking machine unit liners with hot water (90°C) after milking of infected cows[Chapter 4]. In herds where post-milking teat disinfection and use of antibiotics are limited, e.g. in organic dairy farms, segregation can be used to reduce the incidence of new infections. To be able to treat, segregate, or cull infected animals, the infection status of animals must be known. Routine screening of cow milk SCC and bacteriological culture of milk from cows with elevated SCC is possible through the Royal Dutch Dairy Syndicate and the Dutch Animal Health Service. Although some infections with low SCC may go undetected, screening of SCC is a cost-effective manner to select samples for culture. If new infections continue to occur when all high SCC cows have been identified and dealt with, other possible sources of infection need to be investigated, including cows that do not show SCC elevation, the milking machine, teat dips, water and bedding material. For early detection of infected animals, herd screening must take place frequently, e.g. monthly. Early detection and treatment of infected animals improves cure rates. As a result, prevalence of infection and subsequent spread to other animals in the herd are limited.

When deciding whether an infected cow should be treated or culled, factors that affect the probability of cure, factors that affect the susceptibility of the cow to infection, and the value of the cow should be taken into account. Older animals have a lower probability of cure and a higher risk of new infection than first or second parity animals. Unless all quarters of a cow are cured, infected quarters may reinfect the cured quarters. Recovery from infection does not protect against reinfection. On the contrary, quarters that recover from *Staph. aureus* or *Strep. uberis* infection are subsequently more susceptible to

infection with either pathogen. Quarters with rough or extremely callused teat ends are at an increased risk of infection with *Staph. aureus* compared to teat ends that are less severely callused. To prevent extreme teat end callosity, proper maintenance, function and use of the milking machine are important[108].

Many udder health problems can be solved with our current understanding of mastitis epidemiology[59, 60, Chapter 4]. To promote the use of existing knowledge, continued education of farmers, veterinarians and herd advisors is necessary[12]. For future improvement of udder health in dairy herds, incentives to implement known control measures are needed, together with further research into cost-effective ways to prevent and cure mastitis.

Future studies of mastitis

Many gaps exist in our knowledge and understanding of mastitis. Any study raises at least as many questions as it answers. In each chapter of this dissertation, unanswered questions have been identified. A few areas of future research will be discussed here.

The ability to identify strains within bacterial species, coupled with a willingness to let go of existing paradigms, may give nuance to our understanding of the epidemiology of pathogens or strains that are contagious, environmental, in between, or neither. Within the coagulase negative staphylococci and the so-called "environmental streptococci", bacteria must be identified at the species level if we want to move beyond our current understanding of their epidemiology and pathobiology. As a first step, species identification in veterinary practice can be based on phenotypic characteristics. The next step is species identification by means of genotypic methods performed by veterinary microbiology laboratories or specialized diagnostic labs. Genotyping is preferred, because genotypic traits are less variable than phenotypic traits. Strain identification will be an essential elements of attempts to understand why existing management programs fail to eradicate disease, even when implemented.

Research on the epidemiology of different genotypes within a bacterial species will permit quantification of strain specific potential for spread, virulence, probabilities of cure, and other characteristics. Identification of strains from intramammary infections, bulk tank milk, farmers and food handlers, the cow's environment, and processing plants allows for identification of sources of infection or contamination, and for quantification of the relative importance of sources. The use of molecular techniques should be integrated into studies of mastitis epidemiology and food safety, including population studies, clinical trials, and quality control procedures. Interpretation and comparison of results from strain typing studies would be facilitated if library typing techniques were developed and used, such as binary typing systems or multilocus sequence typing. Widespread use of strain specific treatment decisions or management advice in dairy farms will only be possible

once typing techniques have been implemented in routine diagnostic laboratories and veterinary practices.

It is conceivable that the majority of isolates of *Staph. aureus* that cause very mild or very severe disease of short duration are genetically different from isolates that cause chronic subclinical disease in dairy cows. The first group of strains may be better suited to survival in the environment, while the second group is adapted to survival in the mammary gland. Differences in virulence could be associated with differences in epidemiology, as strains that survive in the udder for prolonged periods of time are more likely to spread from cow-to-cow than strains with limited presence. At the herd level, presence of environmental *Staph. aureus* could manifest as a problem of high incidence rate of clinical mastitis in combination with low BMSCC. Presence of cow-adapted *Staph. aureus* is more likely in herds with high BMSCC and low incidence rate of clinical mastitis. Isolation and characterization of *Staph. aureus* isolates from low and high BMSCC herds could reject or support this hypothesis. If the hypothesis is supported by strain typing studies, experimental studies may be needed to establish whether strains differ in virulence and potential for spread. A similar scenario can be envisaged for *Strep. uberis*. The proportion of cow-adapted strains with contagious transmission is probably much smaller for *Strep. uberis* than for *Staph. aureus*. Environmental sources of *Strep. uberis* strains that cause intramammary infections need to be identified to improve our understanding and control of *Strep. uberis* mastitis. In addition, it is necessary to establish how *Strep. uberis* is introduced to the cow's environment, i.e. from infected cattle, or from outisde sources.

Another area where more work is needed is understanding risk factors for mastitis. Some risk factors are known, but often the underlying pathobiology is poorly understood. For example, older cows are more likely to become infected and less likely to cure than younger cows. What mechanism causes this difference? So far, risk factor studies at the cow and quarter level provide limited information on how to prevent mastitis. This is primarily because many risk factor cannot be prevented (e.g. stage of lactation, parity, quarter position), and also because known risk factors only explain part of the variability between cows and quarters. Additional insight may be gained from immunological and genetic studies, or from studies on the interaction between pathogens and milk (growth of pathogens in milk) or udder tissue (adhesion, invasion, intracellular survival and replication). At herd level, many management factors associated with increased risk of clinical mastitis have been identified in recent years. Unfortunately, the causal relationship between mastitis and management factors is often unknown, and painstaking longitudinal studies will be necessary to identify causal associations and to formulate evidence-based mastitis control strategies.

Dairy farming is an economic activity. From an economic point of view, a control strategy is only successful when it is cost-effective. Statistical and mathematical models can be used to identify risk factors for infection and cure, to test hypotheses on mechanisms of spread of mastitis in a population, and to quantify the effect of risk factors and control measures on disease dynamics. Costs and benefits of multiple scenarios for sampling,

testing, treatment, culling, and management intervention can be examined in optimization models or in stochastic simulation models. The more our knowledge of mastitis expands, the more complex models will become. For example, to determine the economic viability of treatment of subclinical mastitis, a model would need to include the likelihood of detection of infection under different sampling and culture strategies, the probability of cure with or without treatment as determined by clinical trials, the distribution of host-level risk factors for cure, the probability of spread given the herd management and the pathogen strain, and the costs of diagnosing or not diagnosing mastitis and its consequences. Characteristics of cows and strains may be taken into account when making treatment and management decisions, and ideally, a model would incorporate this. For example, a herd manager may decide to treat only those cows with an expected probability of cure of more than 50% and above average production, and to cull other infected cows. Of the models that are currently available to simulate mastitis dynamics and control strategies, including costs and benefits, discrete event simulation modeling seems most versatile and most suitable for incorporation of our vast and expanding scientific knowledge base. Future studies of mastitis pathobiology and epidemiology with molecular, statistical, and mathematical techniques will enhance our understanding of prevention and control options, but economic modeling will be needed to determine their feasibility.

