UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

BOVINE MASTITIS DUE TO COAGULASE-NEGATIVE STAPHYLOCOCCI AND THE ROLE OF MINOR PATHOGENS ON MASTITIS

JOSÉ RICARDO DIAS BEXIGA

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JOSÉ RICARDO DIAS BEXIGA

TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS
ESPECIALIDADE DE SANIDADE ANIMAL

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BOVINE MASTITIS DUE TO COAGULASE-NEGATIVE STAPHYLOCOCCI AND THE ROLE OF MINOR PATHOGENS ON MASTITIS

Abstract

Bovine mastitis is the most common disease of dairy cows. To implement efficient control measures, it is generally necessary to diagnose the relevant aetiologic agents. Coagulase-negative staphylococci (CNS) and *Corynebacterium* spp. are considered minor mastitis pathogens because of the mild impact they have on udder health. Despite that, they are the most common agents isolated from milk samples in several large scale surveys worldwide. The objectives of this study were to evaluate if there were differences in pathogenicity between individual CNS species, to evaluate if alternative sampling or diagnostic techniques could more accurately determine in which cases the aforementioned minor pathogens were responsible for mastitis, and to determine if treatment of CNS infected quarters was a cost-effective control measure.

We followed 111 intramammary infections due to CNS for up to 48 weeks in four commercial dairy farms. Duration of infection had a mean of 188 days and was not significantly different between CNS species; geometric mean quarter somatic cell count (SCC) overall was 132,000 cells/ml and was also not significantly different between CNS species. There were differences in diversity between CNS species, with *Staphylococcus epidermidis* and *Staphylococcus simulans* showing less diversity than *Staphylococcus haemolyticus,* the epidemiological significance of which is debated.

Freezing milk samples overnight at -20ºC did not increase detection of intramammary bacteria in milk samples. Use of a real-time PCR-based test allowed for detection of udder pathogens beyond the ones identified using conventional bacteriology in milk samples with a high SCC that were culture negative or that yielded CNS or *Corynebacterium bovis* shown by conventional bacteriology.

Single quarter milk samples were collected in duplicate from 132 dairy cows in a commercial dairy farm, with the standard technique and by use of a cannula surpassing the teat canal. There was a significant difference between the two sampling techniques for recovery of *Corynebacterium* spp. and for culture-negative samples. The observed difference could not be attributed to a particular sampling order and no significant change was observed in quarter SCC between the sampling day and seven days later, indicating iatrogenic IMI following use of the alternative technique was not an issue.

Use of a deterministic economic model allowed to determine that in most situations lactational treatment of subclinical mastitis due to CNS would result in a net financial loss, on average of €38.74 per treated quarter.

Taken together, these results indicate that CNS have a low impact in terms of udder health, with little differences between individual species. Treating CNS subclinical mastitis during the lactation is not cost-efficient and should therefore not be advised. Using a RT-PCR and sampling with a teat cannula might improve diagnosis of mastitis etiology.

Keywords: minor mastitis pathogens, diagnosis, economics, coagulase-negative staphylococci, *Corynebacterium* spp., diversity, PFGE, impact, udder health, bovine

MASTITE BOVINA POR STAPHYLOCOCCI COAGULASE-NEGATIVOS E O PAPEL DE AGENTES PATOGÉNICOS MENORES NA MASTITE

Resumo

A mastite bovina é a doença mais comum das vacas leiteiras. Para implementar medidas de controlo eficientes, é geralmente necessário identificar os agentes etiológicos relevantes. Os staphylococci coagulase-negativos (SCN) e *Corynebacterium* spp. são considerados agentes patogénicos menores porque têm um impacto ligeiro na saúde do úbere. Apesar disso, são os agentes mais frequentemente isolados a partir de amostras de leite em vários estudos de larga escala, em diversos pontos do mundo. Os objectivos deste estudo foram avaliar se havia diferenças em termos de patogenicidade entre espécies individuais de SCN, avaliar se um método de colheita e de diagnóstico alternativos podiam determinar mais precisamente em que casos os agentes mencionados acima eram responsáveis por mastite, e determinar se o tratamento antimicrobiano durante a lactação de quartos infectados com SCN era uma medida de controlo eficiente em termos de custos.

Acompanhámos 111 infecções intramamárias por SCN durante até 48 semanas em quatro explorações leiteiras comerciais. A duração média de infecção foi de 188 dias e não foi significativamente diferente entre espécies de SCN; a média geométrica de contagens de células somáticas (CCS) global foi de 132.000 células/ml de leite e também não foi significativamente diferente entre espécies de SCN. Houve diferenças na diversidade entre espécies de SCN, tendo *Staphylococcus epidermidis* e *Staphylococcus simulans* mostrando menor diversidade do que *Staphylococcus haemolyticus,* sendo discutida o possível significado epidemiológico destas observações.

A congelação de amostras de leite durante a noite a -20ºC não permitiu o aumento da detecção de bactérias em amostras de infecções intramamárias. A utilização de um PCR em tempo real permitiu a detecção de agentes patogénicos mamários para além dos identificados após utilização de técnicas bacteriológicas convencionais em amostras de leite com CCS elevadas que se mostraram negativas após cultura ou que levaram ao isolamento de SCN ou *Corynebacterium bovis.*

Foram colhidas amostras de leite de quartos individuais em duplicado de 132 vacas leiteiras numa vacaria comercial, com a técnica convencional e com a utilização de uma cânula permitindo ultrapassar o canal do teto. Observou-se uma diferença significativa entre as duas técnicas de colheita para a recolha de *Corynebacterium* spp. e para amostras sem crescimento após cultura. A diferença observada não era atribuível a uma ordem de colheita particular e não foram observadas alterações significativas na CCS dos quartos entre o dia de colheita e sete dias mais tarde, indicando que a infecção intramamária iatrogénica após a utilização da técnica alternativa não constituiu um problema.

A utilização de um modelo económico determinístico permitiu determinar que na maioria das situações o tratamento durante a lactação de mastites subclínicas por SCN resultaria numa perda finaceira líquida, em média de €38.74 por quarto tratado.

Considerados em conjunto, estes resultados indicam que os SCN têm um impacto baixo em termos de saúde do úbere, com poucas diferenças entre espécies individuais. Tratar mastites subclínicas por SCN durante a lactação não é eficiente em termos de custos e não deve portanto ser aconselhado. A utilização de um RT-PCR e a colheita de amostras com uma cânula de tetos pode melhorar o diagnóstico das causas de mastite.

Palavras-chave: agentes patogénicos menores de mastites, diagnóstico, estudo económico, staphylococci coagulase-negativos, *Corynebacterium* spp., diversidade, PFGE, impacto, saúde do úbere, bovino

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List of abbreviations

AFLP………………………………………………….. amplified fragment length polymorphism
CCS…………..contagens de células somáticas
CFU...colony forming units
CNS……………………………………………………….....coagulase-negative staphylococci
CT..cycle threshold
IMI…………………………………………………………………….intramammary infection
ITS-PCR………………………….internal transcribed spacer region polymerase chain reaction
MLST……………………………………………………………..multilocus sequence typing
NAS…………………………………………………………………...non-aureus staphylococci
NMC………………………………………………………………National Mastitis Council
PCR……………………………………………………………….polymerase chain reaction
PFGE………..pulsed-field gel electrophoresis
RT-PCR………………………………………………..real time polymerase chain reaction
SCC…………………………………………………………………………somatic cell count
SCN………………………………………………………..staphylococci coagulase-negativos
SD……………………………………………………………………………..standard deviation
SDI…………………………………………………………………….Simpson diversity index
SNP…………………………………………………………single nucleotide polymorphism
TSST-1…. …………………………………………………………….toxic shock syndrome toxin-1
UPGMA…. …………………………...unweighted pair group method using arithmetic averages

Introduction

Mastitis is an inflammation of the mammary gland, most often of infectious origin. It is considered the most frequent disease in dairy farms and one of the main reasons for culling dairy cows (Gröhn, Eicker, Ducrocq & Hertl, 1998; Hortet & Seegers, 1998). It may be clinical if it can be detected on examination by the animal handler, or subclinical if an ancillary diagnostic test is needed for its diagnosis. Mastitis has a great economic impact for farmers: the average cost of a clinical mastitis episode has been estimated to be around 210€ and the average cost of a subclinical mastitis, for a farm with a bulk tank somatic cell count (SCC) between 250 and 400,000 cells/ml, to be around 94€ (Huijps, Lam & Hogeveen, 2008). Intrammammary infections (IMI) are infections of the mammary gland, detected more often through culture of milk samples, but not necessarily associated with inflammation.

Traditionally, microorganisms causing mastitis were divided into major and minor mastitis pathogens. Major mastitis pathogens included microorganisms that frequently led to clinical mastitis episodes, had low cure rates or were easily transmitted from cow to cow, hence having a high impact on udder health. These comprised *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli* and *Mycoplasma bovis* (Hassan, Samarasinghe & Lopez-Benavides, 2009). Minor mastitis pathogens had none of the aforementioned attributes and included *Corynebacterium bovis* as well as other species of the genus *Corynebacterium* and a group collectively termed as coagulase-negative staphylococci (CNS).

The latter group has progressively been acquiring more attention from researchers and milk quality advisors, with seminars being dedicated exclusively to it (Seminar on coagulase-negative staphylococci, Ghent, Belgium, 15-16 September 2010) as well as whole issues of scientific journals (Veterinary Microbiology 2009, volume 134, issues 1-2, dedicated to heifer and CNS mastitis). In the late 1960's the five-point mastitis control plan started to be applied (Neave, Dodd & Kingwill, 1966), including postmilking teat disinfection, antimicrobial treatment of clinical mastitis, dry cow therapy, culling chronically infected cows and correctly maintaining the milking machine. With its effective implementation in most farms, prevalence of many of the major pathogens has been greatly reduced and continues to be so (Makovec & Ruegg, 2003; Pitkälä, Haveri, Pyörälä, Myllys & Honkanen-Buzalski, 2004). This led to more emphasis being put on the role of minor mastitis pathogens. Over the last decade or so, several large scale studies around the world have identified CNS as the most frequently isolated microorganisms from milk samples submitted either from clinical or subclinical mastitis cases or for mastitis surveillance programmes (Wilson, Gonzalez & Das, 1997; Makovec & Ruegg, 2003; Pitkälä et al., 2004; Bexiga, Cavaco & Vilela, 2005; Tenhagen, Koster, Wallmann & Heuwieser, 2006; Bradley, Leach, Breen, Green & Green, 2007). As for *C. bovis* and other species from the same genus, not as much attention has been devoted to them, despite their frequent isolation in the same studies.

Despite CNS being frequently isolated from milk samples, their role as mastitis pathogens is not clearly defined, with some authors giving them little importance (Schukken et al., 2009) whereas others question if their role in terms of udder health is significantly different from that of major pathogens (Taponen & Pyörälä, 2009).

Common to both CNS and *C. bovis* is the fact that they seldom lead to clinical episodes of mastitis (Bradley et al., 2007) and that they lead to fairly low SCC in quarters where they are causing intramammary infections (Djabri, Bareille, Beaudeau & Seegers, 2002).

The current thesis aims to better characterise the impact of these minor pathogens on udder health and mastitis diagnosis. Aspects related to CNS and to *C. bovis* are under different headings for convenience.

Literature review

Coagulase-negative staphylococci

Identification

Historically, CNS were not always identified to species level because that distinction was not easily or reliably performed, and because different species within this group were viewed as having very similar characteristics and clinical relevance.

There are several ways of identifying CNS to species level. The method commonly used to discriminate between this group of microorganisms and *S. aureus* is the coagulase test. This test detects the presence of coagulase, an enzyme commonly expressed by *S. aureus*, *S. intermedius*, *S. pseudintermedius*, *S. delphini*, *S. schleiferi* subsp. *coagulans* and by some strains of *S. hyicus* (Blaiotta, Fusco, Ercolini, Pepe & Coppola, 2010). Coagulase forms an active molecular complex with prothrombin, which converts fibrinogen to a fibrin clot. For detection of coagulase, rabbit plasma is generally inoculated with isolated colonies and tested for gel or clot formation (Roberson, Fox, Hancock, Gay & Besser, 1996).

In terms of mastitis herd investigations it is often viewed as sufficient and more economical to know what is the incidence of *S. aureus* and of CNS as a group, because clinical relevance, expected cure rates and control measures differ between the two groups (Taponen & Pyörälä, 2009). The following review focuses on the identification of CNS, not on *S. aureus*.

Use of biochemical reactions to identify species of CNS is the basis of the method described by Kloos and Schleifer (1975) cited as the phenotypic reference method (Watts & Washburn, 1991; De Paulis, Predari, Chazarreta & Santoianni, 2003). This method includes tests for coagulase activity, hemolysis, lysostaphin and novobiocin susceptibility, phosphatase activity, nitrate reduction and aerobic acid production from fructose, xylose, arabinose, ribose, maltose, lactose, sucrose, trehalose, mannitol, and xylitol. This method could only distinguish nine different species of CNS, far from the more than 40 species currently described (NCBI Taxonomy, 2010). Biochemical reactions are the basis of most commercial tests for the species level identification of CNS which include the API Staph, API Staph ID 32 and Vitek (all bioMérieux), the BBL Crystal Gram-positive (Becton & Dickinson) and the Staph-Zym (Rosco).

More recently, genotypic methods have become widespread to either detect the presence of CNS in milk samples (Koskinen et al., 2009) or to identify CNS to species level after isolation in culture (Poyart, Quesne, Boumala & Trie-Cuot, 2001; Drancourt & Raoult, 2002). Some of the latter methods are based on the recognition of species-specific sequences including sequencing of housekeeping genes (Poyart et al., 2001; Drancourt & Raoult, 2002) or amplified fragment length polymorphism (AFLP, Piessens et al., 2010a), while others measure the variation in, or close to, genomic sequences present in all species,

which include internal transcribed spacer-PCR (Couto, Pereira, Miragaia, Santos-Sanches & Lencastre, 2001), tRNA intergenic length polymorphism analysis (Maes et al., 1997), transfer RNA-intergenic spacer PCR (Supré et al., 2009), $(GTG)_5$-PCR (Braem et al, 2010) or ribotyping (Carretto et al., 2005).

Commercial biochemical identification systems often provide unreliable results for CNS species, as a result of the variability of metabolic profiles within species and the subjective nature of their interpretation (Carretto et al., 2005). The fact that commercially available biochemical methods of identification are not generally conceived to identify veterinary pathogens probably contributes to this problem.

Genotypic methods seem to be generally more discriminatory than commercially available biochemical methods in differentiating between CNS species (Zadoks & Watts, 2009). Sampimon et al. (2009), comparing the performance of two biochemical identification systems with that of sequencing a housekeeping gene, found that for *Staphylococcus chromogenes*, the species with the highest number of representatives in their study, 0 and 36.5% of the isolates were correctly identified by the two commercial systems, respectively. Taponen, Simojoki, Haveri, Larsen and Pyörälä (2006) and Taponen, Koort, Björkroth, Saloniemi and Pyörälä (2007) mention an agreement between API Staph ID 32 and AFLP CNS identifications of 66 and 71.9%, respectively. Carretto et al. (2005) were able to identify correctly 93.8% of CNS isolates (n=177) through ribotyping, whereas only 36.7% were identified correctly with an API 20 Staph system. A faster diagnosis has also been claimed for some of the genotypic methods of diagnosis, as well as the identification of genes responsible for resistance to antimicrobials (Koskinen et al., 2009). Economical considerations, the technical possibilities of staff and equipment and the final objective of the diagnostic should also be taken into account in the choice of the diagnostic test. If diagnosing only to CNS group level remains the most useful information, with no need for identification to species level, then phenotypic methods currently in use may suffice (Zadoks & Watts, 2009).

Differentiation between bacterial strains within a certain species is essential for epidemiological surveillance and to identify possible cases of bacterial cross-transmission (van Belkum et al., 2007). Several methods have been proposed to distinguish between different strains of CNS species. These include use of antibiograms, pulsed-field gel electrophoresis (PFGE) or phage typing (Kloos & Bannerman, 1994; Bjorland et al., 2005; Taponen, Björkroth & Pyörälä, 2008).

Effects on udder health

Coagulase-negative staphylococci have been isolated from milk samples from clinical and subclinical mastitis cases. Isolation of CNS in cases of clinical mastitis, defined as mastitis apparent on examination by the animal handler or veterinarian (IDF, 1999), has been reported for CNS as a group (Smith et al., 1985; McDougall, 1998) or for individual species (Jarp, 1991; Birgersson, Jonsson & Holmberg, 1992; Todhunter, Cantwell, Smith, Hoblet & Hogan, 1993; Waage et al., 1999; Taponen et al., 2006). Simojoki et al. (2009) performed an experimental infection with *S. chromogenes* in six primiparous cows and all developed mild signs of clinical mastitis. All but one of the animals cleared the infection spontaneously within a week of being infected and there were no significant differences in average daily milk yields pre and post challenge, despite a 16.3% reduction in production for the challenged quarters during the seven-day observational period.

Likewise, isolation of CNS in cases of subclinical mastitis, defined as inflammation of the mammary gland that is not visible and requires a diagnostic test for detection (IDF, 1999), has also been reported for CNS as a group (Timms & Schultz, 1987; Rainard, Ducelliez & Poutrel, 1990; Chafer et al., 1999; Borm et al., 2006) and for individual species (Jarp, 1991; Birgersson et al., 1992; Taponen et al., 2006). Several criteria are used to define subclinical mastitis including increased SCC (Djabri, Bareille, Beaudeau & Seegers, 2002), increased N-acetyl-β-D-glucosaminidase (NAGase) activity (Myllys, 1995) or increased conductivity (McDougall, 1998). Generally papers seem to indicate a small increase in quarter SCC for infected quarters, when compared to uninfected quarters. Djabri et al. (2002), in their meta-analysis reporting on the effects of several intramammary pathogens on SCC, mention a mean quarter value of 138,000 cells/ml for staphylococci other than *S. aureus*, with 68,000 cells/ml being reported for culture-negative samples.

Besides being isolated from clinical and subclinical mastitis cases, several CNS species have also been found in samples from animals with no clinical signs and a low cell count. Birgersson et al. (1992) observed no significant differences in frequency of isolation for most CNS species between the three categories they cultured: clinical mastitis, subclinical mastitis and non-mastitic quarters, even though their case definition for non-mastitic was no clinical signs of inflammation and a SCC<300,000 cells/ml, a somewhat high threshold to define presence of inflammation.

It is well established that increases in SCC beyond a certain level lead to decreased milk production (Green, Schukken & Green, 2006), so milk production losses would be expected for CNS infected quarters. Timms and Schultz (1987) report a mean loss of 821 kg for 305-day milk production when comparing CNS infected animals with controls, whereas Wilson et al. (1997) and Schukken et al. (2009) actually report higher milk production for animals with IMI due to CNS, which seems somewhat surprising. This could be linked with confounding factors not yet identified: Wilson et al. (1997) suggest that higher producing cows may be

more likely to contract CNS mastitis and avoid major losses in milk production, whereas Schukken et al. (2009) suggest that this effect might be mediated through a protective effect of CNS against clinical mastitis by major pathogens. In fact, Piepers, Opsomer, Barkema, de Kruif and De Vliegher (2010) observed that a high proportion of CNS IMI in their study was short-lasting and that the affected quarters had a lower incidence of clinical mastitis and a higher milk production.