It is very likely that therapeutic options and protocols for treatment of mastitis improve in the future. However, prevention of mastitis is preferable over cure, especially in countries where consumer concern over animal welfare and food safety is an issue. A combination of improved understanding of the epidemiology of mastitis and identification of pathogens at species and strain level, insight in herd, cow- and quarter level risk factors and their causal role in mastitis, improved host resistance through vaccination or breeding, and incentives to implement our knowledge, should make it possible to control mastitis with restricted use of antibiotics, for the health and welfare of humans and cows alike.

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Summary

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Summary

Mastitis is the most common and costly production disease affecting dairy cows. Literally, "mastitis" is "inflammation of mammary gland tissue". This dissertation deals with intramammary infections caused by *Staphylococcus aureus* and *Streptococcus uberis*. In The Netherlands, those bacterial species are among the most prevalent causes of clinical mastitis (udder inflammation accompanied by visible abnormalities of milk or udder, or by systemic signs) and subclinical mastitis (udder inflammation without visible abnormalities). Mastitis is a major concern to dairy producers and the food industry because it affects milk quantity and milk quality, cow health, and possibly food safety and public health (**Chapter 1**). Based on our current understanding of mastitis epidemiology, control programs have been formulated. The choice of control measures is often based on a classification of pathogen species as "environmental" or "contagious". Environmental pathogens are present in the environment of the dairy cow, and the cow may be exposed to such pathogens at any time. Infected udders form the main reservoir of contagious pathogens, and exposure of cows to contagious pathogens is largely limited to the milking process. Existing control programs have contributed to a decrease in bulk milk somatic cell counts, which reflect the prevalence of mastitis. In recent years, progress in mastitis control in the Netherlands has stagnated. The aims of the project described in this dissertation were to improve our understanding of the dynamics of *Staph. aureus* and *Strep. uberis* infections in dairy herds that implement standard control measures, and to identify host and pathogen traits that affect the occurrence and outcome of infection. To achieve this, a combination of molecular and biometrical approaches to mastitis epidemiology is used.

In **Chapter 2**, the use of two molecular typing techniques for identification of bovine *Staph. aureus* isolates is explored. Thirty-eight bovine mammary *Staph. aureus* isolates from diverse clinical, temporal and geographical origins were genotyped by pulsed-field gel electrophoresis (PFGE) after *Sma*I digestion of prokaryotic DNA. After Southern blotting of DNA macrorestriction fragments, isolates were subsequently strain typed by means of binary typing (BT) with the use of 15 DNA probes that had been developed for typing of human *Staph. aureus* strains. PFGE identified seven main types with four subtypes and BT identified 16 types. Genetic relatedness of isolates as documented by the two techniques was concordant. Binary types of bovine isolates were compared to binary types of 55 human *Staph. aureus* strains, including methicillin-resistant isolates. Clusters of genetically related strains were predominantly associated with one host species, although a mixed cluster was observed. For 28 bovine *Staph. aureus* infections, clinical and epidemiological observations in vivo were compared to strain typing results. Associations were found between distinct genotypes and severity of disease, suggesting strain-specific virulence. Circumstantial evidence supported an environmental origin of one strain. It was concluded that PFGE and BT can be used successfully for genotyping of *Staph. aureus* isolates from bovine mammary secretions. BT in particular seemed a promising tool for strain

characterization, for comparison of strains from different host species and different databases, and for identification of sources and transmission routes of bovine *Staph. aureus.*

In **Chapter 3,** PFGE is implemented to examine the role of reservoirs and fomites of *Staph. aureus* mastitis. In addition, the usefulness of BT for large-scale studies of bovine mastitis is assessed through comparison to epidemiological data and PFGE, which is currently the gold standard for *Staph. aureus* typing. PFGE patterns of 225 *Staph. aureus* isolates from healthy bovine teat skin, human skin, milking machine unit liners, and milk were compared. PFGE of *Sma*I digested DNA identified 24 main types and 17 subtypes among isolates from 42 herds, and discriminated between isolates from healthy bovine teat skin and bovine milk. Human skin isolates belonged to the same PFGE types as bovine skin isolates. Milking equipment harbored strains from teat skin and from milk. It is concluded that the milking machine is a fomite for the spread of *S. aureus* strains from bovine teat skin and for strains from milk, but that bovine teat skin and skin of milkers' hands are not important sources of bovine intramammary *S. aureus* infections. Phage typing (Fox, L. K., M. Gershmann, D. D. Hancock, and C. T. Hutton. 1991. Cornell Vet. 81:183-193) was less discriminatory than PFGE and failed to differentiate between isolates from skin and milk. A subset of 142 isolates was identified by binary typing with probes for human *S. aureus*. Typeability, discriminatory power and concordance with site of isolation were lower than for PFGE. Therefore, BT with human probes is not suitable as stand-alone technique for typing of bovine *Staph. aureus*. Within several PFGE types, binary typing discriminated between main types and subtypes, and between isolates from different herds or different sources. Thus, BT may be useful in combination with PFGE to refine strain differentiation. Development of probes that are specific for bovine *Staph. aureus* will be necessary to improve typeability and discriminatory power.

In addition to molecular approaches, mathematical approaches were used to study *Staph. aureus* epidemiology. An ordinary differential equation model to describe the dynamics of *Staph. aureus* mastitis in dairy herds is presented in **Chapter 4**. Herds were characterized as populations of udder quarters that were never infected, subclinically infected, clinically infected, or recovered from infection. Data to estimate model parameters were obtained from a longitudinal observational study in three commercial dairy herds. For 18 months, milk from all udder quarters of all lactating cows was sampled upon entry into the population (purchase or calving), routinely at 3-week intervals, when clinical mastitis was observed, and upon exit from the population (dry-off or culling). A deterministic simulation model was constructed to estimate the value of the basic (R_0) and effective (R_t)

reproductive number in each herd, and to simulate the effect of changes in herd management on mastitis control. In all herds, R_0 was below the threshold value 1, which indicates that cow-to-cow transmission of *Staph. aureus* was successfully controlled. R_t was higher than R_0 because recovered individuals were more susceptible to infection than individuals without a prior infection history. Simulations showed that the model described the dynamics in two herds adequately, and that treatment of subclinical mastitis and prevention of influx of infected individuals into the population contribute to fast decrease of *Staph. aureus* prevalence to low levels. For one herd, the model failed to mimic field observations. Possible causes for this failure are discussed, and include the inability of a deterministic model to capture random variability, the assumptions underlying the model, e.g. the assumption that management did not change over time, and the possibility that new infections do not result from cow-to-cow-transmission.

In compartmental models, the subpopulation that constitutes a compartment is assumed to be homogeneous. In reality, cows differ in their susceptibility to mastitis. Knowledge of factors that increase or decrease the probability of infection can contribute to mastitis prevention. Cow and quarter level risk factors for *Staphylococcus aureus* and *Streptococcus uberis* mastitis are described in **Chapter 5**. The quarter milk samples from the longitudinal observational study in three dairy herds were used for bacteriology and somatic cell counting. For each animal, data on parity, lactation stage, and bovine herpesvirus-4 serology were recorded. During the last year of the 18-month study, body condition and teat end callosity were scored at 3-week intervals. In total, 100 new infections with *Staph. aureus* were detected in 22,593 observations on lactating quarters at risk of *Staph. aureus* infection, and 93 new infections with *Strep. uberis* were detected in 22,665 observations on lactating quarters at risk of *Strep. uberis* infection. Multivariable Poisson regression analysis with clustering at herd and cow level was used to identify risk factors for infection. Rate of *Staph. aureus* infection was higher in cows of parity 3 or above, in bovine herpesvirus-4 positive cows, in right quarters, in quarters that had recovered from prior *Staph. aureus* or *Strep. uberis* infection, in quarters exposed to other *Staph. aureus* infected quarters in the same cow, in quarters with SCC > 250,000 cells/ml prior to infection, and in quarters with extremely callused teat ends. Infection with coagulase negative staphylococci was not significant as a risk factor. The effect of infection with corynebacteria on rate of infection with *Staph. aureus* depended on herd, stage of lactation, and teat end roughness. Rate of infection with *Strep. uberis* was higher in older animals than in first and second parity animals, and depended on stage of lactation in one herd. Quarters that were infected with *Arcanobacterium pyogenes* or enterococci, quarters that had recovered from prior *Strep. uberis* or *Staph. aureus* infection, and quarters that were exposed to another *Strep. uberis* infected quarter in the same cow had a higher rate of *Strep. uberis* infection. Teat end callosity, previous quarter milk SCC and infection with coagulase negative

staphylococci or corynebacteria were not significant as risk factors. Body condition score and change in body condition score were not associated with the rate of *Staph. aureus* or *Strep. uberis* infection.

In one herd, an outbreak of *Strep. uberis* mastitis occurred. This outbreak is analyzed in **Chapter 6**. During the 18-month observation period, 54 infections with *Strep. uberis* were observed, and the majority occurred during a 21-week period that constituted the disease outbreak. The infection dynamics are described by an ordinary differential equation model. In the *Strep. uberis* model, the non-infected population is subdivided in one of two ways. First, the susceptibility of recovered individuals is examined in a model with a compartment of uninfected individuals that don't have a history of *Strep. uberis* infection and a recovered compartment. Next, the impact of minor pathogens on subsequent infection with *Strep. uberis* is examined in a model where the susceptible population is split into a subpopulation with other pathogen infection (mostly minor pathogens) and a subpopulation without other pathogen infection, irrespective of recovery history. The expected number of new *Strep. uberis* infections per 3-wk interval was described by means of a Poisson logistic regression model. Significant predictor variables in the model were the number of existing *Strep. uberis* infections in the preceding time interval (i.e. the prevalence of quarters that shed *Strep. uberis* in milk), the phase of the study (early phase vs. post-outbreak phase), and recovery from prior *Strep. uberis* infection, but not infection status with respect to other pathogens. Results with respect to recovery and minor pathogens from the population-based mathematical model in Chapter 6 were in agreement with the results from the host-based statistical model in Chapter 5. The finding that the number of existing infections was a significant predictor for the number of new infections, is in agreement with the hypothesis that the outbreak was the result of contagious transmission of *Strep. uberis* mastitis. This hypothesis could be supported or refuted by molecular typing of strains involved in the outbreak.

Clinical, epidemiological and molecular characteristics of *Strep. uberis* infections are described in **Chapter 7**, based on 84 intramammary infections that were detected in 70 quarters of 46 cows in two dairy herds. The majority of infections were subclinical and occurred during lactation. Chronic infections and infections with non-elevated SCC (< 250,000 cells/ml) were observed in both herds. Out of 348 *Strep. uberis* isolates, 152 isolates originating from 52 quarters were genotyped by means of random amplified polymorphic DNA (RAPD) fingerprinting. In each herd, multiple strains were identified but one RAPD type predominated. Persistent infections within a quarter were usually caused by one strain, while recurrent infections could be caused by the same or by different strains. When multiple quarters of a cow were infected, infections were mostly caused by

the same strain. No associations were found between strains and SCC elevation or presence of clinical signs. In one herd, infections with the dominant strain were significantly longer than infections with other strains. The majority of infections during an outbreak of *Strep. uberis* mastitis in the other herd (see also Chapter 6) was attributable to one strain. Infections with this strain had all started during lactation, while the majority of infections with other strains started during the non-lactating period. The association between strain and time of onset was highly significant. *Strep. uberis* could be isolated from milking machine unit liners after an infected cow had been milked, and after one or two subsequent non-infected cows had been milked. The strains isolated from the liners were identical to the strains isolated from the infected cows. Taken together, the epidemiological and molecular data support within-cow and between-cow transmission of specific *Strep. uberis* strains i.e. contagious transmission, with possible transfer of bacteria via the milking machine, and infection from environmental sources for other strains.

In **Chapter 8**, combined results from the preceding chapters are discussed, and recommendations with respect to udder health management and future research are given. The main conclusion with respect to udder health management is that not all *Staph. aureus* spreads through contagious transmission, and not all *Strep. uberis* spreads through environmental transmission. Therefore, control of contagious transmission is essential to prevent spread of both *Staph. aureus* and *Strep. uberis*, but insufficient to eradicate either pathogen. The terms "contagious" and "environmental" transmission should not be interpreted at the level of the bacterial species, but at the level of the bacterial strain and prevention and control measures should be chosen based on the epidemiology of the bacterial strains that occur in a herd. Because virulence characteristics that play a role in the pathobiology and epidemiology of mastitis can be strain specific, future studies of mastitis pathogens, including population based studies, clinical trials, and studies of pathogenesis, must consider identification of bacteria at species level, as well as identification at strain level.

Samenvatting

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Mastitis is de meest vóórkomende ziekte bij melkkoeien in Nederland. "Mastitis" betekent letterlijk "ontsteking van het melkklierweefsel". Dit proefschrift gaat over uierontsteking ten gevolge van infecties met *Staphylococcus aureus* en *Streptococcus uberis*. Deze bacteriën behoren in Nederland tot de belangrijkste verwekkers van klinische mastitis (uierontsteking met zichtbare afwijkingen aan melk, uier, of koe) en subklinische mastitis (uierontsteking waarbij geen afwijkingen te zien zijn met het blote oog). Mastitis is van groot belang voor veehouders en voedselproducenten omdat de ziekte nadelige gevolgen heeft voor melkhoeveelheid en melkkwaliteit, diergezondheid en dierwelzijn, en mogelijk voor voedselveiligheid en volksgezondheid (**Hoofdstuk 1**). Op basis van de huidige kennis over mastitis zijn programma's opgesteld voor het bewaken en verbeteren van uiergezondheid. De keuze van maatregelen in dergelijke programma's wordt vaak gebaseerd op de classificatie van mastitisverwekkers als "omgevingskiemen" of "koegebonden kiemen". Omgevingskiemen zijn aanwezig in de omgeving van de koe. De koe kan op elk willekeurig moment aan zulke bacteriën blootgesteld worden. Koegebonden bacteriën bevinden zich voornamelijk of uitsluitend in geïnfecteerde uierkwartieren. Koeien worden tijdens het melken blootgesteld aan deze bacteriën. Toepassing van de bestaande mastitis-preventieprogramma's heeft bijgedragen tot een sterke verlaging van het gemiddelde tankmelkcelgetal in Nederland tussen 1980 en 1995. Het tankmelkcelgetal is een weerspiegeling is van de mate van vóórkomen van mastitis. De afgelopen jaren heeft de verbetering van uiergezondheid in Nederland niet doorgezet. Sinds het voorjaar van 2000 wordt zelfs een stijging van het tankmelkcelgetal gezien. Daarnaast blijft het optreden van klinische mastitis een probleem op bedrijven in elke celgetalklasse. Doel van dit promotie-onderzoek was het vergroten van het inzicht in de verspreiding van *Staph. aureus* en *Strep. uberis* infecties op bedrijven die standaard- preventiemaatregelen toepassen, en het identificeren van koe- en bacterie-eigenschappen die het aanslaan en de ernst van uierinfecties beïnvloeden. De epidemiologie van mastitis is in dit project bestudeerd met een combinatie van moleculaire en biometrische (statistische en wiskundige) technieken.