Several pathogenic changes have been reported in mammary quarters infected with CNS including leukocyte infiltration and increased connective tissue stroma. One study on nuliparous heifers with naturally acquired infection with CNS concluded that there were significant differences in stroma percentage and leukocyte infiltration, but not in epithelium or lumen percentages (Trinidad, Nickerson & Alley, 1990). Another study analysed microbiological results and histopathological changes of udders collected at abattoirs (Benites, Guerra, Melville & Costa, 2002), which are of debatable significance considering that some of the histological changes might have been produced by other pathogens that had previously infected the mammary gland. The authors concluded that no differences were observed between the main histopathological results associated with CNS and coagulase-positive staphylococci.

Persistence of intramammary infection has been studied for CNS, with reported values of 46% (Taponen et al., 2007), 66% (Seymour, Jones & McGilliard, 1989), 75.6% (Rainard et al., 1990), 84.5% (Chaffer et al., 1999) and 85% (Timms & Schultz, 1987) of infections persisting until dry-off or until the cows left the herd. Todhunter et al. (1993) mention an average length of IMI with CNS of 222 days.

Several studies addressed differences in pathogenicity between CNS species. Myllys (1995) found *Staphylococcus simulans* and *S. hyicus* more frequently associated with clinical infections and with increased inflammatory response than other CNS species. Myllys, Honkanen-Buzalski, Virtanen, Pyörälä and Müller (1994) also mention that *S. hyicus* was more effective in causing teat canal infections than *Staphylococcus epidermidis*. Jarp (1991) found that *Staphylococcus haemolyticus* was the CNS species more often associated with clinical mastitis, even though no significant difference was observed in clinical severity between different species. A Danish study (Aarestrup & Jensen, 1997) indicated that *S. simulans* produced more persistent and stable infections than the other CNS species, whereas a Finnish study (Taponen et al., 2007) found no significant differences between clinical characteristics and persistence of infection between the two most frequently isolated CNS species causing bovine mastitis. Hogan, White and Pankey (1987) observed no significant differences among SCC for quarters infected with different CNS species, indicating that the same type of inflammatory response is elicited by the several species that constitute the group. Chaffer et al. (1999) similarly found no differences in SCC or differential cell counts between CNS species. To the author's knowledge there have been no published

studies on differences in impact on udder health between strains of individual CNS species. Immunity to CNS at the level of the mammary gland is also not sought on the aforementioned studies focusing on differences in impact between CNS species. Both these factors could influence results of these studies and have not been investigated.

The overall impact of CNS on bulk tank SCC has also been addressed. Rainard et al. (1990) found that quarters infected by CNS contributed to 18.1% of bulk tank SCC, whereas Schukken et al. (2009) found that CNS IMI contributed 12% to the bulk tank SCC in herds in which this was between 200 and 400.000 cells/ml and 8% in herds with a bulk tank SCC above 400,000 cells/ml. Of the 4,200 full herd samplings analysed in this study, less than 2% of the herds would implicate CNS IMI as an important contribution to a bulk tank SCC higher than 400,000 cells/ml. The financial impact of mastitis caused by CNS has been estimated to be lower than mastitis caused by other pathogens (Ruegg & Dohoo, 1997).

Epidemiology

Prevalence of CNS is generally highest at calving for all parities (Mathews, Harmon & Langlois, 1992; Todhunter et al., 1993; Honkanen-Buzalski, Myllys & Pyörälä, 1994) and is higher in primiparous than in multiparous cows (Mathews et al., 1992; Rajala-Schultz, Smith, Hogan & Love, 2004). Their role as aetiological agents of mastitis in heifers has been highlighted, especially before parturition and in the peripartum period (Trinidad, Nickerson & Alley, 1990; Pankey, Drechsler & Wildman, 1991; Myllys, 1995; Nickerson, Owens & Boddie, 1995; Fox et al., 1995; Aarestrup & Jensen., 1997).

Due to the fact that many such studies address the isolation of CNS prior to calving, mostly they either report results on bacteriology of gland secretions (Trinidad et al., 1990a; Birgersson, et al., 1992; Aarestrup, Larsen & Jensen, 1999) or from skin and teat canal (Woodward, Ward, Fow & Corbeil, 1988; White, Harmon, Matos & Langlois, 1989; Matthews et al., 1992), often making no correlation between isolation of the organisms and pathologic effects. It is debatable if the terms mastitis or intramammary infection should be used in these circumstances. However, Trinidad et al. (1990a) found that the species more frequently involved in clinical mastitis were also the most frequently isolated from teat canal keratin samples: *S. chromogenes* and *S. hyicus* besides *S. aureus*.

Coagulase-negative staphylococci have been found on the skin and other body sites of cattle (White et al., 1989) and on the skin and mucous membranes of humans (Kloos & Bannerman, 1994), with no deleterious effects in these circumstances. They have also been isolated from several environmental sources (Matos, White, Harmon & Langlois, 1991) including several feeds and bedding material. There is evidence that the most frequently isolated CNS species from extramammary sites are not the same as the most frequently isolated from milk samples. Taponen et al. (2008) found that *Staphylococcus equorum*, *Staphylococcus sciuri*, *Staphylococcus saprophyticus*, and *Staphylococcus xylosus*

predominated in extramammary samples but were not isolated from milk samples. The same authors also found that for *S. chromogenes*, common PFGE pulsotypes were observed for environmental and intramammary isolates and that *S. simulans* was more often found in milk samples than isolated from environmental samples. This could be an indication that *S. chromogenes* IMI is acquired more often from environmental sources and that *S. simulans* is more often transmitted from cow to cow. Thorberg et al. (2006) observed that the most common *S. epidermidis* PFGE pulsotype isolated from milk samples for each of the two farms in their study, was also found on the milkers' hands, suggesting that the *S. epidermidis* IMI observed originated from humans. Aarestrup et al. (1999) found that in seven out of nine herds from which multiple *S. simulans* were isolated from milk samples, more than one ribotype was present, which can be seen as an indication that several sources of infection are present.

Despite the aforementioned evidence that at least some CNS IMI may originate from environmental sources, the most frequent form of infection acquisition is still not known. The classical differentiation between environmental and contagious mastitis pathogens no longer makes sense for several microorganisms (Bradley & Green, 2001; Zadoks et al., 2003; Rato et al., 2008), but it is still important to understand what are the main sources of infection in a particular farm when performing milk quality advisory work, so that control measures can be applied to reduce new infections. To our knowledge there is no evidence in the literature of what the most frequent source of infection for CNS as a group is, or if there are differences between CNS species.

There is some evidence of a low (Østerås, Sølverød & Reksen, 2006) or moderate (Barkema et al., 1997) herd-level clustering effect for CNS. Scientific evidence of a herd-level clustering effect for individual CNS species is still missing and the identification of herd and cow-level risk factors for IMI with CNS has not been addressed by many. Sampimon et al. (2009) found that an increased prevalence of CNS IMI was associated with the source of drinking water not being tap water, housing of dry cows in one group instead of multiple groups, monthly milk recording, udder health monitoring by the veterinarian, pasturing during outdoor season, percentage of stalls contaminated with milk and bulk tank SCC>250,000 cells/ml. It needs to be reminded that this was an epidemiological study and therefore no causality can be established from the observed associations.

Another subject that may contribute to our understanding of the epidemiology of CNS mastitis is the occurrence of seasonal variation of CNS IMI, which has been addressed by two publications, both originating in Norway. Waage et al. (1999) found a significantly lower percentage of cases from September to December, which agrees with Østerås et al. (2006) who found significantly higher prevalences of CNS IMI in April through to July. Seasonal variation might be linked with a number of factors (Østerås et al., 2006): feed quality when grazing (vitamin E intake), climatic and hygienic conditions.

Most CNS IMI would appear to be self-limiting. Aarestrup and Jensen (1997) found that *S. chromogenes* was isolated from 15% of heifers before parturition, but from only 1% four weeks after parturition. Different CNS species displayed different patterns of infection: *S. epidermidis* was seldom isolated from the same quarter for two consecutive weeks, whereas *S. simulans* persisted for several weeks. In this study no molecular fingerprinting method was used to try to understand if the presence of the same bacterial species corresponded to the same infection or to a newly acquired infection with the same species. The same authors (Aarestrup et al., 1999) used ribotyping for that purpose and were able to demonstrate that *S. simulans* can cause persistent infections. Similarly, Thorberg, Danielsson-Tham, Emanuelson and Persson Waller (2009) observed that *S. epidermidis*, *S. chromogenes* and *S. simulans* were the species more frequently isolated from the same quarters in two months in succession. Piepers et al. (2010) found that nearly half of CNS IMI detected at 1 to 4 days post-partum, could not be detected 5 to 8 days after calving. Several other authors have reported a decrease in infection levels after parturition (Hogan, Pankey & Smith, 1987b; Mathews et al., 1992; Myllys, 1995). It would appear therefore that different species have different spontaneous cure rates, but that this is probably influenced by lactational stage.

There is still some debate as to whether CNS might have a protective role against IMI caused by major pathogens, with some authors reporting evidence of such an effect (Schukken, Van de Geer, Grommers, Smit & Brand, 1989; Matthews, Harmon & Smith, 1990; Green et al., 2005), and some reporting evidence of the opposite effect (Hogan, Smith, Todhunter & Schoenberger, 1988; Davidson, Dohoo, Donald, Hariharan & Collins, 1992; Myllys, 1995; Aarestrup & Jensen, 1997). Matthews et al. (1990) performed a trial in which 35 quarters were challenged by infusion of *S. aureus* into the teat sinus, 18 that were culture negative and 17 that were infected with *S. chromogenes*. The outcome was that 100% of the previously non-infected quarters got infected, whereas 47% of the quarters harboring *S. chromogenes* became infected. Several theories have been proposed to justify this potential protective effect. One is the production of inhibitory substances with antagonistic effect for *S. aureus* or other major pathogens. A study by De Vliegher et al. (2004) seems to support this theory. They assessed the *in vitro* growth of mastitis pathogens in the presence of ten strains of *S. chromogenes*. Two of the *S. chromogenes* strains showed inhibitory activity against all *S. aureus*, *S. dysgalactiae* and *S. uberis* isolates, but not against *E. coli*. Another study (Nascimento, Fagundes, Paiva Brito, Santos & Freire Bastos, 2005) demonstrated that 6.4% of 188 CNS strains isolated from bovine mastitis cases exhibited antimicrobial substance production against an indicator strain of *Corynebacterium fimi*. The same study showed that up to 78% of *S. agalactiae* indicator strains were inhibited by one strain of CNS. Another possible explanation would be the protective effect of an increased SCC against infection by major pathogens. This is not a well-established theory, largely based on the again not well-established theory that the incidence of clinical mastitis in low SCC herds is higher in cows

with a lower SCC (Peeler, Green, Fitzpatrick & Green, 2002). A study that tried to explore this hypothesis introduced inert devices into glands leading to increases in SCC; however this showed no protective effect against experimental infection with *S. uberis* (Nickerson, Boddie, Owens & Watts, 1990). The significance of these findings should be questioned because even if a protective role is proven, the deleterious effects would have to be measured and a cost-to-benefit analysis performed. Maintaining or inducing a protective teat apex flora should be preferred over causing IMI as an IMI with the protective pathogen would result in an increased SCC and potential production losses (De Vliegher et al., 2004). In this sense, Woodward et al. (1988) tested the persistence of teat apex colonisation by several bacteria that had been previously proven to inhibit growth of mastitis pathogens *in vitro*. A strain of *S. hominis* that inhibited the growth of several Gram-positive bacteria was recovered from the same animals for up to 28 days. Conversely, if the risk of new infections is greater for CNS infected quarters, this could be due to their alteration of normal protective effects against other pathogens (Hogan et al., 1988), although these effects are not well understood. Overall, the current literature seems far from clear as to whether CNS infection is, or is not, protective against major pathogen IMI in the cow in field conditions.

Antimicrobial treatment

Irrespective of the discussion on their impact in terms of udder health, the fact is that both clinical and subclinical CNS IMI are treated with antimicrobials.

The bacteriologic cure rates reported for clinical mastitis therapy (McDougall, 1998; Waage et al., 2000; McDougall, 2003; McDougall, Arthur, Bryan, Vermunt & Weir, 2007a; McDougall, Agnew, Cursons, Hou & Compton, 2007b) vary between 53.3% and 100% depending on the study and on the antimicrobial used. Reported bacteriologic cure rates (McDougall, 1998; Wilson, Gonzalez, Case, Garrison & Gröhn, 1999; Taponen et al., 2006) for subclinical mastitis therapy vary between 81% and 88.9%. Cure rates for CNS infected quarters with dry cow therapy are not frequently reported in the literature. Rajala-Schultz, Torres, DeGraves, Gebreyes and Patchanee (2009) report a cure rate of 82% for quarters infected with CNS after dry cow therapy.

Several papers report results for antimicrobial treatments performed on pre-calving heifers (Oliver, Lewis, Gillespie & Dowlen, 1992; Oliver et al., 2003; Oliver et al., 2004; Borm et al., 2006) with cure rates for CNS infected quarters varying from 73 to 100% for intrammamary treatment. Despite the fairly high cure rates obtained, there is no agreement between these authors as to whether such a type of treatment should actually be performed. For Oliver et al. (2003) a net revenue of $200.64 per treated heifer was obtained when treating heifers pre-calving with intramammary cloxacillin or cephapirin, whereas for Borm et al. (2006) pre-calving treatment with intramammary cephapirin led to no significant improvements in milk production or linear somatic cell count. It should be mentioned that

these studies report results mainly on CNS infected quarters but also on IMI due to other pathogens. Parker, Compton, Anniss, Heuer and McDougall (2008) reporting on pre-calving treatment of heifers with parenteral tylosin found no difference between treated and untreated quarters with regards the proportion of quarters that underwent bacteriological cure.

Results of parenteral treatment of CNS IMI are also available in the literature. McDougall et al (2007b) reporting on cure rates for clinical mastitis mention a 75.9% cure rate for penethamate hydriodide and 90.5% for tylosin and Pyörälä and Pyörälä (1998) report cure rates of 78.8 and 58.3% for parenteral treatment with penicillin G and spiramycin respectively.

Cure rates for antimicrobial treatment seem therefore to be fairly high. This is partly explained by a low resistance to antimicrobials. Several papers address antimicrobial susceptibility testing either through the Kirby-Bauer method (Costa, Benites, Guerra & Melville, 2000; Cattell, Dinsmore, Belshner, Carmen & Goodell, 2001; Makovec & Ruegg, 2003) or by determination of minimum inhibitory concentrations (Rajala-Schultz et al., 2004; Lüthje & Schwarz, 2006; Pol & Ruegg, 2007). Antimicrobial resistance seems mostly to be low but some exceptions have been reported including one study that reports 90.9% of penicillin resistant strains (Costa et al., 2000). Other potentially important issues are the observation of changes in resistance patterns through time (Makovec & Ruegg, 2003), differences in resistance levels seen with different production systems (Pol & Ruegg, 2007) or the existence of methicillin-resistant strains of CNS (Sawant, Gillespie & Oliver, 2009; Fessler, Billerbeck, Kadlec & Schwarz, 2010). Besides resistance to antimicrobials, there is also the possibility of resistance to quaternary ammonium compounds among CNS (Bjorland et al., 2005), which could have an impact on udder health as several pre and post milking teat sanitizers are based on these compounds.

Control

Despite the literature on risk factors for CNS IMI being scarce, there are other options besides antimicrobial treatment for mastitis control. Initially mastitis control programmes targeted contagious mastitis pathogens. The increasing frequency of isolation of CNS led to the discussion about the causes of such a change in relative frequency of isolation (Zecconi, Piccinini & Fox, 2004). After an effective control of contagious pathogens, did environmental pathogens simply became relatively more prevalent, or did control measures aimed at contagious pathogens favour the environmental pathogens, causing an actual increase in their infection level? Zecconi et al. (2004) tested the hypothesis that strict adoption of contagious mastitis control practices by nine commercial dairy herds would result in an increase in the prevalence of non-contagious mastitis pathogens. There was no significant increase in the prevalence of coliforms, environmental streptococci or CNS at the end of the

trial period, even though the risk for CNS mastitis was significantly lower at the beginning of the adoption of the contagious pathogens control programme.

Included in most control programmes to prevent infection with udder pathogens is the use of post-milking teat disinfection. Different germicide solutions seem to have different effects on the prevalence of CNS IMI: both Goldberg et al. (1994) and Foret, Corbellini, Young and Janowicz (2005) found significant differences in the reduction of new IMI between two iodine-based formulations. The relative distribution of different CNS species can also be influenced by the type of sanitizer used (Hogan et al., 1987a). The effect of pre-milking teat sanitization has also been addressed. Ruegg and Dohoo (1997) observed no difference in the isolation of CNS between the group of animals submitted to pre-dipping and the group where this was not performed and likewise, Oliver et al. (1993) found no difference in new CNS IMI between performing pre and post-milking teat disinfection and post-milking disinfection alone.

Vaccination has also been addressed as a possible tool in the control of CNS IMI. Development of vaccines against mastitis problems is a considerable technical challenge because of the idiosyncratic immunological nature of the mammary gland and the multitude of microorganisms causing the disease (Denis, Wedlock, Lacy-Hulbert, Hillerton & Buddle, 2009). In 1994, Amorena, Baselga and Albizu (1994) reported on results of an experimental challenge following immunization against *S. aureus* and CNS in ewes. Despite showing some promising results for certain CNS species, this study was not followed up by a field trial. More recently, Middleton, Luby and Adams (2009) reported on results of a commercial vaccine against *S. aureus* mastitis (Lysigin®, Boehringer Ingelheim Vetmedica, Inc.) in field conditions. No significant differences were observed between vaccinated cows and unvaccinated controls with regards to number of quarters that developed new CNS IMI, time to CNS IMI or milk SCC. To the author's knowledge, currently there is only one vaccine licensed in Europe for the prevention of CNS mastitis (Startvac®, Laboratorios Hipra, S. A.), which is also indicated for the prevention of coliform and *S. aureus* mastitis. The manufacturer reports a significant difference in the number of CNS IMI between the placebo and vaccinated groups both for primiparous and multiparous cows. A benefit-to-cost analysis of the use of this vaccine is not yet available.

Virulence factors

Evidence of the production of a number of different virulence factors, or of the possibility to express them, has been evaluated for several CNS species. These factors could contribute to their infective or damaging potential to the mammary gland, to their capability of escaping the host's natural defences or the effects of antimicrobial therapy, or to negative consequences in terms of Public Health.

Being frequently found on the skin, the ability to establish themselves in the mammary gland is what confers CNS their infectious potential. This ability depends on the capacity to

bind tissue matrix and plasma proteins, which may be exposed in the traumatized or toxin-damaged mammary epithelium (Mamo, Froman & Wadstrom, 1988), but probably also on the capacity to avoid host defence mechanisms by way of biofilm production (Oliveira et al., 2006). Watts, Naidu and Wadstrom (1990) studied the abilities of several CNS species of bovine origin to bind collagen, degrade elastin (elastase production) and produce biofilm. Comparison between animals infected by different strains showed no significant difference in SCC between animals affected by strains producing biofilm, elastase or binding collagen and the remaining strains. Jarp (1991) suggested that haemolysin could be another important virulence factor owing to the fact that *S. haemolyticus* was more often associated with clinical mastitis in his study. Watts and Owens (1987) reported production of a delta-like toxin (cytolysin) by several species of CNS. Production of this toxin was associated with a higher increase in SCC for *S. epidermidis*, *S. hominis* and *S. warneri* but not for *S. hyicus* or *S. chromogenes*. Other potentially virulent toxins and enzymes produced by CNS strains include leucocidin, lipase, proteases and DNAse (Zhang & Maddox, 2000). Surface characteristics such as hydrophobicity and capsule formation have also been described as potential virulence factors (Matthews, Oliver and Guidry, 1991; Birgersson et al., 1992). Adhesion to and internalisation into bovine mammary epithelial cells could also contribute to the CNS infective potential. Different species display different levels of adhesion and internalization, with *S. xylosus* showing the highest values for both. *S. epidermidis* was the least efficient. Distinct pathways for internalization may be involved for different CNS species (Almeida & Oliver, 2001).