In **Hoofdstuk 2** wordt de bruikbaarheid van twee moleculaire typeringstechnieken voor de identificatie van bovine *Staph. aureus* beschreven. Achtendertig *Staph. aureus* isolaten zijn onderzocht met behulp van pulsed-field gel electroforese (PFGE) en binaire typering (BT). De isolaten waren afkomstig van diverse dieren en representeerden variatie in tijd, plaats en ernst van het klinisch beeld. Na *SmaI* digestie van bacterieel DNA werd PFGE gedaan, gevolgd door Southern blotting van macrorestrictiefragmenten en binaire typering (BT) met 15 DNA probes (herkenningsfragmenten) die ontwikkeld waren voor typering van humane *Staph. aureus* stammen. Met PFGE werden zeven hoofdtypen en vier subtypen onderscheiden, terwijl met BT 16 typen onderscheiden werden. De genetische verwantschap tussen isolaten die werd aangetoond met PFGE was vrijwel hetzelfde als de genetische verwantschap op basis van BT. Binaire types van bovine isolaten werden

vergeleken met binaire types van 55 humane *Staph. aureus* isolaten, inclusief methicilline-resistente stammen (MRSA of "ziekenhuisbacterie"). Clusters van genetisch verwante isolaten waren overwegend gastheerspecifiek, maar er werd ook een cluster van gemengde herkomst gevonden. Voor 28 bovine *Staph. aureus* isolaten waren klinische en epidemiologische gegevens bekend over de dieren en bedrijven van herkomst. De ernst van het klinisch beeld bij de levende koe was significant geassocieerd met de *Staph. aureus* stam die uit de melk geïsoleerd werd. Ook werden aanwijzingen gevonden voor stam-specifieke verspreidingsmechanismen. Uit deze verkennende studie blijkt dat zowel PFGE als BT met succes kan worden gebruikt voor de genotypering van bovine *Staph. aureus* isolaten uit uiersecretum. Vooral BT leek een veelbelovende techniek voor het vergelijken van stammen afkomstig van verschillende gastheersoorten en uit verschillende gegevensbestanden en voor het identificeren van bronnen en verspreidingswegen van bovine *Staph. aureus.*

Hoofdstuk 3 beschrijft het gebruik van PFGE bij onderzoek naar de rol van reservoirs en passieve vectoren van *Staph. aureus*. Ook werd de bruikbaarheid van BT voor grootschalig onderzoek naar rundermastitis getest. Daartoe werden BT resultaten vergeleken met epidemiologische gegevens en met de resultaten van PFGE. PFGE is op dit moment de gouden standaard voor typering van *Staph. aureus*. PFGE patronen van 225 *Staph. aureus* isolaten, afkomstig van gezonde speenhuid, handen van melkers, tepelvoeringen en melk, werden met elkaar vergeleken. De isolaten waren afkomstig van 42 bedrijven. Na *SmaI* digestie van DNA werden met PFGE 24 hoofdtypen en 17 subtypen onderscheiden. In melk werden andere typen gevonden dan op de speenhuid. Isolaten van de handen van melkers waren vergelijkbaar met isolaten van speenhuid. Een deel van de isolaten uit tepelvoeringen was van hetzelfde type als de isolaten uit melk en een deel was verwant aan de typen afkomstig van speenhuid. Er wordt geconcludeerd dat de melkmachine zowel melkstammen als speenhuidstammen van *Staph. aureus* kan overbrengen en dat speenhuid en menselijke huid (handen van melkers) geen belangrijke bronnen van mastitisverwekkende *Staph. aureus* zijn. Faagtypering (Fox, L. K., M. Gershmann, D. D. Hancock, en C. T. Hutton. 1991. Cornell Vet. 81:183-193) had een lager onderscheidingsvermogen dan PFGE en differentieerde niet tussen melkisolaten en speenhuidisolaten. Een deel van de isolaten (n=142) werd binair getypeerd met herkenningsfragmenten voor typering van humane *Staph. aureus*. BT had een lager typerings- en onderscheidingsvermogen dan PFGE. Ook toonde BT minder overeenstemming met epidemiologische gegevens dan PFGE. BT met probes voor humane *Staph. aureus* is dus niet geschikt als zelfstandige typeringstechniek voor bovine *Staph. aureus*. Binnen een aantal PFGE typen werd met behulp van BT onderscheid gemaakt tussen hoofdtypen en subtypen en tussen isolaten die afkomstig waren van verschillende bedrijven of bronnen (huid, melk). In combinatie met PFGE kan het gebruik van BT dus

wel zinvol zijn voor verfijning van de stamtypering. Door herkenningsfragmenten te ontwikkelen voor bovine *Staph. aureus* kan het typerings- en onderscheidingsvermogen van BT waarschijnlijk worden verbeterd.

Naast moleculaire technieken zijn ook mathematische technieken gebruikt om de epidemiologie van *Staph. aureus* te bestuderen. In **hoofdstuk 4** wordt een wiskundige model van de infectiedynamiek beschreven. Een melkgevende koppel wordt weergegeven als een populatie van uierkwartieren die met betrekking tot *Staph. aureus* ongeïnfecteerd, subklinisch geïnfecteerd, klinisch geïnfecteerd of hersteld zijn. Parameters voor het model werden geschat op basis van gegevens uit een longitudinale observationele studie op drie commerciële melkveebedrijven. Gedurende 18 maanden werden kwartiermelkmonsters genomen van alle koeien, 1. bij binnenkomst in de populatie (afkalven of aankoop); 2. routinematig met 3-weekse intervallen; 3. als klinische mastitis werd gezien, en 4. bij vertrek uit de populatie (droogzetten of afvoer). Met behulp van een deterministisch simulatiemodel werd de waarde van de basale reproductieratio (R_0) en de effectieve reproductieratio (R_t) voor ieder bedrijf berekend. R_0 was lager dan 1 voor de drie bedrijven. Dat betekent dat contagieuze (koegebonden) transmissie effectief werd onderbroken. R_t was hoger dan R_0 doordat genezen kwartieren gevoeliger waren voor infectie dan kwartieren die niet eerder een *Staph. aureus* infectie hadden doorgemaakt. Uit de simulaties bleek dat het model een goede weergave was van de waargenomen dynamiek op twee bedrijven. Voor die bedrijven kon het effect van veranderingen in bedrijfsmanagement worden gesimuleerd. Behandeling van subklinische mastitis en preventie van instroom van geïnfecteerde kwartieren in de melkgevende koppel droegen volgens de simulatie bij aan een snelle en sterke daling van de *Staph. aureus* prevalentie. Voor één bedrijf werd de waargenomen situatie slecht nagebootst door het model. Mogelijke oorzaken voor deze discrepantie zijn o.a. gelegen in het deterministisch karakter van het model (geen toevalsvariaties), in onjuistheid van de aannames die zijn gedaan bij het construeren van het model en in het ontstaan van nieuwe infecties vanuit bronnen van *Staph. aureus* in de omgeving van de koe.