Coagulase-negative staphylococci have also been found to produce toxins that pose a potential risk for Public Health. Staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST-1) are toxins produced by staphylococcal species responsible for food poisonings. Their production by 40 mastitis isolates was investigated by Orden et al. (1992). Enterotoxins were only produced by two strains of *S. xylosus* and TSST-1 was only produced by five strains of the same species, one strain of *S. sciuri* and two strains of *S. epidermidis*. Another aspect contributing to CNS virulence and potentially to Public Health concerns is transfer of antimicrobial resistance among mastitis isolates. Muhammad, Hoblet, Jackwood, Bech-Nielsen and Smith (1993) mention that of nine strains representing three species of CNS, two could transfer streptomycin resistance. A *S. hominis* strain transferred streptomycin resistance to a *S. chromogenes* lacking resistance, and to a *S. aureus* strain carrying penicillin and tetracycline resistance.

There is a potential for differences in expression of virulence factors according to *in vitro* or *in vivo* conditions experienced. Therefore, more recently a number of genotypic studies have addressed several potential virulence factors: presence of the *bap* (biofilm-associated protein) gene and the capability of its horizontal transfer (Tormo, Knecht, Götz, Lasa & Penadés, 2005), presence of staphylococcal superantigen genes (Nemati et al., 2008; Park

et al., 2010), and presence of genes responsible for antimicrobial resistance (Lüthje & Schwarz, 2006), which may all contribute to competitive advantages for the strains expressing these genes.

Despite the multitude of studies on virulence factors, there are very few studies on the correlation of these with clinical characteristics *in vivo*. Haveri, Taponen, Vuopio-Varkila, Salmenlinna and Pyörälä (2005), Fournier et al. (2008) and Graber et al. (2009) found that certain *S. aureus* genotypes were more often associated with certain clinical characteristics such as persistence or contagiousness. However, Haveri, Roslöf, Rantala and Pyörälä (2007) in a follow up study, failed to correlate persistence or other clinical signs with presence of a particular gene or group of virulence genes. Specifically for CNS, Simojoki, Hyvönen, Taponen and Pyörälä (2010) observed no correlation between biofilm production and persistence of CNS IMI.

Table 1 displays a summary of CNS characteristics and relevant references that support those characteristics.

Table 1. CNS characteristics as a group or for individual species and respective supportive references.

Characteristic	CNS/individual species	Reference
Isolated from cases of clinical mastitis	CNS	Smith, Todhunter & Schenberger, 1985 McDougall, 1998
	Individual species	Jarp, 1991 Birgersson et al., 1992 Todhunter et al., 1993 Waage et al., 1999 Taponen et al., 2006 Simojoki et al., 2009
Isolated from cases of subclinical mastitis	CNS	Timms and Schultz, 1987 Rainard et al, 1990 Chafer et al., 1999 Borm et al., 2006
	Individual species	Jarp, 1991 Birgersson et al., 1992 Taponen et al., 2006
Isolated from clinically normal samples with low cell count	Individual species	Birgersson et al., 1992
Isolated from extramammary sites	Individual species	White et al., 1989 Matos et al., 1991 Thorberg et al., 2006 Taponen et al., 2008
Infected cows had lower milk yield	CNS	Timms and Schultz, 1987
Infected cows had higher milk yield	CNS	Wilson et al., 1997 Schukken et al., 2009 Piepers et al., 2010
Leads to histopathological changes	CNS	Trinidad, Nickerson & Adkinson, 1990 Benites et al., 2002
Observed differences in pathogenicity between species	*S. haemolyticus* more often associated with clinical mastitis	Jarp, 1991
	S. hyicus more effective in causing teat canal infections than *S. epidermidis*	Myllys et al., 1994
	S. simulans and *S. hyicus* more often associated with clinical infections and increased inflammatory response	Myllys, 1995
	S. simulans produced more persistent infections	Aarestrup & Jensen, 1997
	No difference among SCC for quarters infected with different species	Hogan et al., 1987a Chaffer et al., 1999
	No difference in clinical characteristics and persistence between two most frequent species	Taponen et al., 2007

Table 1 (continued).

Characteristic	CNS/individual species	Reference
Protective effect against mastitis by major pathogens	CNS	Schukken et al., 1989 Green et al., 2005
	Individual species	Matthews et al., 1990 De Vliegher et al., 2004
No protective effect against mastitis by major pathogens	CNS	Hogan et al., 1988 Davidson et al., 1992 Myllys, 1995 Aarestrup & Jensen, 1997

Table 2. Bacteriological cure rates for clinical and subclinical CNS mastitis observed after antimicrobial treatment and spontaneous cure rates.

Characteristic	Observed cure rates	Reference
Response to antimicrobial treatment - clinical	53.3 and 91.7%*	McDougall, 1998
	89.6%	Waage et al., 2000
	80 and 100%*	McDougall, 2003
	84.3%	Taponen et al., 2006
	76, 92.9 and 93.8%*	McDougall, 2007a
	75.9 and 90.5%*	McDougall, 2007b
Response to antimicrobial treatment - subclinical	81.8%	McDougall, 1998
	68, 75, 76, 87 and 89%*	Wilson et al., 1999
	88.9%	Taponen et al., 2006
Spontaneous cure for subclinical mastitis	64.5%	McDougall, 1998
	72%	Wilson et al., 1999
	39.5%	Taponen et al., 2006
Response to antimicrobial treatment – dry period	82%	Rajala-Schultz et al., 2009

* Depending on which antimicrobial was used.

Corynebacterium spp.

Effects on udder health

Like CNS, *Corynebacterium* spp. have been isolated from cases of both clinical and subclinical mastitis. Isolation from clinical mastitis cases is not very frequent, with authors reporting percentages of 2.5% (Botrel et al., 2010) or 3.5% (Bradley et al., 2007) of the total number of clinical mastitis samples. Typically, *Corynebacterium* spp. are isolated from cases of clinical mastitis that are less severe than cases from which other pathogens are isolated, but that are slightly more severe than mastitis cases that are culture-negative (Morin & Constable, 1998).

Isolation from subclinical mastitis cases is not very frequently reported in the literature. The author found *Corynebacterium* spp. in 12.4% of samples from sublinical mastitis cases (Bexiga et al., 2005). Most large scale studies refer to bacteriology results of clinical and subclinical mastitis samples without differentiating between them; sometimes including samples which are not necessarily mastitic, but that were submitted to laboratories as part of mastitis control programmes. This type of study reports isolation frequencies of 2.7% (Makovec & Ruegg, 2003), 7.2% (Wilson et al., 1997), 7.3% (Tenhagen et al., 2006) and 11.5% (Pitkälä et al., 2004) for *C. bovis* as a percentage of all samples (including culture-negative samples).

The impact of *C. bovis* IMI in terms of SCC is low. Djabri et al. (2002) on a meta-analysis of individual quarter SCC resulting from IMI with different pathogens, report a value of 105,000 cells/ml for *C. bovis* infected quarters. This was the lowest value for the pathogens studied and not so distant from the geometric mean obtained for culture-negative samples which was 68,000 cells/ml.

This may be because not all *C. bovis* isolated from milk samples will actually be causing mastitis (Honkanen-Buzalski and Bramley, 1984). The term intramammary infection is sometimes used interchangeably with subclinical mastitis and there is great variability between authors in defining criteria for these (Andersen et al., 2010). Reports on the isolation rates of *C. bovis* from milk samples are therefore not always linked to a measure of inflammation of the mammary gland (Wilson et al., 1997; Tenhagen et al., 2006). Teat canal colonisation with *C. bovis* would lead to contamination of milk samples submitted for mastitis diagnosis. There is evidence of this in the scientific literature for a long time. Black, Marshal and Bourland in 1972, observed that when sampling milk directly from the teat cistern by use of a transdermic needle, large differences were observed in the recovery of *C. bovis*: only 5 out of 20 samples previously positive with the conventional sampling technique were positive for *C. bovis* when sampling directly from the teat cistern and the average number of colony forming units (cfu) per ml in those 5 samples was 66, whereas it was 1900 for the 20 samples collected with the standard technique. Honkanen-Buzalski and Bramley in 1984

report on the results of an experimental infection with *C. bovis* on 72 quarters. No clinical mastitis was recorded, even though the microorganism was being excreted three days after the initial challenge in 73.6% of quarters. Again using a transdermic needle to collect milk samples from quarters that were excreting *C. bovis*, 44.4% of the samples were negative, indicating that colonisation did not go further than the teat canal.

Evidence about duration of infection is scarce. Sordillo, Oliver, Doane, Shull and Maki (1989c) performed experimental infections with *C. bovis*. In one of the trials, infection during the lactation persisted for 93 days, the duration of the study, and in the other study, infection just before drying off led to infections that persisted through the whole dry period and into early lactation. Rainard and Poutrel (1982) performed three-weekly milk sampling for one year on three herds and found that of 161 IMI with *C. bovis*, only 27 were eliminated. Approximately one third (34.8%) of *C. bovis* IMI were detected at calving, another third (33.5%) in the first three months post-calving and another third (31.7%) from three months onwards.

The impact of *C. bovis* infection on milk yield has been addressed by LeVan, Eberhart and Kesler (1985). These authors compared the milk yield of 53 quarters experimentally infected with *C. bovis* with their contralateral quarters and observed a reduction in yield of 0.18kg/day, a non-significant difference.

Corynebacterium bovis contribution to the bulk tank SCC is also low, with estimated values varying between 7.1 and 8.6% of the total SCC, depending on the overall level of bulk tank SCC (Schukken et al., 2009).

There are at least two descriptions of the pathological changes associated with IMI due to *C. bovis*. Sordillo, Doymaz, Oliver and Dermody (1989a) observed that despite *C. bovis* colonised glands showing higher numbers of most inflammatory cells, the differences observed in the proportions of epithelium, lumen and stroma were not significantly different from control uninfected glands. Ngatia, Jensen and Berg (1991) studied mammary glands naturally infected with *C. bovis* alone and found inflammatory changes affecting teat cisterns, Furstenberg's rosettes and for some, but not all the glands, changes in the mammary parenchyma.

Other species of the *Corynebacterium* genus have been implicated in bovine mastitis including *C. amycolatum*, *C. ulcerans*, *C. pseudotuberculosis*, C. *minutissimum* and *C. xerosis* (Hommez et al., 1999; Watts, Lowery, Teel & Rossbach, 2000). These identifications were confirmed through genotypic methods, since the performance of phenotypic methods seems to be poor for corynebacteria (Watts et al., 2000). No differences in pathogenicity of corynebacteria have been reported regarding udder health.

Corynebacteria causing intramammary infection have been reported to present a low resistance to antimicrobials, with Watts & Rossbach (2000) observing a good *in vitro* activity

for the 15 antimicrobials tested and a much lower resistance than the correspondent human isolates.

There has also been some discussion about the potential protective effects of colonisation with *C. bovis*. Some authors have found evidence of a protective effect (Schukken et al., 1989; Lam et al., 1997; Green et al., 2005), whereas others found no evidence of such an effect or in fact found an opposite effect (Pankey, Nickerson, Boddie & Hogan, 1985; Hogan et al., 1988; Berry & Hillerton, 2002). There is also some *in vitro* evidence to support a possible protective effect of some *Corynebacterium* spp. (Woodward, Besser, Ward & Corbeil, 1987). The stage of the lactation may influence the protective role of *Corynebacterium* spp. as Green, Green, Medley, Schukken and Bradley (2002) observed that its presence at drying off was associated with an increased risk of new clinical mastitis, whereas its presence in early lactation was associated with a reduced risk.

Epidemiology and control

There is not much literature available on the epidemiology of *C. bovis* as an intramammary pathogen. It has been considered a contagious mastitis pathogen (Fox & Gay, 1993) but the scientific evidence to back that statement is scarce and more recent studies, resorting to molecular biology techniques, are not straightforward. García-Crespo, Navas, Medley and Juste (2005) used PFGE to strain type 162 *C. bovis* isolates from 57 dairy herds and observed only seven pulsotypes, which might suggest that the criteria used to discriminate between different strains of *C. bovis* might need to be modified, to be sufficiently discriminatory and a useful tool to study its molecular epidemiology. Pulsed-field gel electrophoresis is generally a useful tool for epidemiological analysis, but interpretation criteria need to be adapted for bacterial species that typically are highly conserved, such as *E. coli* O157, or that have high genetic macrodiversity, such as *Helicobacter pylori* (Goering, 2010). Busato, Trachsel, Schällibaum and Blum (2000) observed that the quarter prevalence of *C. bovis* increased nearly 20% between samples collected in the period 7-100 days in milk and 100-305 days in milk. An increase in the level of infection as the lactation progresses could be seen as more characteristic of a contagious type of infection – the longer the animals are exposed to the milking risk factor, the more they will get infected – but this is just a supposition.

Corynebacterium bovis has also been viewed as occurring mainly in farms where post-milking teat disinfection is sub-optimal (Bramley, Kingwell, Griffin & Simkin, 1976; Hillerton, Staker & Shearn, 1995) or not performed (Brooks, Barnum & Meek, 1983). Certainly there is evidence that use of post-milking teat disinfection leads to a lower number of IMI due to *C. bovis* being acquired in naturally challenged quarters (Oliver et al. 1989; Erskine, Sears, Bartlett & Gage, 1998). Likewise, use of a backflush system at milking with an iodine solution significantly reduced the number of new IMI due to *C. bovis* (Smith et al., 1985a).

There are not many references to cure rates obtained after antimicrobial treatment during the lactation, probably because the impact on udder health is most often low and such treatment not recommended. Spontaneous lactational cure rates appear to be high, with Wilson et al. (1999) reporting a bacteriological cure rate of 95%. As for cure rates during the dry period, reported values are often close to 100% (Harmon, Crist, Hemken & Langlois, 1986; Hogan et al., 1994), with some authors also recording values close to 80% (Shepard, Burman & Marcun, 2004). Spontaneous cure rates during the dry period are somewhat lower, with Harmon et al. (1986) observing 47.6% and Hogan et al. (1994) observing 63.9%. Contradicting these results, Green et al. (2005) observed that approximately two thirds of the quarters from which *Corynebacterium* spp. was isolated in the late dry to calving period also had the bacteria cultured from the milk sample taken at drying off, even after antimicrobial therapy.

If *C. bovis* are more often transmitted from cow to cow, then the control measures that are effective in the control of other contagious mastitis pathogens (Neave et al., 1966) would likely have succeeded in eliminating the infection from most herds. That is, the five-point mastitis control plan should succeed in reducing its prevalence. This does not always seem to be the case with some authors reporting a decrease in its prevalence with time (Makovec & Ruegg, 2003), but with others reporting just the opposite (Pitkälä et al., 2004).

Main objectives of the study

Some of the main conclusions drawn from the previous literature review are that CNS and *Corynebacterium* spp. are isolated very frequently from milk samples, that their impact on udder health is not considerable and that there are probably some differences between individual CNS species in terms of impact on udder health. This poses a problem to veterinary practitioners and milk quality advisors – what is the significance of finding these pathogens in milk samples and what should be the course of action when they constitute a big proportion of bacteriology results. As Morin and Constable (1998) put it, with regards to clinical mastitis and *Corynebacterium* spp., “Dairy producers face a dilemma when no bacteria or bacteria commonly regarded to be of minor pathogenicity such as *Corynebacterium* spp. are cultured from the milk of cows with clinical mastitis”.

The starting point for the current study was thus to investigate:

1) if all the CNS species have the same impact in terms of udder health;
2) if CNS and *Corynebacterium* spp. are truly responsible for high somatic cell counts or if they are simply easier to isolate than the pathogens that are the true culprits;
3) what control measures are advisable or not, as part of a mastitis control programme when CNS are isolated in a large proportion of milk samples.

To answer these questions, several specific objectives were drawn:

To compare individual quarter somatic cell counts in milk samples from which different CNS species had been isolated; to compare the duration of IMI for different CNS species and to gain insight into their most likely forms of transmission (study I).

To determine the effect of freezing on the detection rate of certain bacterial groups in milk samples and to determine if a real-time PCR test could detect mastitis pathogens in culture-negative samples or in samples that yielded minor pathogens with conventional bacterial culture techniques (study II).

To evaluate the difference in bacteriology results from milk sampled with the standard milk sampling technique and by use of a teat cannula surpassing the teat canal, potentially avoiding sample contamination with teat canal microbiota (study III).

To evaluate the potential economic benefit in performing lactational antimicrobial therapy of subclinical mastitis caused by CNS (study IV).

Studies

Study I

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Bovine intramammary infection due to coagulase-negative staphylococci in four farms: impact of individual species

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Abstract

The objectives of this study were to compare the impact of different coagulase-negative staphylococci (CNS) species on udder health measured in terms of individual quarter somatic cell count and duration of intramammary infection, and to get some insight into their epidemiology. This longitudinal observational study was performed in four farms, sampled at four week intervals for 12 visits each. Quarters infected with CNS were followed through time with milk samples being submitted for bacteriology and somatic cell count (SCC) determination. PCR amplification of the internal transcribed spacer region was used for species allocation with sequencing of the *sodA* and *rpoB* genes being used when the PCR was not completely informative. Pulsed-field gel electrophoresis was performed to assess strain identity. The most frequently isolated CNS species was *S. epidermidis*, followed by *S. simulans*, *S. chromogenes* and *S. haemolyticus*. It was possible to follow 111 CNS intramammary infections through time. Duration of infection had a mean of 188 days and was not significantly different between species. Geometric mean quarter SCC overall was 132,000 cells/mL and did not differ significantly between species. Estimation of the Simpson diversity index showed significant differences between species when all farms were considered, with *S. epidermidis* and *S. simulans* showing less diversity than *S. haemolyticus*. When comparing *S. epidermidis* diversity between two of the farms a significant difference was found. Despite the similarity in terms of udder health impact observed for different CNS species, this study supports the possibility of a different epidemiology of infection.

Keywords: CNS, individual species, mastitis, impact, diversity

Introduction

In recent years much attention has been devoted to the role of coagulase-negative staphylococci (CNS) in bovine mastitis. Traditionally, individual CNS species were grouped together and it was assumed that they all presented the same characteristics in terms of pathogenicity. Several authors have studied the impact of different CNS species on mastitis, with evidence of some being more frequently isolated from clinical cases (Waage et al., 1999; Taponen et al., 2006) and others found to be more persistent during lactation (Taponen et al., 2007). It is well established that CNS are present in the environment (White et al., 1989; Matos et al., 1991). However, the most frequent form of infection might differ for different CNS species. Taponen et al. (2008) found *Staphylococcus chromogenes* pulsotypes that were shared between isolates from mastitic milk samples and extramammary sites, whereas *Staphylococcus simulans* was very seldom isolated from extramammary sites despite frequently being isolated from mastitic milk samples, indicating a stronger possibility of cow to cow transmission.

The objectives of the present study were to compare intramammary infections (IMI) due to different CNS species in terms of quarter somatic cell count (SCC) and duration of infection, and to gain insight into their most likely forms of transmission.

Materials and methods

Animals and bacterial isolates

This longitudinal observational study was performed in four commercial dairy farms. All animals were Holstein-Friesian, zero-grazed, with year-round calving and housed in cubicles with sand bedding. Farm A milked three times per day and was a closed farm, whereas the other farms milked twice daily and would occasionally buy

in primiparous animals. Post-milking teat dipping and antimicrobial dry cow therapy were routinely performed on every animal across farms. Mean data for SCC, milk production and number of animals in each farm is displayed on Table 1. Quarter milk samples were taken according to NMC protocols (NMC, 1999) every four weeks in each farm for 12 visits, and a total of 48 weeks. In the initial visit, 12 cows were randomly selected from those with individual cow SCC showing an increase from below to above 200,000 cells/mL in the previous two milk recordings, to tentatively detect new infections at cow level. On subsequent visits, cows from which CNS had been isolated in one of the two previous visits, were resampled at quarter-level; if these animals were not enough to total 12 cows, others were selected according to the criterion used in the first visit, to obtain samples from 12 cows per visit. A total of 2302 quarter milk samples from 264 cows were collected. None of the samples originated from clinical mastitis cases. Individual quarter SCC were determined through flow cytometry (CombiFoss, Foss). Records of treatments performed during the study were analysed to exclude animals that received antimicrobial treatment during the study from the analysis or for right censoring of data.

Samples were refrigerated, transported to the laboratory and processed in the sampling day. From each milk sample, 0.01 mL of milk were plated onto sheep blood agar (Columbia, bioMérieux), incubated at 37 ºC and observed after 24 and 48 hours of incubation. Samples yielding more than two morphologically different bacterial colonies were considered contaminated. A single colony representative of colonies with similar morphology was selected for isolation and identification if there was evidence of growth of ≥500 cfu/mL. Gram-positive catalase-positive cocci were initially identified to species level by use of a biochemical identification system (ID 32 Staph, bioMérieux). Isolates with an identification probability <90% were

excluded from the study. Milk samples in which CNS were found concurrently with a major mastitis pathogen were also excluded from the analysis.

Strain typing

Molecular fingerprinting of staphylococci to strain level was performed by pulsed-field gel electrophoresis (PFGE) as described previously (Chung et al., 2000). Staphylococcal isolates submitted to PFGE (n=467) included all the CNS isolated from each quarter when these were found in four or fewer sampling visits in succession, and every other isolate when these were found in five or more sampling visits in succession. The PFGE patterns were analysed with BioNumerics v. 4.61 (Applied Maths NV). Levels of similarity between profiles were estimated with the Dice coefficient and an unweighted pair group method using arithmetic averages (UPGMA) was used for clustering, to produce band-based dendrograms, with a band position tolerance of 1.25% and optimization of 0.5% according to McDougal et al. (2003). Cut-off values of 70 and of 80% similarities were used to differentiate clusters of PFGE types.

Genotypic species identification

Genotypic identification was sought using an internal transcribed spacer PCR (ITS-PCR), as described previously (Couto et al., 2001) with modifications. Briefly, the disks containing DNA used to perform the PFGE were melted at 65 ºC for 10 minutes in 1x TE buffer. A 1.25 µL aliquot of bacterial DNA was combined with 10 µL of reaction buffer, 6 µL of 25mM $MgCl_2$, 1 µL of 10mM dNTP mix, 0.25 µL of DNA polymerase, 29 µL of nuclease free water (all reagents from Promega) and 1.25 µL of the G1 (5'-GAAGTCGTAACAAGG) and of the L1 (5'-CAAGGCATCCACCGT) primers (Stabvida). PCR amplification products, obtained using a MyCycler thermal

cycler (Bio-Rad Laboratories), were resolved in a 3% Sea Plaque agarose (Lonza) for 6 hours at 80 volts. A 100 bp DNA step ladder was used as a molecular weight marker (Promega). All the isolates with characteristic PFGE profiles were submitted to the ITS-PCR, with at least two representatives of each profile being selected whenever available. The ITS-PCR profiles of 94 isolates were compared with 19 type strains (Table 2) and with a *Staphylococcus pseudintermedius* isolate previously identified and characterized genotypically (Couto et al., 2011). The 19 type strains were selected based on phenotypic identification results. The profile analysis was performed visually with the aid of the BioNumerics software v. 4.61 (Applied Maths NV). Whenever ITS-PCR results did not allow for a clear differentiation between species or did not match type strain profiles, isolates (n=27) were submitted to PCR amplification of both *sodA* and *rpoB* housekeeping genes, as described previously by Poyart et al. (2001) and Drancourt and Raoult (2002), respectively. PCR products were sequenced at Macrogen (Seoul, Korea), and sequence data proofread using Bioedit v. 7.0.5 (Hall, 1999), and compared with publicly available sequence data using nucleotide-nucleotide BLAST[1].

Data analysis

Unless otherwise stated, only results based on genotypic identification were used in the data analysis. An IMI with a CNS was defined by isolation of CNS from at least one quarter milk sample. Spontaneous cure was defined as the non-detection of that strain in two subsequent visits in succession. Duration of IMI was defined by the period of time a CNS with the same PFGE profile, was found in successive or alternate months and analysed by way of a Kaplan-Meier survival curve and a log-rank survival test. Right censoring was performed whenever an infection was still

[1] See: http://www.ncbi.nlm.nih.gov/blast/.

present or a single culture-negative quarter was present either at the end of the study, at drying-off or if an antimicrobial treatment occurred, not allowing for the criteria that define continuation of the infection or spontaneous cure to be observed. Individual quarter SCC was normalized using a $\log_{10}$ transformation and comparisons were made between individual CNS species with a general linear model considering clustering at sampling number, quarter, cow and herd level by use of nested random effects, with an adjusted R-square of 0.60 being obtained for the model.

The Simpson diversity index (SDI) and its confidence intervals were calculated based on PFGE profiles for different CNS species from all farms, and for each species within a farm. A significant difference between results was defined as non-overlapping confidence intervals according to Grundmann et al. (2001).

Data were analysed using SPSS version 15.0 (survival analysis) and in Minitab 16.0 (general linear model). A significant difference was defined as a probability value of $P<0.05$.

Results

Considering only the first time a cow was sampled, 147 out of 1021 sampled quarters (14.4%) had an IMI due to CNS according to phenotypic identification. The proportion of quarters with CNS IMI among sampled quarters, ranged from 34.7% in farm A to 6.4% in farm B (Table 1). It was possible to follow 111 infections due to CNS in 51 cows through time. Several infections (n=36) were excluded from the analysis because they could not be followed for at least three successive samplings. Several others (n=63) were right-censored in order to be used in the analysis. This occurred because: a) antimicrobial treatments were performed during the study; b) it

was not possible to follow the animals through time (some died, were culled or were missed by the operator during sampling visits); c) some animals included in the final visits did not stay long enough in the study to allow for cure or maintenance of infection to be effectively evaluated. Duration of infections caused by different CNS species was not significantly different (P=0.179) as observable in Fig. 1. Quarter SCC was also not significantly different between CNS species. The relationship of $\log_{10}$ SCC with both sampling number and cow were species-dependent (P=0.014 and P=0.015, respectively).

Staphylococcus epidermidis was the most frequently isolated CNS despite having the lowest impact on SCC, whereas *S. chromogenes* had the greatest impact on SCC and *S. simulans* was found in the same quarters in more successive visits than other species (Table 3). The geometric mean SCC for infections with different duration or caused by different CNS species was not significantly different.

Calculation of SDI showed that *S. epidermidis* and *S. simulans* had lower values, when compared with *S. chromogenes* and *S. haemolyticus*. Comparison of SDI values for *S. epidermidis* and *S. simulans* in farm A and D, showed that the former had different SDI values, whereas the latter had similar values between the two farms considered. Simpson diversity indices for each of the four most prevalent CNS species and for the two species-farm combinations with the highest number of isolates are shown on Tables 4 and 5 respectively. Dendrograms showed that clusters within *S. epidermidis* and *S. simulans* isolates were generally grouped per farm, whereas for *S. chromogenes* and *S. haemolyticus* this separation was not noticeable. Cows with two or more quarters affected by CNS IMI (n=29) had the same species in multiple quarters (n=10), different species between quarters (n=9) or both (n=10). In cases

where the same species was isolated in more than one quarter per cow (n=20), shared pulsotypes between quarters of the same cow were found in 17 animals.

The ITS-PCR method did not allow for confident identification of several isolates, namely it was difficult to distinguish between *S. epidermidis* and *S. chromogenes* (Fig. 2), and some isolates that had been identified phenotipically as *S. intermedius* had profiles that did not match any of the type strains used. These were clarified through sequencing of housekeeping genes.

Discussion

Coagulase-negative staphylococci were the most frequently isolated bacteria from milk samples in several studies worldwide (Makovec and Ruegg, 2003; Pitkälä et al., 2004). In our study, the proportion of CNS infected quarters was 14.4% of all sampled quarters. Some CNS species were only found in two herds, notably *S. epidermidis* and *S. simulans*, the most frequently isolated, were found in farms A and D, whereas *S. chromogenes* and *S. haemolyticus*, which had the third and fourth greatest number of CNS isolates respectively, were isolated from milk samples in all four farms. Most studies on prevalence and persistence of CNS infection have focused on single farms, not allowing for clustering effect evaluation of CNS species per farm. There is evidence in the literature of a low (Østerås et al., 2006) or moderate (Barkema et al., 1997) herd-level clustering effect for CNS, but not for individual CNS species. Despite the low number of farms in the current study, the predominance of two species in two of the farms suggests a clustering effect for individual CNS species. However, the low number of farms involved did not allow for specific risk factors to be evaluated for CNS IMI at herd level.

Mean duration of infection for all CNS was 188 days, corresponding to 6.7 successive monthly visits. There were no significant differences in mean duration of infection between CNS species, ranging from 123 days for *S. haemolyticus* to 213 days for *S. simulans*. Persistence of CNS IMI has been reported by several authors, with 46% (Taponen et al., 2007), 66% (Seymour et al., 1989), 75.6% (Rainard et al., 1990), 84.5% (Chaffer et al., 1999) and 85% (Timms and Schultz, 1987) of infections persisting until dry-off or until the cows left the herd. Todhunter et al. (1993) mentioned an average length of CNS IMI of 222 days and Rainard et al. (1990) of 236 days, despite neither resorting to molecular biology techniques for confirmation of IMI with the same strain. It is impossible to establish more accurately the exact duration of the IMI followed in our study because there is no exact knowledge of when the infections were acquired or cleared. A lower SCC threshold at cow level could have been used for detection of new CNS IMI: in the current study, sampling on the initial visit aimed at detecting recent infections at cow level, but in fact with the quarter SCC value obtained for CNS IMI, the use of the 200,000 cell/mL threshold at cow level would likely have poor sensitivity to detect new cases of CNS IMI.

Geometric mean SCC for CNS IMI followed through time was 132,000 cells/mL, with no significant differences observed between CNS species. *Staphylococcus chromogenes* was associated with the highest mean SCC, with a geometric mean of 202,000 cells/mL and *S. epidermidis* with the lowest mean, with a SCC of 95,000 cells/mL. The geometric mean obtained in this study is similar to the 138,000 cells/mL reported by Djabri et al. (2002) in a meta-analysis for quarter milk samples infected with staphylococci other than *S. aureus.* Literature on the impact of individual CNS species on quarter SCC is scarce. Nickerson et al. (1995) also observed that *S. chromogenes* led to the highest quarter SCC and, similarly to our

work, Hogan et al. (1987) did not find significant differences in quarter SCC between CNS species. In the current study, the impact of sampling number and of cow on $\log_{10}$ SCC were both species-dependent. The reduced number of observations and the fact that not every species had the same average duration of IMI, render the sampling occasion relationship of questionable significance. The significant relationship for cow would be expected since individual animal have been shown to respond differently to infections with CNS (Simojoki et al., 2009) or with other udder pathogens (Burvenich et al, 2003).

PFGE has been used as a tool to study mastitis outbreaks in single farms (Munoz et al., 2007), the possible origin of intramammary isolates (Taponen et al., 2008) and the distribution of certain characteristics such as antimicrobial resistance of strains over several farms (Fessler et al. 2010). In the current study, PFGE was used to evaluate duration of infection and strain diversity. Choice of a cut-off value to differentiate between different PFGE pulsotypes is not unanimous, with some authors resorting to the lowest reproducibility value to define the cut-off (Silva et al, 2008) and others defining similarity coefficients after reviewing epidemiological data associated with each cluster of isolates (McDougal et al., 2003). The criteria for PFGE profile interpretation that seem to gather more agreement were defined by Tenover et al. (1995). In the current study, the 80% cut-off value was used according to a publication by the same research group, analysing the same genus and using the same restriction enzyme (McDougal et al., 2003). However, the 70% cut-off value could be viewed as more appropriate because not all the criteria defined by Tenover et al. (1995) are fulfilled in the current study; namely the analysed strains were obtained from different geographical areas and over a time span close to a year in which natural variation would likely add to the observed DNA fingerprint differences.

A significant difference was found between the SDI for *S. epidermidis* and *S. simulans*, and *S. haemolyticus*, with both the cut-off set at 70 and at 80%. This indicates a greater diversity within *S. haemolyticus* than within the other two species. A significant difference was also observed for the SDI of *S. epidermidis* between farms A and D when using 80% as the cut-off value, suggesting that this species' diversity varied between these two farms (Fig. 3). On the contrary, the SDI for *S. simulans* in the same farms showed no difference, suggesting that this species is less affected by herd factors. Nevertheless, the low number of isolates per farm did not allow for a more extensive inter-species comparison within each farm. The use of such methodologies across different farms raises the question whether the differences observed in diversity between species are a feature of the species, a feature of the farm (including host and farm management factors) or a combination of both. Coagulase-negative staphylococci are frequently found in nuliparous animals (Aarestrup and Jensen, 1997), suggesting an environmental source of infection, as exposure to the milking parlour is yet to take place. However, infections established pre-calving may derive from other animals through ingestion of contaminated milk, such as might occur for mastitis due to *Mycoplasma* spp. (Fox et al., 2008; Roy et al. 2008). Literature on the source of CNS IMI after calving supports the view of an environmental origin for some species. Taponen et al. (2008) found the same pulsotypes of *S. chromogenes* isolated from milk samples and extramammary sites suggesting an environmental source of infection. They also found that *S. simulans* was very commonly isolated from milk samples but seldom found in environmental samples, indicating that it is likely to be a specific mastitis pathogen. An environmental point source can still be responsible for the same strain being regularly isolated from milk samples: Thorberg et al. (2006) found common *S. epidermidis*

PFGE pulsotypes in milk and on milkers' skin, in which case it might be difficult to classify infection as being of environmental or contagious origin.

Conclusions

There were no statistically significant differences between individual CNS species in terms of duration of IMI and mean quarter SCC. Mean duration of IMI was approximately six months and mean quarter SCC was 132,000 cells/mL. Some differences in diversity of CNS species were observed. These need to be further characterised to better elucidate the predominant mode of infection for different species, especially in farms in which CNS mastitis constitute the main problem.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1

Arithmetic mean for the number of lactating animals and milk yield, geometric mean for individual cow SCC for the duration of the study; number of cows and quarters sampled, number of cows and quarters with CNS IMI and proportion of quarters affected

Parameter	Farm			
	A	B	C	D
Lactating animals	228	178	533	375
Milk production at 305 days	11923	9496	9569	9330
SCC ($x10^3$ cells/mL)	228	244	465	601
Sampled cows	30	88	88	58
Cows with CNS IMI	19	20	20	31
Sampled quarters	118	343	329	231
Quarters with CNS IMI	41	22	24	60
% quarters with CNS	34.7%	6.4%	7.3%	26.0%

Table 2

List of *Staphylococcus* sp. used for comparison with ITS-PCR isolate profiles and respective DSM numbers

Staphylococcus sp.	DSM number
S. lugdunensi	*4804*
S. sciuri subsp. *carnaticus*	*15613*
S. sciuri subsp. *rodentium*	*16827*
S. epidermidis	*20044*
S. saprophyticus subsp. *saprophyticus*	*20229*
S. aureus subsp. *aureus*	*20231*
S. haemolyticus	*20263*
S. xylosus	*20266*
S. warneri	*20316*
S. simulans	*20322*
S. capitis subsp. *capitis*	*20326*
S. hominis subsp. *hominis*	*20328*
S. intermedius	*20373*
S. chromogenes	*20454*
S. hyicus	*20459*
S. caprae	*20698*
S. auricularis	*20609*
S. gallinarum	*20610*
S. pseudintermedius	-

Table 3

Number of infections followed per farm, geometric mean SCC and presence in successive months of CNS identified genotypically

CNS	Farm					SCC[1]	Presence in successive months[2]
	A	B	C	D	Total		
S. epidermidis	11	0	0	27	38	95	5.6
S. simulans	29	0	0	7	36	132	7.6
S. chromogenes	3	3	7	3	16	202	5.4
S. haemolyticus	3	1	1	2	7	182	4.4
Other CNS	0	1	6	7	12	171	6.0
Geometric means					111	132	6.7

[1]Geometric mean; [2]Kaplan-Meier survival analysis

Table 4

Number of isolates and of clusters, Simpson diversity indices and confidence intervals for each of the four most prevalent CNS species isolated in the study, according to a cut-off value of 80 and of 70%

CNS species	Number of isolates	Cut-off 80%			Cut-off 70%		
		Number of groups	SDI	CI	Number of groups	SDI	CI
S. epidermidis	82	15	0.82	0.77-0.88	9	0.77	0.71-0.82
S. simulans	73	14	0.84	0.79-0.89	8	0.78	0.72-0.83
S. chromogenes	28	11	0.92	0.88-0.96	7	0.84	0.78-0.90
S. haemolyticus	26	15	0.96	0.93-0.98	12	0.92	0.88-0.96

Table 5

Number of isolates and of clusters, Simpson diversity indices and confidence intervals for the species-farm combinations with the highest number of isolates, according to a cut-off value of 80 and of 70%

Species - Farm	Number of isolates	Cut-off 80%			Cut-off 70%		
		Number of groups	SDI	CI	Number of groups	SDI	CI
S. epidermidis - A	25	11	0.89	0.81-0.96	7	0.75	0.65-0.85
S. epidermidis - D	57	6	0.68	0.61-0.75	4	0.59	0.53-0.66
S. simulans - A	55	8	0.74	0.67-0.82	5	0.66	0.59-0.74
S. simulans - D	18	6	0.78	0.67-0.90	4	0.63	0.52-0.75

Fig. 1. Duration (four week intervals) of quarter infections with different CNS species on all four farms (Kaplan-Meier survival analysis).