Eén van de aannames in het wiskundig model is dat alle kwartieren binnen een compartiment dezelfde eigenschappen hebben. In werkelijkheid verschillen koeien en uierkwartieren in hun gevoeligheid voor mastitis. Risicofactoren voor *Staph. aureus* en *Strep. uberis* infecties worden beschreven in **Hoofdstuk 5**. De kwartiermelkmonsters uit het veldonderzoek op drie bedrijven zijn gebruikt voor bacteriologisch onderzoek en voor bepaling van het celgetal. Voor ieder dier zijn gegevens over pariteit, lactatiestadium en serostatus ten aanzien van bovine herpesvirus-4 geregistreerd. Gedurende 12 maanden van

het veldonderzoek werden de conditiescore en de speenpuntscore (mate van vereelting en rafeligheid van eeltring) van alle melkgevende dieren elke drie weken beoordeeld. In totaal werden 100 nieuwe infecties met *Staph. aureus* geconstateerd in 22.593 observaties "at risk" en 93 nieuwe infecties met *Strep. uberis* in 22.665 observaties "at risk". Met een multivariaat Poisson regressiemodel werden risicofactoren voor infectie geïdentificeerd, rekening houdend met het feit dat koeien binnen een bedrijf of herhaalde waarnemingen van kwartieren bij één koe niet onafhankelijk zijn van elkaar. De kans op *Staph. aureus* infectie was groter voor oude koeien (3e kalfs of ouder) dan voor jonge dieren, voor koeien die seropositief waren voor bovine herpesvirus-4, voor rechter kwartieren, voor kwartieren die genezen waren van een eerdere *Staph. aureus* of *Strep. uberis* infectie, voor kwartieren die blootgesteld zijn aan een ander *Staph. aureus*-geïnfecteerd kwartier van dezelfde koe, voor kwartieren met een hoog celgetal (> 250.000 cellen/ml voorafgaand aan de *Staph. aureus* infectie) en voor kwartieren met sterk vereelte speenpunten. Voorafgaande infectie met coagulase-negatieve staphylococcen was geen significante risicofactor. Voorafgaande infectie met corynebacteriën kon risicoverhogend, risicoverlagend of neutraal zijn, afhankelijk van het bedrijf, het lactatiestadium en de ruwheid van de speenpunt. De conditiescore of verandering van conditiescore had geen invloed op de kans op *Staph. aureus* of *Strep. uberis* mastitis. De kans op infectie met *Strep. uberis* was voor 3e-kalfs of oudere dieren hoger dan voor jonge dieren, en hing op één bedrijf samen met het lactatiestadium. Kwartieren die waren geïnfecteerd met *Arcanobacterium pyogenes* (de wrangbacterie) of met enterococcen, kwartieren die eerder waren genezen van *Strep. uberis* of *Staph. aureus* infectie en kwartieren die blootstonden aan een ander *Strep. uberis* geïnfecteerd kwartier van dezelfde koe hadden een hogere kans op *Strep. uberis* mastitis dan kwartieren uit de bijbehorende referentiecategorie. Voorafgaand celgetal, vereelting of ruwheid van de speenpunt, en infectie met coagulase-negatieve staphylococcen of corynebacteriën waren geen significante risicofactoren.

Op één bedrijf trad een uitbraak van *Strep. uberis* mastitis op. Die uitbraak is beschreven in **Hoofdstuk 6**. Gedurende het veldonderzoek, dat 18 maanden duurde, werden 54 infecties met *Strep. uberis* waargenomen. Het grootste deel daarvan trad op binnen een periode van 21 weken. De *Strep. uberis* dynamiek werd beschreven met twee variaties op een wiskundig model. De gevoeligheid van kwartieren die hersteld waren van infectie werd onderzocht met een model waarin de kwartieren die niet geïnfecteerd waren met *Strep. uberis* werden ingedeeld naar het wel of niet doorgemaakt hebben van een *Strep. uberis* infectie in het verleden. De invloed van andere pathogenen op infectiegevoeligheid werd onderzocht met een model waarin de kwartieren die niet met *Strep. uberis* geïnfecteerd waren, werden onderverdeeld op basis van de aan- of afwezigheid van andere pathogenen (voornamelijk corynebacteriën en coagulase-negatieve staphylococcen). Het te verwachten aantal nieuwe infecties met *Strep. uberis* werd voorspeld met een Poisson regressiemodel.

Het aantal bestaande *Strep. uberis* infecties in de melkgevende koppel (ofwel: de prevalentie van kwartieren die *Strep. uberis* uitscheiden in melk) was een statistisch significante voorspeller voor het aantal nieuwe infecties. Onderzoeksperiode (eerste of tweede deel van de observatieperiode) en de genezingsgeschiedenis van de kwartieren (wel of niet eerder *Strep. uberis* gehad) waren ook significant. De aan- of afwezigheid van andere pathogenen had geen invloed op het aantal nieuwe infecties, net als in het statistische model uit hoofdstuk 5. Omdat het aantal bestaande infecties voorspellend was voor het aantal nieuwe infecties, is de verspreiding van *Strep. uberis* mogelijk het gevolg geweest van koegebonden transmissie. Moleculaire typering van stammen die tijdens de uitbraak geïsoleerd zijn zou die hypothese kunnen steunen of verwerpen.

In **Hoofdstuk 7** worden klinische, epidemiologische en moleculaire eigenschappen van *Strep. uberis* infecties nader bestudeerd. Hiervoor zijn gegevens gebruikt van 84 uierinfecties in 70 kwartieren van 46 koeien op twee melkveebedrijven. Het grootste deel van de mastitisgevallen was subklinisch. De infecties begonnen meestal tijdens de lactatie. Op beide bedrijven kwamen chronische infecties voor met *Strep. uberis* en infecties waarbij geen celgetalverhoging werd gemeten (<250.000 cellen/ml). Van de 348 *Strep. uberis* isolaten zijn er 152 gekarakteriseerd met behulp van random amplified polymorphic DNA (RAPD) typering. Deze isolaten waren afkomstig uit 52 uierkwartieren. Op beide bedrijven kwam een aantal *Strep. uberis* stammen voor, maar beide bedrijven hadden een eigen dominante stam. Uit persisterende infecties werd meestal bij herhaling dezelfde stam geïsoleerd. Bij herinfectie (nieuwe infectie na een periode van genezing) werd soms, maar niet altijd, dezelfde stam aangetoond als bij de eerste infectie. Als een koe meer dan één geïnfecteerde kwartier had, dan behoorden isolaten uit die kwartieren meestal tot één stam. Er was geen verband tussen *Strep. uberis* stam en de mate van celgetalverhoging of de aanwezigheid van klinische verschijnselen. Op één bedrijf bestond wel verband tussen *S. uberis* stam en infectieduur. De uitbraak van *Strep. uberis* mastitis die beschreven is in hoofdstuk 6 werd veroorzaakt door één bacteriestam. Infecties met deze stam begonnen altijd tijdens de lactatie, terwijl infecties ten gevolge van andere stammen meestal begonnen in niet-melkgevende kwartieren (bij vaarzen voor afkalven, of tijdens droogstand). Nadat een geïnfecteerd kwartier machinaal gemolken was, kon *Strep. uberis* geïsoleerd worden uit de desbetreffende tepelvoering. Dit was ook mogelijk als één of twee andere, niet-geïnfecteerde koeien gemolken waren na de geïnfecteerde koe. De *Strep. uberis* stam die uit een tepelvoering geïsoleerd werd, was steeds identiek aan de *Strep. uberis* stam uit het kwartier van de bijbehorende geïnfecteerde koe. De epidemiologische en moleculaire gegevens zijn in overeenstemming met de hypothese dat koegebonden transmissie optreedt van specifieke *Strep. uberis* stammen. Deze transmissie vindt waarschijnlijk plaats via de melkmachine. Daarnaast treden er *Strep. uberis* infecties op vanuit bronnen buiten de uier.

De resultaten uit de voorafgaande hoofdstukken worden besproken in **Hoofdstuk 8**. Zij monden uit in een aantal aanbevelingen voor uiergezondheidsmanagement en voor toekomstig onderzoek. Voor mastitispreventie is de belangrijkste conclusie dat niet alle *Staph. aureus* mastitis koegebonden is en dat niet alle *Strep. uberis* mastitis afkomstig is uit omgevingsbronnen. Dat betekent dat preventie van koegebonden transmissie een wezenlijk onderdeel is van de beheersing van zowel *Staph. aureus* als *Strep. uberis* mastitis, maar ook dat preventie van koegebonden transmissie niet afdoende is om de bacteriën uit te roeien. De begrippen "koegebonden" en "omgevingsgebonden" zouden niet toegepast moeten worden op bacteriesoorten, maar op bacteriestammen, en preventie- en bestrijdingsmaatregelen moeten gekozen worden op basis van het verspreidingsmechanisme van die bacteriestam(men) die op een bedrijf aanwezig zijn. De eigenschappen die een rol spelen in de pathobiologie en de epidemiologie van mastitis kunnen binnen een bacteriesoort variëren. Daarom moet toekomstig mastitisonderzoek aandacht besteden aan de identificatie van pathogenen op soortniveau en aan identificatie van pathogenen op stamniveau.

Uierontsteking

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Uieronsteking – een hoofdstuk voor veehouders en hun adviseurs

Uierontsteking is één van de belangrijkste ziekten bij melkkoeien in Nederland. Uierontsteking, ook wel mastitis genoemd, komt voor in twee verschijningsvormen: klinische mastitis en subklinische mastitis. Bij klinische mastitis ("zichtbare uierontsteking") zijn afwijkingen te zien aan de melk (vlokjes, waterig, geel of rood), de uier (rood, gezwollen, hard, pijnlijk) of de koe (koorts, uit de melk, geen eetlust, liggen). Bij subklinische mastitis ("onzichtbare uieronsteking") zien we met het blote oog niets, maar bij onderzoek van de melk kunnen ziektekiemen worden aangetoond. Meestal is er ook een verhoging van het celgetal. Juist die celgetalverhoging maakt subklinische mastitis tot een belangrijk probleem: de melkgift daalt bij celgetalverhoging, en het tankmelkcelgetal stijgt. De belangrijkste verwekkers van onzichtbare uierontsteking zijn de bacteriën *Staphylococcus aureus* (op uitslagen van de Gezondheidsdienst aangegeven met SAU) en *Streptococcus uberis* (op GD-uitslag: SUB). Op de derde plaats komt de groep van coagulase-negatieve staphylococcen soorten (vergelijkbaar met GD-uitslag STC = staphylococ, niet-aureus). De belangrijkste verwekker van zichtbare uierontsteking is *Escherichia coli* (op GD-uitslag: ECO). Ook *Staph. aureus* en *Strep. uberis* zijn vaak de oorzaak van zichtbare uierontsteking. Dit boekje gaat over *Staph. aureus* en *Strep. uberis*, en over de manier waarop die kiemen zich binnen een bedrijf verspreiden. Bij de verspreiding van uierontsteking spelen bacterie-factoren, koe-factoren en bedrijfsfactoren een rol.

Uit het onderzoek blijkt dat niet alle *Staph. aureus* hetzelfde is: binnen de bacteriesoort *Staph. aureus* bestaan verschillende stammen. Dat is te vergelijken met rassen binnen een diersoort. MRIJ koeien hebben andere eigenschappen dan Fries-Hollandse koeien, Holsteins of Piemontesen. Zo hebben verschillende stammen binnen een bacteriesoort ook verschillende eigenschappen. De meeste *Staph. aureus* stammen zijn besmettelijk: ze worden van koe naar koe overgedragen. Dat gebeurt vooral tijdens het melken. Er zijn daarnaast ook *Staph. aureus* stammen die in de omgeving vóórkomen: op de huid van mens en dier, in het strooisel, in het voer, enz. Daardoor is het onmogelijk om *Staph. aureus* uit te roeien, zelfs als we overdracht van koe naar koe beperken. Ook *Strep. uberis* is een bacteriesoort met verschillende stammen. En ook *Strep. uberis* stammen kunnen verschillen in hun eigenschappen. Er zijn veel *Strep. uberis* stammen die in de omgeving van de koe vóórkomen, maar er zijn daarnaast *Strep. uberis* stammen die van koe naar koe overgaan. In de praktijk betekent dit dat preventieve maatregelen nodig zijn om mastitis verspreiding van koe naar koe tegen te gaan, zowel voor de bestrijding van *Staph. aureus* als voor de bestrijding van *Strep. uberis*. De wetenschappelijke resultaten die uit dit promotie-onderzoek naar voren zijn gekomen, zijn verzameld in het hoofdstuk "Samenvatting". De maatregelen die de veehouder kan nemen, worden hieronder op een praktijkgerichte manier op rijtje gezet. Daarbij wordt informatie uit ons eigen onderzoek gecombineerd met algemene kennis op het gebied van uiergezondheid.

- Zorg voor een goed functionerende melkmachine en gebruik de melkmachine op de goede manier. Lang blindmelken is ongewenst, omdat het tot speenpuntvereelting kan leiden. Laat twee maal per jaar de machine doormeten ("droge meting") en vervang de tepelvoeringen tijdig (rubberen tepelvoeringen moeten gemiddeld na 2500 melkingen vervangen worden, siliconen voeringen na 3000-5000 melkingen of 3-5 maanden). Laat zonodig tijdens het melken ("natte meting") het functioneren en het gebruik van de melkmachine controleren. Uiteindelijk gaat het erom dat de koe goed gemolken wordt, dus de gebruikssituatie van de melkmachine is belangrijker dan de rustsituatie. Specifieke redenen om een natte meting te laten uitoveren zijn: te hoog tankmelkcelgetal, veel klinische mastitis, lastige koeien, ernstige speenpuntvereelting. Bij een goede melktechniek hoort onder andere het gebruik van een schone doek voor iedere koe. Soms wordt gezegd dat een rol goed papier te duur zou zijn om voor elke koe een nieuwe doek te pakken. Een rol duur papier is altijd nog goedkoper dan de gedwongen afvoer van een koe met mastitis, of een tank melk die geweigerd wordt vanwege een te hoog celgetal of verontreiniging met antibiotica die gebruikt zijn voor behandeling van mastitis.