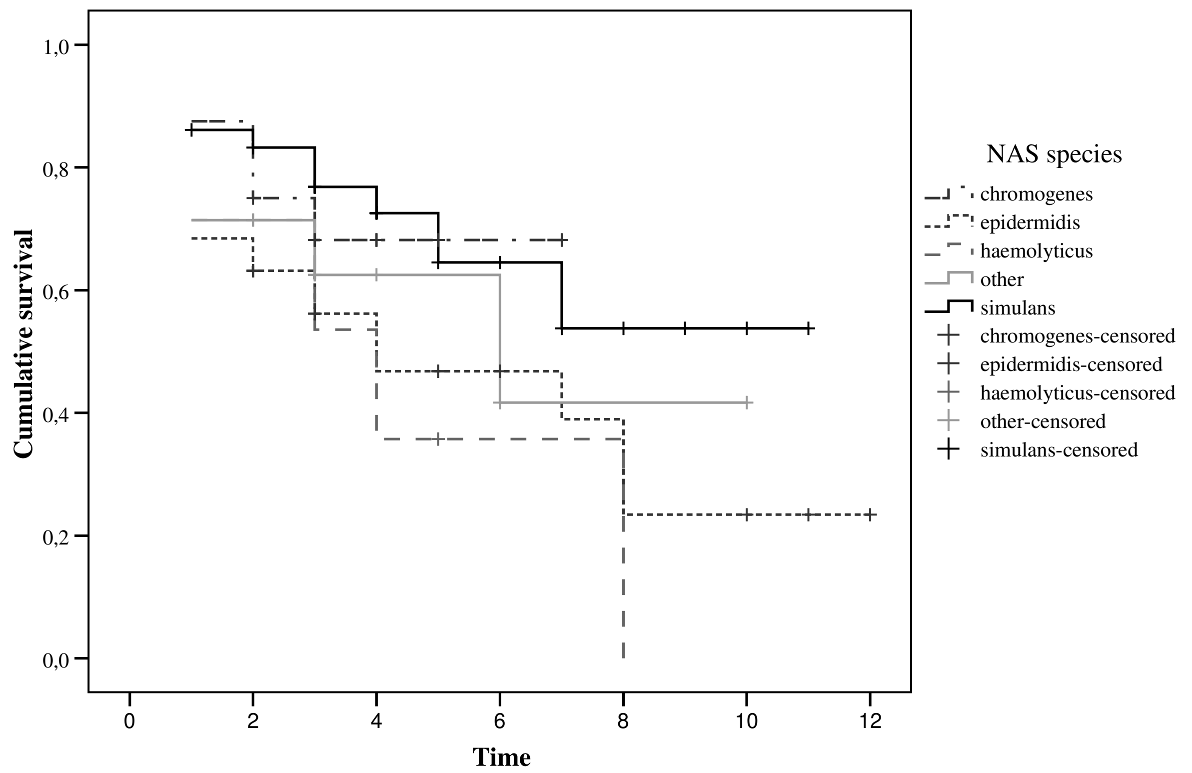
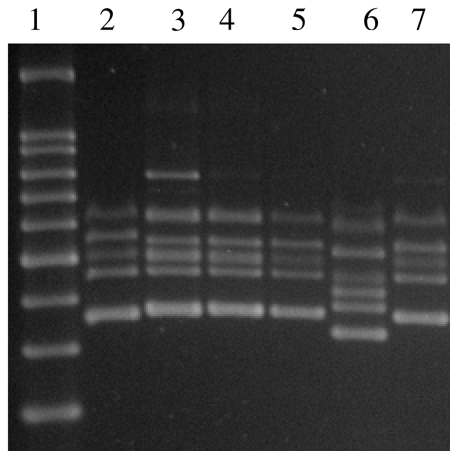


Fig. 2. ITS-PCR amplification patterns for several staphylococci: lane 1 - 100-bp ladder molecular size markers; lane 2 - *S. epidermidis* type strain; lane 3 through 5 – *S. chromogenes* isolates; lane 6 – *S. capitis* type strain; lane 7 – *S. chromogenes* type strain.

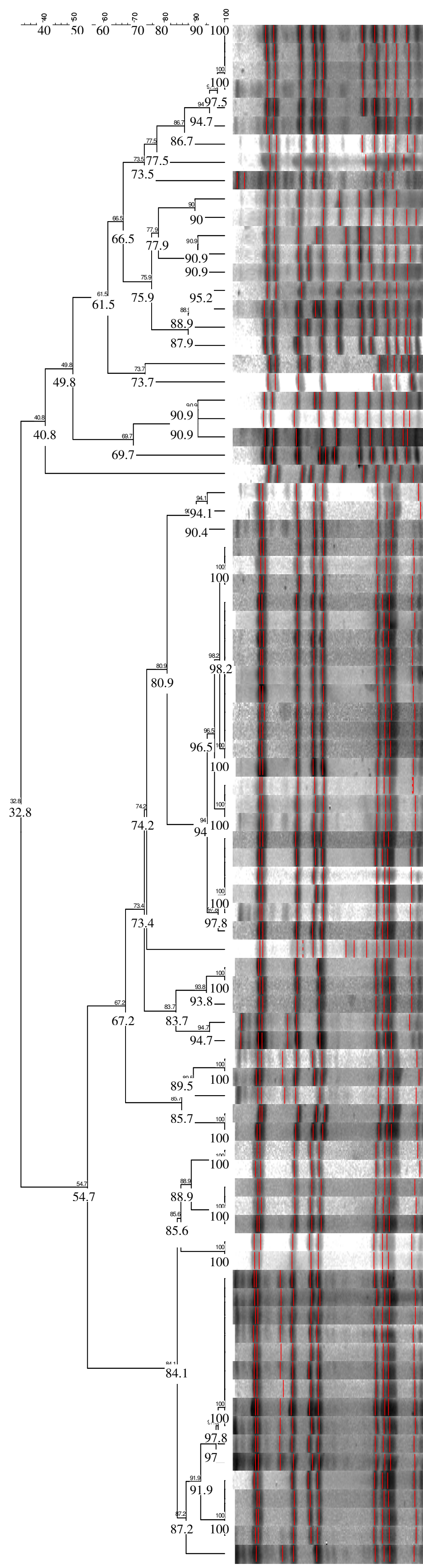


1 Fig. 3. Dendrogram with PFGE profiles for *S. epidermidis* found on farms A and D. A

2 greater diversity is observable for *S. epidermidis* in farm A (SDI = 0.89, with an 80%

3 cut-off) when compared with farm D (SDI = 0.68).

4



Farm	Cow/Quarter	Sampling occasion
Q	1220 RH	10
Q	1220 RH	12
A	1045 RF	2
A	1045 RF	5
A	688 RH	1
A	1137 RH	1
A	1220 LF	10
A	1220 LH	10
A	1143 LF	11
A	1288 RF	7
A	1283 LF	9
A	1288 RH	10
A	1288 RH	2
A	1283 LH	9
A	1288 RH	10
A	1288 RH	2
A	1283 LH	9
A	979 RH	11
A	1336 RH	1
A	897 RF	4
A	897 RH	1
D	3111 LH	5
Type strain		
A	1283 RF	10
A	1143 LF	12
A	1143 RH	12
A	633 RH	3
A	979 RH	2
D	512 LH	10
D	480 RF	6
D	634 LF	8
D	321 RH	8
Q	1215 RH	9
D	666 LH	8
D	3104 LF	10
D	5136 RH	12
D	535 RF	12
D	304 LF	2
D	3129 LF	5
D	3129 RF	5
D	3104 LF	1
D	3104 LH	1
D	3104 RH	1
D	5136 RH	1
D	5127 LF	5
D	5136 LF	3
D	5136 LF	10
D	3104 LF	5
D	649 RH	10
D	321 LH	8
D	504 RH	8
D	321 LH	9
D	3164 LH	1
D	5136 RF	7
D	535 LH	12
D	343 RF	6
D	3104 RF	7
D	535 LF	12
D	630 LH	12
D	666 RH	10
D	612 RH	2
D	630 LF	12
D	5127 RH	2
D	5127 LH	2
D	5118 LH	3
D	480 LF	3
D	5127 LF	5
D	480 RH	3
D	5127 LF	2
D	223 RH	12
D	3157LH	12
D	634 RH	11
D	612 RH	12
D	634 LH	12
D	589 RF	3
D	589 RH	3
D	593 LH	4
D	504 RH	5
D	612 LF	5
D	612 RF	8
D	593 RH	2
D	634 LH	7
A	1275 RH	11
D	5127 RF	1

Study II

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Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing

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Up to half of quarter milk samples submitted for mastitis diagnosis are culture-negative results or lead to identification of coagulase-negative staphylococci or *Corynebacterium bovis* in conventional culturing, the so-called minor pathogens. The interpretation and usefulness of these results in terms of udder and animal health management is limited, even though the amount of resources spent is relatively high. This work aimed to test two methods of analysis of milk samples with the goal of increasing detection of intramammary pathogens. In the first study, 783 milk samples were processed in duplicate: before and after freezing at −20 °C for 24 h, using standard bacteriological techniques. There was a significant difference between the two methods with samples frozen for 24 h yielding significantly fewer Gram-positive catalase-positive cocci, Gram-negative bacilli, Gram-positive bacilli and significantly more samples leading to no growth, than samples before freezing. The number of samples yielding Gram-positive catalase-negative cocci was not significantly affected by freezing. In the second study, a real-time PCR-based test was performed on milk samples with an individual quarter somatic cell count above 500 000 cells/ml that were either negative ($n=51$ samples) or that led to the isolation of minor pathogens in culturing: *Corynebacterium bovis* ($n=79$ samples) or non-aureus staphylococci (NAS, $n=32$). A mastitis pathogen, beyond the result obtained with standard bacteriology, was detected on 47% of the no-growth samples, on 35% of the samples from which *C. bovis* had been isolated and on 25% of the samples from which NAS had been isolated. The most commonly detected major pathogen was *Escherichia coli*, followed by *Streptococcus uberis, Arcanobacterium pyogenes/Peptoniphilus indolicus* and *Streptococcus dysgalactiae*. These results suggest that simply freezing milk samples for 24 h does not increase the detection of intramammary bacteria in milk samples and therefore should not be recommended. However, use of the real-time PCR-based test may be useful in diagnosing intramammary infections when milk samples with high somatic cell counts are culture-negative or when culturing results in the detection of minor pathogens.

Keywords: Freezing, real-time PCR, no-growth samples, minor pathogens.

When performing udder health investigations, it is important for veterinarians to know the mastitis pathogens involved in a particular problem. Knowledge of the most prevalent pathogens is essential for practitioners to propose targeted corrective measures that will truly lead to an improvement in a situation. A large proportion of milk samples submitted for bacteriology from cases of clinical/subclinical mastitis, lead to no-growth results: a value of 30% is commonly cited for the 'no-growth samples' (Taponen et al. 2009) with cited values ranging from 26·5% (Bradley et al. 2007) to 49·7% (Makovec & Ruegg, 2003). In addition to the no-growth results, practitioners are often confronted with a high number of samples yielding non-aureus staphylococci (NAS) and *Corynebacterium bovis*, so-called minor pathogens. In fact, several large-scale studies around the world mention that NAS and *C. bovis* are the most frequently isolated bacteria from milk samples submitted for detection of pathogens

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responsible for intramammary infection (IMI) (Wilson et al. 1997; Makovec & Ruegg, 2003; Pitkälä et al. 2004). The somatic cell count (SCC) recorded for milk samples that yield these pathogens tends to be low in comparison with samples yielding major pathogens (Wilson et al. 1997; Pitkälä et al. 2004). Therefore, practitioners investigating a high bulk tank SCC problem find it difficult to ascribe appropriate significance to a high proportion of NAS and *C. bovis* isolates. It is difficult to identify the minor pathogen responsibility share, as they may simply be easier to isolate than, for example, *Staphylococcus aureus*, owing to its intermittent shedding pattern (Sears et al. 1990) or intracellular location (Brouillette et al. 2003).

To overcome the difficulty of major pathogen isolation, freezing milk samples prior to culture has been suggested as a means of improving detection rates (Dinsmore et al. 1992; Sol et al. 2002). However, this method is controversial because some authors have found that freezing led to no effect or to a decrease in detection rates (Schukken et al. 1989; Dinsmore et al. 1992), whereas others reported an increased detection of major pathogens (Sol et al. 2002). Other methods have been studied in the past to tentatively increase the detection rate of major mastitis pathogens, including centrifugation of milk samples prior to culturing (Zecconi et al. 1997) or use of Petrifilm for the increased detection of *Staph. aureus* (Silva et al. 2005).

Recently a polymerase chain reaction (PCR)-based diagnostic test showed promising results in the detection of intramammary pathogens in milk samples that showed no bacterial growth with conventional culturing (Taponen et al. 2009).

The objectives of the present study were a) to determine the effect of freezing on the detection rate of certain bacterial groups in milk samples and b) to determine whether a real-time PCR test could detect mastitis pathogens in culture-negative samples or samples yielding minor pathogens.

Materials and Methods

Study 1

The study was performed on four commercial dairy farms selected on the basis of their geographic location (close to the Faculty of Veterinary Medicine, Technical University of Lisbon) and on the farmers' willingness to participate in the study. Three-month rolling geometric means for the first 3 months of the study were 231, 180, 568 and 407 milk recorded animals per farm, SCC of 255, 246, 334 and 766×10^3 cells/ml and 305-d milk production of 11 891, 9513, 9589 and 9600 kg, respectively. All four farms were performing the 5-point plan mastitis control programme. Farms were visited every 4 weeks for a total of 20 weeks. On the initial visit, 12 cows were randomly selected from those with individual cow SCC that increased from below to above 200 000 cells/ml in the previous two milk recordings. Quarter milk samples were taken according to National Mastitis Council protocols (NMC, 1999) and kept refrigerated, transported to the laboratory and processed on the sampling day. On subsequent visits, cows from which NAS had been isolated were resampled at quarter-level; other animals were selected according to the above criteria to total 12 cows per visit. None of the samples included in the study came from clinical mastitis cases (i.e. gross changes to milk, udder or to the condition of the cow).

From each milk sample, 0·01 ml of milk was plated onto sheep blood agar (Columbia™, bioMérieux, France) and MacConkey agar (Liofilchem, Italy), incubated at 37 °C and observed after 24 h and 48 h of incubation. Colonies of similar morphology were selected for isolation and identification if there was evidence of growth of $\geqslant$500 cfu/ml. Samples yielding more than two morphologically different bacterial isolates were considered to be contaminated. Individual quarter SCC were determined through flow cytometry (CombiFoss™, Foss, Denmark).

To study the effect of freezing on the isolation rates of IMI pathogens, defined groups of microorganisms were classified based on Gram stain characteristics and catalase test results into: Gram-positive, catalase-positive cocci which would include the staphylococci; Gram-positive, catalase-negative cocci, which would include the streptococci; Gram-positive bacilli, which would include *Corynebacterium* spp.; Gram-negative bacilli, and yeasts, besides culture-negative samples. Immediately after plating, milk samples were frozen at −20 °C and replated 24 h later after thawing at room temperature. Subsequent colony identification was made according to the protocol described above. To clarify whether samples evidencing growth of staphylococci post freezing but not in the corresponding samples pre-freezing could yield *Staph. aureus*, post-freezing isolates were identified to species level by use of a biochemical identification system (ID 32 Staph™, bioMérieux, France). Pre and post-freezing bacteriology was performed on 783 milk samples.

Study 2

In the second study, a commercial real-time PCR test (PathoProof™ Mastitis PCR Assay, Finnzymes Oy, Espoo, Finland) was used to investigate whether further microorganisms could be detected in quarter milk samples with negative culture results or yielding only *C. bovis* or NAS with conventional bacteriology. Identification of staphylococci was made with a biochemical identification system (ID 32 Staph™, bioMérieux, France) and *C. bovis* identification was made through the evaluation of growth of Gram-positive bacilli in trypticase soy agar and trypticase soy agar supplemented with 1% Tween 80, according to Watts et al. (2000). Samples with a SCC >500 000 cells/ml that were culture-negative or from which a NAS or *C. bovis* had been isolated as single isolates, were subjected to real-time PCR analysis (n = 162). These included 51 no-growth samples, 79 samples from which *C. bovis* was isolated as a single pathogen and 32 samples from which NAS were isolated as single pathogens. Milk samples were frozen at −20 °C on the sampling

Table 1. Bacteriology results for 783 paired samples pre-freezing and post freezing and respective *P* values according to a McNemar test for paired samples

Bacteriology results		Post freezing								
Pre-freezing	*n*	Gram −ve bacilli	Gram +ve bacilli	Gram +ve, Catalase −ve cocci	Gram +ve, catalase +ve cocci	No growth	Yeast	Contami-nated	Total	*P* value
		6	131	75	164	402	1	4	783	—
Gram −ve bacilli	20	5 (25%)	4 (20%)	0	0	11 (55%)	0	0	20	<0·05
Gram +ve bacilli	161	1 (0·6%)	100 (62%)	2 (1%)	5 (3%)	52 (32%)	0	1 (0·6%)	161	<0·05
Gram +ve, Catalase −ve cocci	82	0	4 (5%)	70 (85%)	1 (1%)	7 (8%)	0	0	82	0·09
Gram +ve, catalase +ve cocci	225	0	11 (5%)	3 (1%)	152 (68%)	57 (25%)	0	2 (1%)	225	<0·05
No growth	292	0	12 (4%)	0	6 (2%)	272 (93%)	1 (0·3%)	1 (0·3%)	292	<0·05
Yeast	3	0	0	0	0	3 (100%)	0	0	3	0·32
Contaminated	0	0	0	0	0	0	0	0	0	<0·05

day and stored until further analysis by PCR. All PCR protocol details were as described earlier (Koskinen et al. 2009). This PCR assay allowed the detection of 11 microorganisms or groups of microorganisms: 1) *Staph.*, 2) *Staphylococcus* spp., including *Staph. aureus* and the most relevant NAS (*Staph. chromogenes, Staph. epidermidis, Staph. haemolyticus, Staph. hyicus, Staph. lugdunensis, Staph. saprophyticus, Staph. simulans, Staph. warneri* and *Staph. xylosus*), 3) *Streptococcus agalactiae*, 4) *Str. dysgalactiae*, 5) *Str.*, 6) *Escherichia coli*, 7) *Enterococcus* spp., including *Ent. faecalis* and *Ent. faecium*, 8) *Klebsiella* spp., including *K. oxytoca* and *K. pneumoniae*, 9) *C. bovis*, 10) *Arcanobacterium pyogenes* and *Peptoniphilus indolicus*, and 11) *Serratia marcescens*. Cycle threshold (Ct) values, i.e. the number of PCR cycles at which excitation fluorescence detected by the instrument exceeded a pre-defined threshold, were used to score a reaction as positive ($Ct \leqslant 37$) or negative ($Ct > 37$).

Statistical analyses

Data were analysed using SPSS version 15.0 (USA). For the first study a McNemar test for paired samples was used to determine whether there were differences in bacteriology results between samples processed with the standard procedure and the same samples after freezing for 24 h. Only samples that led to single isolates were used in the analysis. A Wilcoxon signed-rank test was applied to test for differences between log-transformed individual quarter SCC between samples that yielded the same bacteriology result pre-freezing and post freezing. A Wilcoxon signed-rank test was also used to test for differences in mean genome copies of NAS and of *C. bovis* between samples that with conventional bacteriology led to their isolation or not. A significant difference was defined as a probability value of $P < 0{\cdot}05$.

Results

A total of 973 samples were processed pre-freezing and post freezing. Only culture-negative samples or samples yielding single isolates were included in the analyses, which totalled 783 samples. A significant difference in the frequency of isolation of bacteria was observed in several of the microorganism groups between pre- and post-freezing samples (Table 1). Gram-positive catalase-positive cocci, more specifically staphylococci, were detected in 12 samples post freezing while not being detected in the same samples pre-freezing. None of these 12 isolates was identified as *Staph. aureus*. There was a significant increase in the log-transformed SCC between culture-negative samples pre-freezing and post freezing (Table 2).

Results of the PCR testing of the 162 quarter milk samples are displayed in Table 3. Out of the 51 no-growth samples, no pathogens could be detected by the PCR assay in 26 samples. Overall, 1 or 2 pathogens were detected in 47% of the no-growth samples (23/49).

Out of the 79 samples from which *C. bovis* had been isolated in conventional culturing, 3 or more pathogens were detected in 5 samples. Excluding these samples, the presence of *C. bovis* was confirmed in 59 samples, in 41 samples as a single pathogen and in 18 samples with another pathogen. Overall, detection of a pathogen other than *C. bovis* was made in 35% of the samples that with conventional bacteriology yielded *C. bovis* (26/74).

Out of the 32 samples from which NAS had been isolated in conventional culturing, 3 or more pathogens were detected in 2 samples. Excluding these samples, the presence of NAS was confirmed in 28 samples, in 21 samples as a single pathogen and in 7 samples with another pathogen. On a sample where *Staph. intermedius* was identified with conventional bacteriology, *Staph. aureus* was detected with

Table 2. Bacteriology results before and after freezing, number of milk samples submitted for cell count determination pre-freezing, geometric mean individual quarter somatic cell count (SCC), geometric mean log-transformed SCC and *P* values for the difference between log_{10}SCC of samples yielding the same bacteriology result pre-freezing and post freezing

Bacterial group isolated	Processing	*n*	Geometric mean SCC ($\times 10^{-3}$)	Mean log_{10}SCC	*P* value
Gram +ve, catalase +ve cocci	Pre-freezing	218	151·7	2·1	0·06
	Post-freezing	157	186·9	2·2	
Gram +ve, catalase −ve cocci	Pre-freezing	74	434·8	2·6	0·22
	Post-freezing	67	510·2	2·6	
Gram +ve bacilli	Pre-freezing	158	98·5	1·9	0·52
	Post-freezing	125	108·2	2·0	
Gram −ve bacilli	Pre-freezing	18	160·5	1·9	0·27
	Post-freezing	6	880·3	2·7	

Table 3. Microorganisms detected with a real-time PCR assay on milk samples with an individual quarter somatic cell count (SCC) >500 000 cells/ml that with standard bacteriology were culture-negative or led to the isolation of *Corynebacterium bovis* as a single isolate or to non-aureus staphylococci (NAS) as a single isolate

Type of sample (defined by conventional bacteriology)	Result of PCR test		Percentage of samples
No growth (*n* = 51)	Negative		51%
	Other bacterial pathogen	NAS	22%
		Esch. coli	12%
		C. bovis	12%
		A. pyogenes/P. indolicus†	2%
		Str. uberis	2%
		Str. dysgalactiae	2%
	More than 3 pathogens detected		4%
C. bovis (*n* = 79)	*C. bovis* as a single pathogen		52%
	C. bovis and another pathogen	*Esch. coli*	11%
		NAS	8%
		Str. uberis	4%
	C. bovis not found	NAS	6%
		Esch. coli	4%
		A. pyogenes/P. indolicus†	1%
		Negative	9%
	More than 3 pathogens detected		6%
NAS (*n* = 32)	NAS as a single pathogen		66%
	NAS and another pathogen	*C. bovis*	9%
		Esch. coli	6%
		Str. uberis	3%
		Enterococcus faecalis/ faecium	3%
	NAS not detected		6%
	More than 3 pathogens detected		6%

† *Arcanobacterium pyogenes/Peptoniphilus indolicus*

the PCR assay. Overall, detection of bacteria other than NAS was possible in 25% of the NAS samples (7/28).

The number of genome copies obtained for each bacterial species detected is displayed in Table 4. The mean number of copies of NAS and of *C. bovis* detected in the samples where these had been found with conventional bacteriology was significantly higher ($P<0·05$) than in the samples where these had not been found.

Table 4. Mean number of genome copies per ml of milk, mean cycle threshold values (Ct) and respective SD for bacterial species identified with a real-time PCR-based kit

Microrganism	Number of identifications	Mean genome copies per ml of milk	SD	Mean Ct value	SD
Non-aureus staphylococci (NAS) (samples yielding NAS with conventional bacteriology)	28	$1·4\times10^4$	$1·6\times10^4$	30·9	1·9
NAS (samples not yielding NAS with conventional bacteriology)	22	$2·1\times10^3$	$4·5\times10^3$	34·4	2·0
Corynebacterium. bovis (samples yielding *C. bovis* with conventional bacteriology)	59	$7·1\times10^2$	$1·5\times10^3$	32·1	2·0
C. bovis (samples not yielding *C. bovis* with conventional bacteriology)	12	$1·8\times10^2$	$2·3\times10^2$	34·0	1·9
Escherichia coli	20	$7·1\times10$	$5·2\times10$	35·9	0·8
Streptococcus uberis	5	$3·9\times10^2$	$5·3\times10^2$	32·6	2·9
Arcanobacterium pyogenes/Peptoniphilus indolicus	2	$3·3\times10$	1·5	36·2	0·1
Staphylococcus aureus†	1	$3·2\times10^3$	NA	29·3	NA
Streptococcus dysgalactiae†	1	$1·7\times10^4$	NA	26·9	NA
Enterococcus faecalis/faecium†	1	$1·5\times10^4$	NA	30·3	NA

† For these 3 pathogens, the values displayed are the ones obtained for single identifications and not true means

Discussion

Study 1

Our results show that freezing milk samples before performing bacteriology with the conventional culture method did not lead to an increased detection of mastitis pathogens.

Many large-scale studies mention that over 50% of milk samples are culture-negative or yield minor mastitis pathogens. Makovec & Ruegg (2003) reporting on the results of 83 650 samples submitted from cases of clinical and subclinical mastitis as well as samples obtained for mastitis surveillance programmes, found 46·6% of samples with either no growth or isolation of *C. bovis* or NAS. Wilson et al. (1997), reporting on bacteriology results of 105 083 composite milk samples submitted from every animal on most farms of the study, found 66·0% of samples with one of the three aforementioned results and Pitkälä et al. (2004), reporting on 12 661 quarter samples representing the totality of the animals on each farm, found 90·5% of samples were culture-negative or yielded *C. bovis* or NAS. Many of the samples from which *C. bovis* and NAS are isolated, may correspond to true cases of IMI due to these agents, because it is well established that they cause mastitis (Honkanen-Buzalski & Bramley, 1984; Simojoki et al. 2009). However, there is evidence that *C. bovis* often leads to teat canal colonization rather than to true IMI (Black et al. 1972; Honkanen-Buzalski & Bramley, 1984). Therefore, frequent isolation of these minor pathogens might simply attest they are easier to find than major mastitis pathogens truly responsible for the deleterious effects of IMI, but more difficult to detect through conventional techniques. The possibility that minor pathogens outnumber major pathogens in the samples could also be part of the problem, potentially occurring with *Staph. aureus* owing to its intermittent shedding pattern (Sears et al. 1990).

It is now well established that *Staph. aureus* and *Str. uberis* are capable of internalizing in mammary epithelial cells and neutrophils (Brouillete et al. 2003; Tamilselvam et al. 2006), and it has been hypothesized that freezing milk samples could lead to the lysis of mammary somatic cells and to higher isolation rates of intracellular pathogens (Dinnsmore et al. 1992). In our study, freezing milk samples at −20 °C for 24 h did not lead to a higher rate of isolation of major pathogens. In fact, there was a net loss of microorganisms, which was significant for most groups of bacteria.

Out of the 783 pairs of samples analysed, a significant reduction in the proportion of samples yielding the following groups of bacteria was observed after freezing: Gram-positive, catalase-positive cocci (27% decrease in proportion); Gram-negative baccilli (70% decrease) and Gram-positive bacilli (19% decrease). Only for the Gram-positive, catalase-negative group was a significant reduction not recorded (9% decrease). Conversely, the proportion of samples that were culture-negative after freezing showed a significant increase (137% increase). There were 12 samples where no growth of staphylococci was recorded prior to freezing, but where their growth was recorded after freezing. None of these was identified as *Staph. aureus* by use of a biochemical identification system, which like other identifications systems, has its limitations (Sampimon et al. 2009). These results disagree with earlier reports. Schukken et al. (1989) reported a decrease in *Esch. coli* isolation and an increase in NAS isolation; however, these authors froze their samples for increased lengths of time (4–16 weeks) and observed that the longer the samples were frozen, the higher was the number of cultures positive for NAS. Dinnsmore et al. (1992) also reported a decrease in *Esch. coli* isolation and an increase in the number of NAS after freezing samples overnight at −20 °C, even though their results were not statistically significant. The work of Sol et al. (2002) is not directly comparable with the current study because although

the authors froze their samples for 24 h at − 20 °C, they also incubated them for 24 h at 37 °C.

An identification of the isolated microorganisms to species level was not performed in the first study, but rather microorganisms were gathered in groups according to their Gram stain and catalase characteristics. Therefore, Gram-positive catalase-positive cocci would include staphylococci but also other genera with the same characteristics (*Micrococcus* spp. and *Kocuria* spp.). Similarly Gram-positive catalase-negative cocci would include not only streptococci, but also *Enterococcus* spp. and *Aerococcus* spp. This approach was chosen because the focus of the study was not on individual species but rather on morphologically similar groups of pathogens. It would appear, however, judging by the geometric mean SCC for each group, that the isolated microorganisms were not contaminants but were being deleterious in terms of udder health. The significant difference observed between samples that led to no growth pre-freezing and post freezing, could suggest that many of the samples that post freezing showed no growth actually had an IMI, and therefore the viability of the pathogens responsible for those infections could have been affected by the process of freezing.

The results of our study showed that simply freezing milk samples overnight did not increase the detection of mastitis pathogens in milk samples and therefore this procedure should not be recommended. In fact, the differences observed could lead to questioning of the usefulness of frozen milk samples for the diagnosis of IMI, even though it can be argued that the numbers of viable bacteria in samples with clinical or subclinical mastitis would be different from the numbers present in some of the samples used in the study. Several anecdotal reports mention that glycerol can be incorporated into milk samples to protect against the effects of freezing on milk samples. There have been studies on the use of glycerol to protect against the negative effects of freezing on the viability of bacteria in other types of samples (Ternent et al. 2004) but to the best of our knowledge the effect of its use on milk samples has not been scientifically tested.

Study 2

We used a real-time PCR test to evaluate whether DNA from mastitis pathogens could be detected in milk samples with a high quarter SCC. After exclusion of samples where PCR detected the presence of DNA from 3 or more pathogens, we analysed the results of a total of 151 samples: 49 samples that were culture-negative with standard bacteriology, 74 samples from which *C. bovis* was isolated as a single microorganism and 28 samples from which NAS was isolated as a single microorganism with standard bacteriology. The decision to exclude samples with 3 or more pathogens from the analysis was made based on the recommendation of considering a sample as contaminated when 3 or more types of colonies are present at > 500 cfu/ml when performing conventional bacteriology (IDF, 1999; NMC, 1999). Applying the same principle when utilizing a PCR test is debatable, especially when quantitative data are available. Still it was decided to apply the same principle as a precautionary measure. Bacterial DNA was detected in 47% of the no-growth samples, a value close to the 43% recently obtained by Taponen et al. (2009) for clinical mastitis samples. For samples from which *C. bovis* or NAS were isolated as single isolates, 35% and 25% of samples, respectively, were positive for another pathogen. Out of the 151 samples, several major pathogens were found: *Esch. coli* was detected in 20 samples, *Str. uberis* in 5 samples, *A. pyogenes/P. indolicus* in 2 samples, *Str. dysgalactiae* in 1 sample. *Staph. aureus* was detected in only 1 sample, which conventional bacteriology had identified as *Staph. intermedius*. The analytical accuracy of the real-time PCR test has been shown to be 100% in identification of *Staph. aureus* and NAS, across a large collection of strains (Koskinen et al. 2009). Hence, it is likely that this single case observed in the current study was due to a biochemical test misidentification, which is not uncommon (Sampimon et al. 2009).

The number of genome copies quantified in our study was generally lower than the numbers found by Taponen et al. (2009), which may be a reflection of the fact that their study focused on samples from clinical mastitis cases, whereas our focused on subclinical cases. The significantly higher number of copies of NAS and of *C. bovis* detected in the samples where these pathogens had been found with conventional bacteriology supports the idea that the number of genome copies detected influences the likelihood of isolating mastitis pathogens with conventional bacteriology. As the test is quantitative, it is possible to identify the bacterial species present in higher numbers, which is potentially useful information when determining the most relevant bacterial species in terms of pathogenicity.

Use of this real-time PCR technique can thus help in the detection of mastitis pathogens in milk samples with high SCC yielding minor pathogens or that are culture-negative. This assay can be used on frozen or bronopol-preserved samples, so culture-negative samples or samples leading to the growth of a minor pathogen can be submitted for further diagnosis when bacteriology is not informative enough.

In Portugal, where the samples were collected, as well as in other parts of the world, detection of *Mycoplasma bovis* is important, as it has been demonstrated to have a large impact on udder health on some farms (L Pinho, personal communication). However, the version of the PCR assay used at the time of the study did not allow DNA from this pathogen to be detected.

Bacteriology results for milk samples with over 50% of samples either culture-negative or leading to the isolation of a minor pathogen are worrying whenever a targeted approach to quarters with mastitis is being used, because significant resources are being used with little information being gained for practitioners or milk-quality advisors. Chronically high-SCC milk samples that are culture-negative or from which only a minor pathogen is isolated may

correspond to false negative results, i.e. negative cultures in cases where there is actually an IMI and false positive results, i.e. detection of minor pathogens not truly responsible for mastitis. On the basis of the findings from the two studies reported here, there seems to be limited benefit in freezing milk samples to enhance detection of pathogens as there was a significant reduction in the number of bacteria isolated in several of the groups tested. However, use of this PCR assay may help to reduce the number of false negatives as it detected bacterial DNA of potential mastitis pathogens in samples that were culture-negative or from which only minor pathogens were isolated with conventional bacteriology.

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Study III

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Observed reduction in recovery of *Corynebacterium* spp. from bovine milk samples by use of a teat cannula

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Although *Corynebacterium bovis* and coagulase-negative staphylococci are frequently the most commonly isolated bacteria from milk samples submitted for identification of pathogens causing intramammary infection, the individual quarter somatic cell count (SCC) from those samples is most often low. The present study aimed at evaluating the difference in bacteriology results from milk sampled by the standard technique (as recommended by the National Mastitis Council) and by the use of a teat cannula surpassing the teat canal, since *C. bovis* is often only found in the teat canal. Single quarter milk samples were collected in duplicate from 132 dairy cows on a commercial dairy farm using the standard milk sampling technique and also using a cannula introduced into the teat. Two groups of quarters were sampled: a group that was selected randomly at cow and quarter level and a group that was selected based on having SCC >200 000 cells/ml at the previous milk recording at cow level and on California mastitis test result at quarter level. Bacteriological culture performed on the samples yielded 29 *Corynebacterium* spp. isolates from the samples collected with the standard technique and 6 isolates from the samples collected with a cannula. Bacteriological culture yielded 73 and 100 culture negative samples respectively with the standard and the alternative sampling technique. A significant difference between the two sampling techniques was observed for recovery of *Corynebacterium* spp. and for no-growth samples. There was no significant difference in the isolation of *Corynebacterium* spp. or other bacterial species when using the standard technique before or after sampling with the cannula; thus the observed difference in bacteriology results could not be attributed to a particular sampling order. No significant change was observed overall in individual quarter SCC measured on the sampling day and 7 d later. Our results agree with several studies showing that *Corynebacterium bovis* often colonizes the teat canal, without causing true intramammary infection.

Keywords: Milk sampling, *Corynebacterium* spp., intramammary infection.

Bovine milk is produced by the epithelial cells of the alveoli, the mammary gland's functional unit, which drain into interlobular ducts of increasing diameter. These drain collectively into a lactiferous sinus, the gland's cistern, proximally to the teat cistern and separated from the latter by an annular fold with an erectile venous plexus. The teat cistern extends distally from that point to the Furstenberg's rosette. The teat canal completes the mammary gland's excretory system (Couture & Mulon, 2005).

Intramammary infection (IMI) is defined as an infection occurring in the secretory tissue and/or the ducts and tubules of the mammary gland, which is diagnosed by microbiological culture of aseptically obtained milk samples (IDF, 1999). Colonization and/or infection of the teat canal is therefore not sufficient to be considered an IMI. True IMI leads to an increase in somatic cell count (SCC), a decrease in milk production and changes in milk composition (Seegers et al. 2003).

Several studies from around the world have found that *Corynebacterium bovis* and coagulase-negative staphylococci are the most commonly isolated bacteria from milk samples submitted for identification of pathogens causing

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IMI (Wilson et al. 1997; Makovec & Ruegg, 2003; Pitkälä et al. 2004). However, these studies also mention that when these microorganisms are isolated from milk samples, the individual quarter SCC from those samples is most often low.

The objective of this study was to evaluate the difference in bacteriology results from milk sampled in two different ways: the standard milk sampling technique and by use of a teat cannula surpassing the teat canal.

Materials and Methods

A total of 132 lactating cows from a commercial dairy farm that were free from gross signs of disease were used in this study, divided into two groups: a group that was selected randomly ($n=65$) and a group with SCC >200 000 cells/ml at the previous milk recording ($n=67$). Individual quarters were selected randomly for sampling in the former group and were selected based on having the highest California mastitis test score in the latter.

The standard milk sampling technique recommended by the National Mastitis Council (NMC) includes: 1) cleaning the teat, by removing visible dirt from the teat e.g with a paper towel; 2) discarding the first milk squirts; 3) predipping, allowing enough time for the solution to act (30 s); 4) drying the teat using an individual paper towel; 5) disinfecting the teat, with cotton wool moistened with 70% alcohol, repeating this operation until no evidence of contamination is evident; 6) milk sampling, removing the lid of the sampling container and keeping it vertical or facing downward so that there is no contamination of the container when the lid is put back on. The top of the container should be kept vertical, away from direct contact with the teat to reduce contamination. A few squirts of milk should be dispensed into the container and it should be immediately closed; 7) sample refrigeration (NMC, 1999). In our study, all the sampled quarters were forestripped, predipped with a chlorine dioxide foam (Valiant Foam-Active®, Genus Breeding Ltd, UK) for 30 s, cleaned with an individual dry udder cloth, swabbed with cotton wool soaked in 70% ethanol and allowed to dry. Each quarter was sampled twice in succession: once by use of the conventional NMC recommended technique, and additionally by insertion of a cannula through the teat canal until the teat cistern was presumably reached and milk flowed into a container held horizontally. The sampling order (conventional or alternative technique first) was selected randomly.

The teat cannula used was a 0·25-ml straw normally used for artificial insemination (IMV technologies, France) from which the plug was removed. The cannulas, 132 mm in length and 2 mm in diameter, were individually packed and sterilized by moist heat in an autoclave (121 °C for 15 min). Evaluation of the most adequate cannula dimensions was first performed by insertion of different models into teat and udder specimens collected at a local abattoir.

Milk samples were transported under refrigeration to the laboratory. From each sample, 0·01 ml of milk was plated onto sheep blood agar (Columbia®, bioMérieux) and MacConkey agar (Liofilchem, Italy), incubated at 37 °C and observed after 24 h and 48 h of incubation. Colonies of similar morphology were selected for isolation and identification if there was evidence of growth of ⩾500 cfu/ml. Samples yielding more than two morphologically different bacterial isolates were considered to be contaminated. Identification was made through biochemical identification systems (ID 32 Staph®, API Coryne®, API 20 Strep® and API 20 NE®, bioMérieux, France). Individual quarter SCC was determined on the sampling day and 7 d later using flow cytometry (CombiFoss®, Foss, Denmark).