- Gebruik na het melken een geregistreerde (REG NL) tepeldip of tepelspray met een desinfecterend middel en een huidverzorgend bestanddeel. Tepeldesinfectie vermindert verspreiding van staphylococcen en streptococcen. Huidverzorgende middelen voorkómen of genezen kloven en wondjes. Sommige huidverzorgende middelen verminderen ook de kans op speenpuntvereelting. Kwartieren met sterk vereelte speenpunten en rafelige speenpunten hebben meer kans om *Staph. aureus* mastitis te krijgen dan kwartieren met een dunne of matig verdikte gladde speenpunt. Alleen op laagcelgetal bedrijven waar *Staph. aureus* en streptococcen niet vóórkomen als mastitisverwekkers, en waar *E. coli* het grootste probleem is, kan overwogen worden om te stoppen met tepeldippen of sprayen. Dat is alleen verantwoord onder de voorwaarde dat celgetalverhoging (subklinische mastitis) en klinische mastitis nauwgezet worden opgespoord. Als dit niet gebeurt, kan een uitbraak van *Staph. aureus* of *Strep. uberis* mastitis het gevolg zijn. Een alternatief voor het stoppen met dippen is het gebruik van een barrièredip. Barrière-middelen zijn voor preventie van *E. coli* mastitis geschikter dan de contactdipmiddelen die tegen *Staph. aureus* of *Strep. uberis* ingezet worden.

- Behandel elke koe die klinische mastitis heeft, tenzij de koe wordt afgevoerd. Neem voorafgaand aan de behandeling een melkmonster, en noteer datum, koe en kwartier op het monsterbuisje. Melkmonsters kunnen in het vriesvak bewaard worden als ze niet meteen worden opgehaald voor onderzoek. Door regelmatig melkmonsters te laten onderzoeken, krijgt u een beeld van de bacteriën die op het bedrijf het meest vóórkomen. Daar kunt u de keuze van behandelingen en preventie maatregelen op afstemmen. Het opstellen van een bedrijfsbehandelplan moet in samenspraak tussen

veehouder en dierenarts gebeuren. De dierenarts kan ook adviseren bij de keuze tussen behandelen en afvoeren. Noteer met welk middel een behandeling uitgevoerd wordt, en hoe vaak en op welke manier (uier, nek) het middel toegediend wordt. Dan kan achteraf geëvalueerd worden of de therapie het gewenste effect heeft. Met die informatie kunt u zonodig de behandelstrategie bij het betreffende dier of het bedrijfsbehandelplan bijstellen. Bij een succesvolle behandeling verdwijnen de zichtbare verschijnselen, de bacterie, en de celgetalverhoging. Om dat laatste te controleren kunt u opnieuw melkmonsters laten onderzoeken, of het celgetal controleren aan de hand van de melkcontrole uitslag of met de vierkwartierenschaal.

- Laat elke 3-4 weken het celgetal van alle koeien controleren. Dat is een goedkope manier om niet-zichtbare uierontsteking snel op te sporen. Een koe die een celgetal boven de 250.000 cellen per ml heeft, heeft mogelijk uierontsteking. Een koe die twee keer een celgetal boven de 250.000 cellen per ml heeft, heeft vrijwel zeker uierontsteking. U kunt dit controleren op de melkcontroleuitslag, of met een vierkwartierenschaal. Met de vierkwartierenschaal kunt u ook vaststellen welk(e) kwartier(en) ontstoken is(en). Neem een melkmonster van de kwartieren met hoog celgetal om vast te laten stellen welke bacterie het probleem veroorzaakt. Hoe eerder uierontsteking ontdekt wordt, hoe eerder u de koe kunt behandelen en maatregelen kunt nemen om verspreiding binnen de koppel te voorkómen. Hoe eerder u met de behandeling van een subklinische mastitis begint, hoe groter de kans op genezing. Door een koe met onzichtbare uierontsteking te behandelen, voorkomt u dat de infectie zich in de koppel verspreidt. Op lange termijn zal het aantal behandelingen dus verminderen als u onzichtbare uierontsteking behandelt. Uiteraard moet behandeling gepaard gaan met goed uiergezondheidsmanagement, anders is het dweilen met de kraan open.

- Soms is behandelen niet verstandig. Dieren die al langdurig of bij herhaling uierontsteking hebben, kunnen beter opgeruimd worden. Laat zo'n dier ook niet insemineren: een drachtige koe die vaak klinische mastitis heeft of een constant verhoogd celgetal wordt meestal niet afgevoerd, en blijft daardoor een besmettingsgevaar voor de rest van de koppel. Door het dier niet te insemineren, maakt u de afvoerbeslissing makkelijker. Ook dieren die een lage kans op genezing hebben, bijvoorbeeld oudere dieren die een sterk verhoogd celgetal hebben en dieren waarbij meerdere kwartieren ontstoken zijn, kunnen beter niet behandeld worden. Als u wel tot behandeling besluit, doe het dan met overtuiging (lang genoeg, en indien mogelijk ook in de nek): een goede behandeling is meestal niet goedkoop, een goedkope behandeling is meestal niet goed.

- Ook zonder gebruik van antibiotica is het mogelijk om verspreiding van mastitisverwekkers te voorkómen. Als u weet welke koeien besmet zijn en u herkent die dieren in de melkpunt, dan kunt u na het melken van een besmette koe het melkstel ontsmetten met heet water (tenminste 85°C). Met een boiler nabij de melkput is dit goed mogelijk. Een emmer warm water is niet afdoende: het water koelt af, en dan is het ontsmettende effect te gering.

- Zet alle dieren droog met antibiotica (droogzetters). Hiermee kunnen bestaande infecties genezen worden, en nieuwe infecties voorkómen worden. De injectoren moet op hygiënische wijze ingebracht worden (schone handen, speentop ontsmetten, niet te ver inbrengen, na het inbrengen dippen of sprayen), anders kunnen juist ziekteverwekkers binnengebracht worden in de uier. Selectief droogzetten van koeien met uierontsteking is ook een mogelijkheid. Helaas leert de ervaring dat vaak te veel koeien die geen mastitis hadden bij droogzetten wel afkalven met uierontsteking als geen droogzetters gebruikt worden. Evalueer in alle gevallen periodiek het resultaat, en kies zonodig een ander middel of een andere behandelstrategie.

- Met de bovenstaande maatregelen kunnen problemen ten gevolge van *Streptococcus agalactiae* (op GD-uitslag: SAG), *Streptococcus dysgalactiae* (op GD-uitslag: SDY) en *Staph. aureus* grotendeels opgelost worden. Ook een deel van de *Strep. uberis* problemen wordt hiermee voorkómen of verholpen. Voor het succes van de aanpak is het belangrijk dat u zorgvuldig te werk gaat bij het opsporen van besmette dieren, en dat u de beschikbare informatie gebruikt: als een celgetal uitslag binnen komt, controleer dan of er dieren zijn met een hoog celgetal en neem zonodig actie (controle celgetal met vierkwartierenschaal, monstername, behandeling of afvoer). Als de uitslag van een melkmonster binnen komt, stel een beslissing over behandeling of afvoer dan niet uit. Elk onderzoek kost geld, en die investering is alleen zinvol als u met de verkregen informatie iets doet. Hoe eerder u actie neemt, hoe groter de kans op genezing, en hoe kleiner de kans op verspreiding.

- Er is ook een aantal bacteriesoorten en bacteriestammen dat niet op de bovenstaande aanpak reageert. *E. coli*, *Klebsiella* en *Pseudomonas* zijn kiemen die vooral in de omgeving van de koe gevonden worden. Ook sommige *Staph. aureus*-stammen en veel *Strep. uberis*-stammen zitten in de omgeving. Bij de preventie van omgevingsmastitis gaat het om twee dingen: het vergroten van de weerstand van de koe (energie-, vitamine en mineralengehalte rantsoen; in de toekomst mogelijk vaccinatie tegen *E. coli*; tochtvrije huistvesting; goede klauwgezondheid), en het verminderen van de blootstelling aan ziekteverwekkers (boxen schoon en droog houden; besmettingsbronnen opsporen, en zo mogelijk verwijderen, bijv. gebarsten tepelvoeringen, melkleidingen met onvoldoende afschot, strooisel). Raadpleeg zonodig een deskundige die bijvoorbeeld ook de voeding en het stalklimaat kan beoordelen.

- Vliegen kunnen mastitis verspreiden. Vliegen kunnen besmet worden met mastitisverwekkers vanuit ontstoken kwartieren, wonden (wrang bacterie), of kalverdrinkemmers (als mastitis melk aan kalveren wordt gevoerd). Zorg dus voor goede vliegenbestrijding in de jongveestal, bij de pinken, en bij de melkkoeien. Voer geen mastitis-melk of antibioticum-melk aan kalveren (ook van belang voor het voorómen van paratuberculose bij kalveren).

- Controleer dieren die aan de koppel toegevoegd worden (afkalven aan het eind van de droogstand, afkalvende vaarzen, aangekochte dieren) op mastitis. Dit kan door de uier te beoordelen, op basis van celgetallen, of met onderzoek van melkmonsters. Als het nodig is om koeien aan te voeren, eis dan dat zij in de laatste lactatie steeds een laag celgetal hebben gehad, en laat voor aankoop kwartiermelkmonsters onderzoeken op aanwezigheid van mastitisverwekkers. Als er veel dieren afkalven met mastitis, overweeg dan een andere droogstandtherapie, of het gebruik van droogzetters bij drachtig jongvee (voor afkalven!). Ook de seleniumvoorziening in het jongveerantsoen beïnvloedt de kans op mastitis.

- Maak een afweging van kosten en baten bij elke maatregel. Als u met de uitslag van een melkmonster niets doet, dan heeft het geen zin om melkmonsters te nemen. Maar als u op basis van de uitslag uw management kunt aanpassen, en een mastitis geval, de afvoer van een koe, of de overschrijding van kwaliteitsgrenzen kunt voorkómen, dan kan bacteriologisch onderzoek, behandeling van subklinische mastitis, of de aanschaf van “duur” papier, tepeldip of een boiler al snel uit.

Het lijkt veel, zo’n hele waslijst van maatregelen. Als de maatregelen onderdeel worden van de dagelijkse, maandelijkse of jaarlijkse routine, dan valt de inspanning op termijn erg mee. En het resultaat dat met deze maatregelen behaald kan worden, is de investering vaak waard. Het niveau van investering (tijd, moeite, geld) en het gewenste resultaat is afhankelijk van persoonlijke en economische overwegingen, de voorkeur van de veehouder, en hoeveelheid tijd, geld en aandacht die nodig is voor andere activiteiten binnen en buiten het bedrijf.

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Dankwoord - Acknowledgements

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The members of my PhD committee are scattered around the world. I'm proud and happy to have Heather Allore on my PhD committee, the person who taught me modeling, cross country skiing, and perseverance. Herman Barkema has been a friend, a father and an example of a researcher rooted in practice. Jos Noordhuizen gave me the opportunity and the support to pursue my scientific ideas and follies, wherever they took me. Finally but foremost, Ynte Schukken has been a guide and inspiration throughout the PhD years. I'm grateful to have you as a mentor and friend, and happy that I'll see more of you and your family when I start my new job at Cornell.

De mensen die het meest hebben bijgedragen, en die het belangrijkst zijn geweest en zullen blijven voor mijn leven voor, tijdens en na de promotie kan ik niet allemaal bij naam noemen. Aan hen allen, mijn ouders, broers, (schoon)zussen en hun kinderen, en aan mijn vrienden, is dit proefschrift opgedragen. And to Roberta, for the design of the cover and for remembering beauty.

Curriculum vitae

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Ruth N. Zadoks werd geboren op 3 april 1968 te Wageningen. In 1986 behaalde zij haar VWO-diploma op het Wagenings Lyceum. In datzelfde jaar werd, vanwege uitloting voor de studie Diergeneeskunde, begonnen met Scheikunde aan de Rijksuniversiteit Utrecht, waar de propedeuse in 1987 werd behaald (cum laude). In 1987 kon alsnog met de studie Diergeneeskunde worden begonnen, waar propedeuse, doctoraal-examen en dierenarts-examen in 1988, 1993 en 1995 cum laude werden behaald. Tijdens de studie was zij lid van het bestuur van de Diergeneeskundige Studenten Kring, van de Faculteitsraad en van de Commissie voor Advies voor de Beroepsuitoefening van de Vrouwelijke Dierenarts van de KNMvD. In 1992/1993 werd een jaar besteed aan onderzoek naar bovine papillomatose bij de afdeling Immunologie van de Faculteit Diergeneeskunde in Utrecht en aan de Faculty of Veterinary Sciences van de University of Zimbabwe in Harare. Dat onderzoek werd in 1994 afgesloten met de graad Master of Veterinary Research. Van 1996 tot 2002 was zij werkzaam als dierenarts, docent en onderzoeker op het gebied van de bedrijfsdiergeneeskunde, epidemiologie, en uiergezondheid bij de Vakgroep Bedrijfsdiergeneeskunde en Voortplanting, later Afdeling Herkauwersgezondheidszorg van de Hoofdafdeling Gezondheidszorg Landbouwhuisdieren. Daar volgde zij tevens een opleiding tot veterinair epidemioloog. Het onderzoek dat beschreven is in dit proefschrift werd verricht in samenwerking met de Gezondheidsdienst voor Dieren. Een deel van het onderzoek werd uitgevoerd bij Cornell University te Ithaca, N.Y., in de Verenigde Staten Zij zal daar het onderzoek in de moleculaire epidemiologie van mastitis voortzetten.

Biography

Ruth N. Zadoks was born on April 3, 1968, in Wageningen, The Netherlands. She graduated from high school (Wagenings Lyceum) in 1986. After one year of studying chemistry at Utrecht University, the Netherlands, she started her studies of veterinary medicine at Utrecht University in 1987. She obtained her MVSc and DVM degrees with honors in 1993 and 1995, respectively. During her student years, she was a member of the executive committee of the Veterinary Students' Society, the Faculty Council, and the Advisory Committee on Women's Affairs of the Royal Dutch Veterinary Association. In 1992/1993, she spent a year on research in the Department of Immunology of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands, and at the Faculty of Veterinary Sciences of the University of Zimbabwe, Harare, Zimbabwe, resulting in a thesis on bovine papillomatosis and the degree of Master in Veterinary Research in 1994. From 1996 to 2002, she was employed as a clinician, lecturer and Ph.D. student focusing on bovine herd health, epidemiology, and udder health in the Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, the Netherlands. In 1998, she obtained the degree of MSc in Veterinary Epidemiology from this school. The research project that is described in this dissertation was conducted in collaboration with the Dutch Animal Health Service. Part of the work was done at the College of Veterinary Medicine, Cornell University, Ithaca, NY, USA. She will continue with her studies of the molecular epidemiology of mastitis pathogens in the Department of Food Science at Cornell University.