Data were analysed using SPSS version 15.0 (USA). A McNemar test for paired samples was used to determine whether there were differences in the bacteriology results between the two sampling techniques. Agreement between results obtained with the two sampling methods was also sought by use of the kappa statistic. A Wilcoxon signed-rank test was applied to test for differences between individual quarter SCC on the sampling day and 7 d later. A non-parametric test was used for this parameter because the difference between quarter milk SCC between the two days did not have a normal distribution (evaluated by a Kolmogorov-Smirnov test). A two-tailed Fisher's exact probability test was used to determine whether the sampling order led to any differences in the bacteriology results. A significant difference was defined as a probability value of $P<0{\cdot}01$.

Results

Out of the 132 quarter milk samples collected in duplicate, 72 had SCC<200 000 cells/ml and 60 had SCC above that value. Ten samples obtained with the standard technique yielded two isolates, whereas none of the samples collected with a cannula yielded more than one isolate. Use of the standard sampling technique led to the isolation of 29 *Corynebacterium* spp., 19 *Staphylococcus* spp., 4 *Streptococcus* spp. and 16 microorganisms from other genera; 73 samples showed no growth and 1 sample was contaminated. *C. bovis* was the most frequently isolated bacterial species, being isolated from 14 samples. Use of a teat cannula to sample led to the isolation of 6 *Corynebacterium* spp., 12 *Staphylococcus* spp., 5 *Streptococcus* spp., 9 microorganisms from other genera; 100 samples showed no growth and no sample was contaminated. *C. bovis* was isolated twice. The numbers of isolates found in samples collected with the standard technique and in samples collected with the cannula are presented in Table 1 (samples with SCC<200 000 cells/ml), Table 2 (samples with SCC>200 000 cells/ml) and Table 3 (all the samples). Out of the 60 quarter milk samples with SCC>200 000 cells/ml, microorganisms were isolated from samples collected with the standard technique but not from

Table 1. Comparison of numbers of isolates obtained with the standard sampling technique (columns) and with a cannula (rows) for samples with SCC<200 000 cells/ml (n=72)

Cannula		Standard						
		Staphylococcus spp.	*Streptococcus* spp.	*Corynebacterium* spp.	Other	No Growth	Contaminated	Total
		9	0	17	3	49	0	
Staphylococcus spp.	4	4	0	1	0	0	0	5
Streptococcus spp.	0	0	0	0	0	0	0	0
Corynebacterium spp.	2	0	0	3	0	0	0	3
Other	0	0	0	0	0	0	0	0
No Growth	66	5	0	13	3	49	0	70
Contaminated	0	0	0	0	0	0	0	0

Table 2. Comparison of numbers of isolates obtained with the standard sampling technique (columns) and with a cannula (rows) for samples with SCC >200 000 cells/ml (n=60)

Cannula		Standard						
		Staphylococcus spp.	*Streptococcus* spp.	*Corynebacterium* spp.	Other	No Growth	Contaminated	Total
		10	4	12	13	24	1	
Staphylococcus spp.	8	7	0	3	0	0	0	10
Streptococcus spp.	5	0	4	0	1	0	0	5
Corynebacterium spp.	4	0	0	3	0	1	0	4
Other	9	1	0	0	9	0	0	10
No Growth	34	2	0	6	3	23	1	35
Contaminated	0	0	0	0	0	0	0	0

Table 3. Comparison of numbers of isolates obtained with the standard sampling technique (columns) and with a cannula (rows) for the total number of samples (n=132)

Cannula		Standard						
		Staphylocoocus spp.	*Streptococcus* spp.	*Corynebacterium* spp.	Other	No Growth	Contaminated	Total
		19	4	29	16	73	1	
Staphylococcus spp.	12	11	0	4	0	0	0	15
Streptococcus spp.	5	0	4	0	1	0	0	5
Corynebacterium spp.	6	0	0	6	0	1	0	7
Other	9	1	0	0	9	0	0	10
No Growth	100	7	0	19	6	72	1	105
Contaminated	0	0	0	0	0	0	0	0

the corresponding samples collected with a cannula on 5 occasions. These microorganisms included *Staph. chromogenes, Staph. hyicus, Kocuria rosae, Aerococcus viridans* and a yeast that was not identified biochemically.

The McNemar test for paired samples showed a statistically significant difference between the two sampling techniques in the number of *Corynebacterium* spp. isolated and in the number of samples that were culture negative, for both SCC groups and in total. There was a difference that approached significance for staphylococci in the group of samples <200 000 cells/ml and for the total samples. Kappa values were lowest for *Corynebacterium* spp. across the three SCC groups, except for the 'Other' category of microorganisms in the <200 000 cells/ml group (Table 4).

Milk sampling was performed on 68 quarters using the teat cannula technique before the standard technique, and on 64 samples using the standard technique ahead of the cannula technique. Performing a two-tailed Fisher's exact probability test to compare the number of samples from which the several groups of microorganisms were isolated with the two alternative sampling orders, revealed that there was no statistically significant difference between them (Table 5).

Individual quarter SCC was measured on the sampling day and repeated 7 d later for 126 out of the 132 quarters

Table 4. Probabaility values (*P*) for the McNemar test and Kappa values (*K*) comparing the two sampling methods in terms of isolated microorganisms, for samples with SCC <200 000 cells/ml, >200 000 cells/ml and for the total number of samples

	SCC<200 000		SCC>200 000		Total population	
Microorganism	*P*	*K*	*P*	*K*	*P*	*K*
Staphylococcus spp.	0·06	0·58	0·50	0·74	0·02	0·67
Streptococcus spp.	—	—	1·0	0·88	1·0	0·89
Corynebacterium spp.	<0·01	0·17	<0·01	0·31	<0·01	0·27
Other	0·25	0·0	0·12	0·78	0·02	0·69
No Growth	<0·01	0·32	<0·01	0·61	<0·01	0·54

Table 5. Comparison of sampling order in terms of number of samples from which *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp. and other microorganisms were isolated and respective *P* values for a two-tailed Fisher's exact probability test

Microorganism	Type of sampling	Sampling order	Number of samples yielding the microorganism considered	Other bacteriology results	*P*
Corynebacterium spp.	Standard	First	15	49	0·671
		Second	13	55	
	Cannula	First	4	64	0·681
		Second	2	62	
Staphylococcus spp.	Standard	First	11	53	0·313
		Second	7	61	
	Cannula	First	7	61	0·765
		Second	5	59	
Streptococcus spp.	Standard	First	3	61	0·354
		Second	1	67	
	Cannula	First	1	67	0·198
		Second	4	60	
Other	Standard	First	10	54	0·29
		Second	6	62	
	Cannula	First	5	63	1·0
		Second	4	60	

sampled in the study. There was an increase from below to above 200 000 cells/ml in 13 quarters between the two sampling dates, and a decrease from above to below that value in 12 quarters. Performing a Wilcoxon signed-rank test showed that there was a significant decrease of the mean SCC recorded between initial sampling and sampling 7 d later for the group of samples above 200 000 cells/ml on the initial sampling day; no significant differences were observed for the group of samples that initially were below 200 000 cells/ml and for the totality of samples (Table 6). No clinical mastitis cases were recorded during the period between sampling dates.

Table 6. Comparison of individual quarter SCC arithmetic means between the sampling day and 7 d later for the groups of quarters that on the initial sampling day had SCC> 200 000 cells/ml, <200 000 cells/ml and for all the samples

Group of samples		Mean	SD	*n*	*P*
>200 000	Day 0	2014	3028	54	0·011
	Day 7	1005	1284		
<200 000	Day 0	70	55	72	0·156
	Day 7	180	376		
Total	Day 0	903	2196	126	0·340
	Day 7	533	973		

Discussion

There was a significant difference in the recovery of *Corynebacterium* spp. from milk samples collected with the standard technique and with a cannula surpassing the teat canal. This difference was observed for all samples, regardless of having SCC values above or below 200 000 cells/ml. There was also a significant difference between the two sampling techniques in the number of samples yielding no growth, probably a reflection of samples yielding *Corynebacterium* spp. when collected with the standard technique, and leading to no growth when sampling with the alternative technique. No significant difference was observed between the two sampling techniques for recovery rates of staphylococci, streptococci and 'other' microorganisms, despite a difference

approaching significance for staphylococci in the group of samples <200 000 cells/ml and overall. Kappa values were lowest for the samples yielding *Corynebacterium* spp., except for the 'other' microorganisms in samples <200 000 cells/ml, which is probably a reflection of a lower environmental contamination when the teat cannula was used. Kappa values for samples yielding staphylococci, 'other' microorganisms and streptococci in samples >200 000 cells/ml were 0·74, 0·78 and 0·88 respectively, corresponding to substantial agreement between the two sampling methods for staphylococci and microorganisms from other genera, and to almost perfect agreement for the streptococci.

The observed differences in bacterial isolation rates are probably due to the fact that collecting milk samples with a cannula that surpasses the teat canal avoids contamination of the milk samples with teat canal flora. This way, only microorganisms present in the teat cistern and beyond, more likely to cause true IMI, are isolated. Several large scale studies have reported *C. bovis* as one of the most frequently isolated bacteria from milk samples submitted for bacteriology (Wilson et al. 1997; Makovec & Ruegg, 2003; Pitkälä et al. 2004). However, Black et al. (1972) reported that *C. bovis* occurred mostly in the teat canal and was seldom recovered from the teat cistern. Honkanen-Buzalski & Bramley (1984) described an experimental infection with *C. bovis* that did not produce any clinical mastitis and in which 44% of the infections were limited to the teat canal. Despite the time that has elapsed since these articles were published, the milk sampling technique to detect IMI-causing pathogens has not changed. This means that potentially, over the years, we have been performing bacteriology to detect both bacteria that populate the teat canal and pathogens that truly cause IMI.

To test the hypothesis that the sampling order could influence the results, we collected approximately half the samples with the standard technique before the alternative technique and the other half using the alternative technique before collecting milk with the standard technique. One could consider that sampling conventionally after sampling with a cannula might lead to higher levels of infection because of a physical action of pushing *Corynebacterium* spp. upwards with a cannula. Alternatively one might think that when the cannula technique was used after the standard technique, the isolation of *Corynebacterium* spp. would be lower because we had previously reduced their level by physically flushing bacteria out with the milk. In fact, no statistically significant difference between the two sampling orders was observed in the number of times that *Corynebacterium* spp. or other groups of microorganisms were isolated. Therefore, the observed difference between the two sampling techniques was not influenced by a particular sampling order.

Another concern with the use of a cannula to collect milk samples was the possibility of the iatrogenic introduction of pathogens into the gland cistern, potentially leading to IMI. The fact that sampling took place immediately before milking, with several litres of milk passing through each gland cistern, was thought to contribute to the safety of the alternative technique. To test that hypothesis we performed individual quarter SCC on the sampling day and 7 d later to determine whether there had been an increase that could be attributed to a recent infection. Overall there was a non-significant decrease in the SCC between the initial sampling day and 7 d later ($P=0·34$). In the group of sampled quarters that were >200 000 cells/ml on the initial sampling day, there was a significant decrease in mean SCC ($P=0·01$), whereas in the group of quarters that was below that value, there was a non-significant increase ($P=0·16$), with the mean SCC on the second sampling date still being <200 000 cells/ml. The significant decrease in SCC between the two sampling dates can potentially be explained by a proportion of animals self-curing during that time period. A similar number of quarter samples crossed the 200 000 cells/ml threshold: 13 samples increased to above that value, whereas 12 samples decreased to below that value. A 7-d interval was considered appropriate to detect iatrogenic increases in SCC, albeit some IMIs might lead to more transient rises in SCC (de Haas et al. 2004). Collecting milk samples with the alternative technique did not have an obvious negative impact on SCC. This issue needs further addressing in a future larger scale study, probably completed with bacteriology on further sampling dates and use of a DNA fingerprinting technique to compare before and after results.

Our results suggest a negligible loss in the recovery of potential pathogens other than *Corynebacterium* spp. with the use of teat cannula. On 5 samples with a SCC>200 000 cells/ml there was no recovery of microorganisms using the cannula, whereas there had been with the standard technique. However, it is difficult to know whether the microorganisms involved (*Staph. chromogenes, Staph. hyicus, Kocuria rosae, Aerococcus viridans* and a yeast) were truly responsible for IMI or if they were merely contaminants.

We do not wish to dispute the fact that *C. bovis* may cause IMI, because there is evidence of that from experimental infection (Honkanen-Buzalski & Bramley, 1984) and the mean SCC recorded in milk samples from which it is isolated, despite being low, is higher than the mean SCC recorded in milk samples yielding no growth (Wilson et al. 1997). However, *C. bovis* infection does not often go beyond the teat canal (Black et al. 1972; Honkanen-Buzalski & Bramley, 1984), which needs to be taken into account when sampling or interpreting bacteriology results. In fact, 35% of milk samples from which only *C. bovis* is isolated by conventional bacteriology, have been shown to harbour other mastitis pathogens (R Bexiga et al. unpublished observations). Collecting milk samples for bacteriology using a teat cannula could help elucidate the relevance of *Corynebacterium* spp. on farms where this microorganism is isolated from a large proportion of milk samples, ruling out possible teat canal colonization rather than true IMI.

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Study IV

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Deterministic model to evaluate the impact of lactational treatment of subclinical mastitis due to coagulase-negative staphylococci

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Short title: Economic model for treatment of CNS mastitis

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Summary

Coagulase-negative staphylococci (CNS) are the most frequently isolated bacteria from milk samples in several studies worldwide. Despite their relative frequency, specific measures aiming at their control are not well established. One possible measure to include in a control programme is lactational antimicrobial treatment. The decision to perform such treatment, as well as other actions on farm, should be based on the likelihood of financial return. A deterministic model was used to evaluate whether performing an antimicrobial treatment during the lactation for quarters infected with CNS was financially justifiable. Input variables for the impact of CNS on udder health were based on a previous study by the same authors and on available literature on the subject. Prices included in the model were based on 2009/2010 conditions in Portugal. The average result per antimicrobial treated quarter was a net loss of €38·74. Performing a sensitivity analysis to evaluate how systematic variation of the input variables of the model would lead to outcome changes, showed that variation in input variables nearly always led to a negative outcome, with the greatest variation in losses observed for variation in the length of treatment and milk withdrawal period (- €46·26 to - €28·49). The situations in which a net benefit was to be expected included the bulk tank somatic cell count decreasing to a level corresponding to a premium payment or to penalties being avoided, and the prevention of transmission of CNS in the milking parlour when the possibility of transmission was at its highest level. For most situations, lactational treatment of CNS subclinical mastitis was not financially justifiable.

Keywords: deterministic model, CNS mastitis, lactational therapy.

Coagulase-negative staphylococci (CNS) are the most frequently isolated pathogens from bovine milk samples in several large scale surveys worldwide (Makovec and Ruegg, 2003; Tenhagen et al., 2006). Despite their relative frequency, few studies have focused on risk

factors that lead to intramammary infection (IMI) with these pathogens, or on the efficacy of specific control measures. There is evidence that post milking teat disinfection with certain products can prevent new CNS infections from occurring (Foret et al., 2005), and recently a vaccine against CNS approved by EMEA showed a significant reduction in the number of new mastitis cases and a significant increase in spontaneous cure rate, when compared with a placebo group (Noguera et al., 2010). Antimicrobial treatment would seem to be an effective way to control CNS IMI as it has been found to lead to high cure rates for clinical mastitis (Waage et al., 2000), subclinical mastitis during the lactation (Wilson et al., 1999), subclinical mastitis during the dry period (Rajala-Schultz et al, 2009) and in pre-calving heifers (Oliver et al., 2004). Despite the high cure rates reported in the literature, it is questionable whether antimicrobial treatment is an efficient way to control CNS IMI, or even if it should be part of a control plan, because the somatic cell count increase that occurs when there is a CNS IMI tends to be low (Djabri et al., 2002), and there is some debate as to whether CNS IMI actually lead to a milk production loss (Schukken et al., 2009). This excludes treatment of clinical mastitis, in which animal welfare issues also need to be taken into account.

Several economic studies that could support decision-making have been published on different areas of udder health including teat disinfection (Ruegg and Dohoo, 1997), antimicrobial treatment (Steeneveld et al., 2007), breeding strategies (Steine et al., 2008) and vaccination (Allore and Erb, 1998). Most mastitis economic studies resort to mathematical models to predict most likely outcomes of different strategies considering various scenarios. These models either rely on set values for the parameters included, in which case they are termed deterministic, or rely on probability distributions to deal with uncertainty in the systems' behaviour, in which case they are called stochastic (Dijkhuizen and Morris, 1997). The initial studies published on lactational treatment of subclinical mastitis focused on blitz therapy of *Streptococcus agalactiae* IMI (Yamagata et al., 1987; Edmonson, 1989), but since then studies addressing other pathogens have been published including *Staphylococcus aureus*

(Swinkels et al., 2005a), *Streptococcus uberis* and *Streptococcus dysgalactiae* (Swinkels et al., 2005b). These publications incorporate information derived from scientific studies and from prices that farmers are confronted with, allowing for a transition from scientific evidence to practice.

Many of the input parameters used in the current study were obtained in a previous study (unpublished observations) by our research team, which followed IMI due to CNS over 48 weeks at 4-week intervals on 4 farms. In that study, genotypic identification was performed to strain level, which allowed for 111 CNS IMI to be followed through time. A mean duration of infection of 188 days and a geometric mean quarter SCC of 132 000 cells/ml for CNS infected quarters were recorded.

It is important for practitioners and milk quality consultants to advise the most efficient mastitis control measures, which are farm-specific and highly dependent of the most prevalent udder pathogens on each farm. Coagulase-negative staphylococci are highly prevalent and therefore, the need for specific measures for their control is often considered. Despite its proven efficacy, antimicrobial treatment for CNS subclinical mastitis may not be cost efficient. The objective of the current study was to evaluate the economic consequence of lactational antimicrobial therapy of subclinical mastitis caused by CNS.

Material and Methods

A partial budget was used to estimate the economic effect of treating subclinical IMI due to CNS during lactation. Partial budgeting is a quantification of the economic consequences of a specific change in farm procedures (Dijkhuizen and Morris, 1997). Partial budgets include only the items of returns and expenses that are predicted to be affected by a particular change in management. As a deterministic approach, partial budgeting uses discrete values instead of the probability distributions used in stochastic modelling. This method includes 4

components: extra revenue, reduced costs, reduced revenue and extra costs. A decision is economically justifiable if by adding extra revenue and reduced costs and subtracting reduced revenue and extra costs, a positive value is obtained.

The model inputs were obtained from the scientific literature, from mean prices for Portugal in 2009-2010, and from mean values obtained for a longitudinal study focusing on CNS (unpublished observations). The input parameters were estimated considering a 100-cow herd. Inputs into the model were not rounded off for the calculations, but are presented in the text as rounded off. A summary of input variables to the model is displayed in Table 1.

Extra revenue

This was considered potentially to include the increase in milk production after cure and the potential benefit of a reduction in the bulk tank somatic cell count (SCC) after treatment.

Studies that measured milk production after treatment for subclinical mastitis have found no significant improvement after treatment of *S. uberis* and *S. dysgalactiae* (St. Rose et al., 2003) or of *S. aureus* and CNS (van den Borne et al., 2010b).

As for the extra revenue potentially derived from a lower bulk tank SCC resulting from cure and reduction in individual quarter SCC, data from our own study were used (unpublished). In that study, the mean proportion of infected quarters was 14·4% across the 4 farms studied, which for a 100-cow herd would be equivalent to 58 infected quarters. Geometric mean SCC for CNS infected quarters was 132 000 cells/ml, for culture-negative quarters was 38 000 cells/ml and for the bulk tank 353 000 cells/ml. Each infected cow had on average two infected quarters with CNS. Considering two quarters with a CNS IMI and two quarters with no IMI and assuming equal contribution from all quarters to the cow SCC, such an animal would have a SCC of 85 000 cells/ml. Cure after antimicrobial treatment has been shown to lead to significant drops in quarter SCC (van den Borne et al., 2010b), therefore one can assume that a cured cow would have a SCC of 38 000 cells/ml, the same as the mean quarter SCC for culture-negative quarters. A decrease of 47 000 cells/ml (85 000 – 38 000)

would therefore be obtained for a cured animal. However, cure rates for subclinical mastitis due to CNS are not 100%. Aggregating data from 3 studies on treatment of CNS subclinical mastitis (McDougall, 1998; Wilson et al., 1999; Taponen et al., 2006), we estimated a cure rate of 81·9% (154 cured quarters out of 188), which means the SCC reduction would only be 81·9% of 47 000 cells/ml on the treated animals or 38 500 cells/ml, considering SCC at cow level. Considering that this reduction would occur on 29 cows (2 quarters affected per animal, for a total of 58 quarters), the expected reduction on bulk tank SCC would be 11 000 cells/ml (0·29 x 38 500). Such a low reduction in bulk tank SCC would probably not lead to improved farm gate milk price in most situations, as this tends to occur over wider SCC differences. Therefore, it was decided to consider extra revenue from improved bulk tank SCC to be zero.

Reduced costs

This was considered to include the prevention of culling and the prevention of new infections.

Mastitis is one of the main reasons for culling dairy cows across several countries, with reported figures of 3·6% (Whitaker et al., 2000), 10·1% (Esslemont and Kossaibati, 1997) or 12·4% (Seegers et al, 1998) of culls occurring due to mastitis. Most culls due to mastitis will probably occur because of incurable clinical mastitis or persistently high SCC. Either situation would be a relatively rare occurrence with CNS, since these microorganisms usually lead to mild clinical mastitis (Taponen et al., 2006) and to low SCC (Djabri et al, 2002). Therefore, it was considered that treatment of subclinical mastitis due CNS would not lead to reduced culling.

Prevention of new IMI would result from a reduced transmission occurring because a proportion of the existing cases of CNS subclinical mastitis would be cured. Transmission of infection can be estimated by the reproductive ratio, R, which is the product of the transmission parameter ß, a measure of a pathogen's contagiousness and the duration of infection τ, which is the inverse of the cure rate α (Swinkels et al., 2005a). ß is the average

number of new infections caused by an infected individual per unit of time and R is therefore the total number of new infections caused by an existing infection during its infectious lifetime. To estimate R we resorted to data from our study: average duration of a CNS IMI was 188 days (unpublished observations). As for ß we used the best case scenario for transmission of *S. aureus* during a steady-state period with post-milking teat disinfection (Barlow et al., 2009) which corresponded to 0·0028. It is well established that *S. aureus* frequently behaves in a contagious manner (Barkema et al., 2006), whereas for CNS there is evidence that the most frequent form of IMI acquisition might be different for different species (Taponen et al., 2008). Therefore the lowest value recorded for transmission of *S. aureus* was used. The R obtained was 0·53 (0·0028 ÷ 1/188), which means that without treatment each infected quarter would on average lead to a new infection in 0·53 other quarters. We considered that if the IMI was cured, duration of infection would be 30 days because it would take that long for the case to be detected, the aetiology to be known and for treatment to be performed. Again, only 81·9% of the cases would be cured, with the remaining cases persisting for 188 days. On average, infection in treated quarters would last 58·6 days (0·819 x 30 days + 0·181 x 188 days), corresponding to an R of 0·16 (0·0028 x 58·6). Hence, with treatment, transmission of infection in one quarter would be reduced from 0·53 to 0·16.

The average cost of a new infection was also estimated. We considered that each new infection would result in mastitis of clinical onset 5% of the times and in mastitis of subclinical onset 95% of the times. These values were used because there is no evidence in the literature as to how often a new IMI due to CNS has clinical onset. Estimated values for clinical onset of new infections are scarce in the literature and include 5% (Jayarao et al, 1999) and 15% (Zadoks et al., 2003) for *S. uberis*, which is more pathogenic than CNS. Out of all the infections that had a subclinical onset, it was considered that 64.5% would self-cure after the first month of infection and the remaining 35·5% would lead to an infection with the

average 188-day infection, based on reports by McDougall (1998). Thus, out of the new infections, 5% would have a clinical onset, 61·3% would have a subclinical onset that would spontaneously cure within one month and 33·7% would have a subclinical onset and last on average 188 days.

These infections would have different costs associated with them. A clinical case would include the costs of antimicrobial treatment (€7·63), of labour associated with the treatment (€0·60) and of discarded milk due to administration of antimicrobials (€34·20), for a total of €42·43. The sections "Extra costs" and "Reduced revenue" contain a full explanation of the costs of treatment and of the value of discarded milk. No milk production loss was considered because CNS normally lead to mild clinical mastitis. A subclinical case that self-cured within the first month was considered to last 15 days. Losses associated with this type of IMI (€1·66) and the longer IMI (€20·85) were due to the impact of increased SCC on milk production: considering the proportion of heifers and cows affected in the herd, the average SCC increase and the milk production losses associated with those increases as described under the section "Reduced Revenue". The average cost of a new infection would then be €10·16 (0·05 x €42·32 + 0·61 x €1·66 + 0·34 x €20·85).

If no treatment was performed, each infected quarter would transmit the infection to 0·53 quarters. Considering 58 infected quarters, this would lead to 30 new infections, representing a cost of €308·38. By comparison, treatment would reduce the infection to only 9 quarters, which would cost €96·11, corresponding to a saving of €212·26.

Extra costs

Extra costs included the costs associated with antimicrobial therapy: cost of antimicrobials, labour, diagnostic testing and the cost of antimicrobial residues.

Cost of antimicrobial treatment was calculated based on data sheet recommendations for the duration of treatment and on prices of 11 intramammary antimicrobials sold in Portugal at the time of the study. An arithmetic mean was estimated for the treatment cost (7·63€), for

number of intramammary tubes (3·36) for duration of treatment (1·9 days) and for duration of milk withdrawal period (3·5 days). Labour costs for the act of treatment were calculated based on employees earning twice the minimum wage in Portugal for the year 2010 (2 x 475€), working 22 days per month and 8 hours per day. If one considers that each treated quarter would take 2 minutes per infusion, a cost of €0·60 (€0·18 x 3·36 tubes) in labour could be assumed per treated mastitis or €34·85 for the whole herd (€0·60 x 58 quarters)

Costs associated with diagnostic testing were considered to be zero because the focus of the current study was the development of a support tool when the aetiology of IMI was already known. However, these diagnostic costs could easily be added to the final value for each quarter. Cost of antimicrobial residues accidentally going into the bulk tank after treatment were also assumed to be zero.

Reduced revenue

This included the value of discarded milk due to treatment. Milk produced during treatment was considered a complete loss, whereas it was considered that milk in the withdrawal period was fed to calves, a current practice among dairy farmers, saving the equivalent volume in milk replacer costs. Two types of milk production were estimated: a baseline milk production, which corresponded to the milk production when no infection was present and that was used for the calculation of the milk production loss associated with new cases of clinical mastitis, and a milk production for animals affected with subclinical mastitis.

Baseline milk production was calculated considering milk recording values for the 4 farms enrolled in our study for its duration (12 months). Animals with a SCC<50 000 cells/ml, considered to be uninfected, had an arithmetic mean daily milk production of 31·5 L for primiparous (35% of the animals) and 36·9 L for multiparous (65% of the animals), which considering the structure of the population led to an overall average of 35 L/day/animal. Milk production for animals with an ongoing subclinical mastitis due to CNS was based on the previous estimated production but discounted for the effect of the increased SCC. According

to Seegers et al. (2003), there is a daily reduction of 0·4 kg of milk for heifers and 0·6 kg for cows, per 2-fold increase in the SCC from a baseline of 50 000 cells/ml at cow level. Considering 2 affected quarters per cow, each with a SCC of 132 000 cells/ml and 2 quarters with no infection, each with a SCC of 38 000 cells/ml, and an equal contribution in terms of milk yield, a cow SCC of 85 000 cells/ml would be defined. This would lead to milk production losses of 0·28L/heifer/day and of 0·42L/cow/day or to a milk production of 34·6 L/day. No effect of stage of lactation on milk yield was considered in this study, despite Hagnestam-Nielsen et al. (2009) observing that the magnitude of daily milk loss associated with increased SCC depended on stage of lactation, being most extensive in late lactation. There is some evidence that CNS infection is not associated with days in milk (Østerås et al., 2006) and therefore we assumed the impact would be equally distributed throughout lactation periods.

The price of a litre of milk (0·299€/L) was based on the rolling 12-month mean in mainland Portugal (Gabinete de Planeamento e Políticas) for the year 2009. The cost of milk replacer was based on the arithmetic mean of prices of 3 milk replacers sold in Portugal at the time of the study, considering 125g/L of milk replacer at a price of €0·178, each litre of milk in the withdrawal period fed to calves would result in a loss of €0.121/L (0·299 – 0·178).

Treatment of subclinical mastitis due to CNS, would lead to a loss of €19·33 (34·6L x 1·9 days x 0·299€/L) through discarded milk plus €14·86 being lost as milk fed to calves during the withdrawal period (34·6L x 3·6 days x €0·12), for a total of €34·19. For the total number of quarters affected, this would be €1969·35.

Treatment of mastitis of clinical onset, which would be a reduced cost with fewer cases being transmitted, would occur in animals with a baseline milk production. Therefore the value of discarded milk would be slightly higher at €34·56 [(35L x 1·9 days x €0·299) + (35L x 3·6 days x €0·12)].

Sensitivity analysis

A sensitivity analysis is performed to evaluate how systematic variation to the input variables of the model (assumptions) leads to outcome changes (Dijkhuizen and Morris, 1997). This helps determine the impact of uncertain estimates on the outcome of decisions and allows consideration of best and worst case scenarios.

Several different inputs to our model were considered possible and their impact estimated, with 2 alternatives considered for most inputs. These included antimicrobial treatment cost and duration, cure rate for antimicrobial treatment, price of milk, different prevalence of infection, a change to the price of milk due to premium payment/avoiding penalties with lowering of the bulk tank SCC and different R values.

Antimicrobial treatment costs included the highest and lowest available price for intramammary antimicrobial treatment according to datasheet recommendations (€3·23 and €9·30 respectively). Duration of antimicrobial treatment and of milk withdrawal period included the shortest (1·5 + 2·0 days) and the longest in duration (2·0 + 5·0 days). Cure rates for antimicrobial treatment of CNS subclinical mastitis seem not to vary much. Reported cure rates of 81·0 (Wilson et al., 1999), 81·8 (McDougall, 1998) and 88·9% (Taponen et al., 2006) are close to the average value of 81·9% we used in our model, so cure rates of 70 and of 90% were used in the sensitivity analysis. Milk prices used in the sensitivity analysis were the highest and lowest recorded values during the time period considered for construction of the model, €0·336 and €0·274 respectively. Different prevalence of infection included the highest and lowest quarter-level prevalence recorded for the farms in the previous study (unpublished observations), 34·7 and 6·4%. The possibility of a change in farm gate milk price due to improvement in bulk tank SCC was considered, with an improvement of €0·005 to the baseline milk price being tested. Two different R values were considered taking into account mean ß values proposed by Barlow et al. (2009) for major Gram-positive mastitis pathogens, including a value for farms performing post-milking teat disinfection (ß=0·00868, R=1·63) and a value for farms not performing it (ß=0·0362, R=6·81).

Results

Net profit for antimicrobial treatment of subclinical mastitis due to CNS was negative at -€38·74 per treated quarter or - €2231·33 for a whole-farm approach, considering the average situation in a 100-cow herd and assuming that the etiological agent is known.

Table 2 displays the results for the sensitivity analysis. This showed that variation to the model inputs would nearly always lead to negative results except in 2 situations: if the decrease in bulk tank SCC was sufficient to result in an improvement in payment class after antimicrobial treatment had been performed, and if the number of new infections caused by an existing infection was set at its highest, in which case prevention of transmission through treatment would also be highest. Different prevalence of CNS IMI at quarter level did not lead to different financial net results per treated quarter as the higher costs would be divided by a higher number of quarters.

Discussion

Considering the input variables used in the present model, antimicrobial treatment during the lactation seems not to be economically advisable for subclinical mastitis due to CNS. For the average situation, such treatment would lead to a net loss of €38·74 per treated quarter. The highest cost would be the reduced revenue that occurs when discarding milk during antimicrobial treatment, and feeding it to calves during the withdrawal period. The cost of the treatment itself was next. These costs were not offset by extra revenue, which was actually zero, or by reduced costs, which corresponded to the predicted reduction in the occurrence of new mastitis cases.

Treatment of subclinical mastitis due to different udder pathogens has been addressed in several studies. Initial studies were performed for *S. agalactiae*, a contagious pathogen with reportedly high cure rates after antimicrobial treatment (Yamagata et al., 1987). These authors report a gain for treatments performed during early and mid lactation, but a net loss for treatments performed in late lactation (over 120 days). Swinkels et al. (2005) studied the economic benefits of treatment of subclinical mastitis due to *S. aureus*, and found a positive or a negative effect depending if the transmission between quarters was high or low respectively. The same authors studied the effect of treating chronic subclinical mastitis due to *S. uberis* during the lactation through 2 different methodologies, a deterministic and a stochastic approach. In the deterministic model, treatment resulted in an average profit of €11·62 over no treatment (Swinkels et al., 2005b), whereas the stochastic model predicted a net loss of 11€ when treatment was performed (Steeneveld et al., 2007). A recently published study on the economics of lactational treatment of subclinical mastitis with different udder pathogens points to different economic ouputs, depending if pathogens involved are transmitted between animals or acquired from the environment (van den Borne et al., 2010a) Therefore, it would seem that even for the so called major pathogens, results of economic studies for lactational therapy are not unanimous and quite often lead to net losses. It is not surprising then that lactational therapy for CNS, which are considered minor mastitis pathogens, results in a net economic loss.

As stated previously, the deterministic approach used in this study resorts to definite predictions for quantities (e.g. mean price of antimicrobials used, mean price of milk, etc). Due to the multifactorial nature of mastitis, use of a stochastic approach, resorting to probability distributions to deal with uncertainty, would probably lead to a more informative result in as far as it would provide a distribution of potential outcomes with the possibility of separating variability and uncertainty. Nevertheless, the quantity and quality of data to parameterise such a model adequately would add considerably to the complexity and duration

of the analysis, if not to render it unworkable. Given the convincing nature of the outcome of the analyses conducted, it is felt that the simple calculations used in this study, with the essential inclusion of the sensitivity analyses, have the advantage of being easily understood and could plausibly be replicated in farm-specific conditions by practitioners.

There is an ongoing debate about the role of CNS as udder pathogens with some authors considering them a potential problem (Taponen et al., 2009), whereas others consider them to be potentially beneficial due to an observed improvement in milk production (Schukken et al., 2009). Two previous studies have approached the subject of the economic benefits of treating CNS IMI, although strictly speaking they also included other pathogens in the analysis (Oliver et al., 2003; Borm et al., 2006). Both these trials studied the effects of pre-calving treatment of heifer subclinical mastitis, with CNS being the most frequently isolated udder pathogens. One such trial concluded there was no economic benefit (Borm et al., 2006), whereas the other led to some economic benefit through increased milk production (Oliver et al., 2003). There are not many studies reporting on milk yield after antimicrobial treatment of subclinical mastitis, but the ones available are suggestive of no improvement in milk yield after lactational treatment for both CNS (van den Borne et al., 2010b) and other species (St. Rose et al., 2003). In the present study, we assumed that no improvement in milk production would result from antimicrobial treatment.

The effects of CNS infection on milk yield are not well established. Some authors have found that CNS IMI led to milk production losses (Timms and Schultz, 1987; Borm et al., 2006), whereas others have found evidence of the opposite effect (Schukken et al., 2009; Piepers et al., 2010). There is a link between increased SCC above a certain threshold and milk production losses (Green et al., 2006). However, publications on this subject generally consider only the SCC level and not the pathogen involved. Some studies have addressed the issue of milk loss associated with specific pathogens. Grohn et al. (2004) observed higher milk production losses associated with clinical mastitis due to *S. aureus*, *Escherichia coli*,

Klebsiella spp. and for samples that were culture-negative. Schukken et al. (2009) studied records of 4200 herds and found that CNS infected cows produced slightly but significantly more milk (0·45 kg/day) than culture-negative cows, whereas cows infected with a major mastitis pathogen produced significantly less milk (between 1·6 and 3·6 kg/day depending on the agent). Reksen et al. (2007) also studied the effect of the isolation of specific pathogens in milk samples and test-day milk yield and found that pluriparous cows infected with *Streptococcus* spp. actually produced more milk than their culture-negative herdmates. Judging from these studies, there is still plenty of scope for improvement of our understanding of species-specific impact on milk production, without forgetting a possible role for increased susceptibility of higher producing cows. This is probably not easily achieved without measurement of quarter milk production and compensatory effects, a logistically complex task.

Another possibility for extra revenue to be gained with treatment for subclinical mastitis due to CNS would be the improvement in bulk tank SCC. In the initial assumptions we considered this to be zero, because we estimated a likely improvement of only 11 000 cells/ml in the bulk tank SCC with treatment, which would only lead to a premium being paid or to avoidance of penalties if the bulk tank SCC was on the verge of a change in payment category. Were that to occur and a premium of €0·005 be paid, then treatment would actually lead to positive results in the conditions considered. Different contributions of CNS to the bulk tank SCC could potentially be included in the model, based on cow-level bacteriology and level of bulk tank SCC (Schukken et al., 2009). This premium payment has a different effect than simply increasing the farm gate milk price. When we performed the sensitivity analysis, increasing the farm gate milk price led to negative results because during antimicrobial treatments, it would lead to milk being discarded at those prices. A premium being paid for better quality milk would mean that the higher prices would come after the treatments were performed, therefore not being affected by discarded milk.

One of the parameters included under reduced costs was prevention of new infections. Several of the assumptions made in estimating this parameter are debatable. We initially considered an R value (total number of new infections caused by an ongoing infection) of 0·53, which is fairly low, implying that infection would not sustain itself on a farm through transmission between animals and would only persist because of an outside reservoir of infection. There is emerging evidence that different CNS species behave differently in terms of the most likely source of infection being the environment or other cows (Taponen et al., 2008), so predominance of a certain CNS species on a particular farm may affect this parameter. Two additional R values were considered in the sensitivity analysis based on ß values cited by Barlow et al. (2009). If we consider the highest of these values, with one infected quarter transmitting infection to 6.81 quarters during its infectious lifetime, lactational antimicrobial treatment would lead to a net profit through prevention of transmission.

The sensitivity analysis revealed that the prevalence of infection at quarter level would not change the outcome of the model. Other factors would have an impact on the level of loss consequent to treatment. Duration of treatment and of withdrawal period would have the largest impact on the economic result of treatment, leading to the highest and lowest loss when considering the longest and shortest duration respectively. Treatment and withdrawal period duration were according to label use of the antimicrobials considered. The duration of treatment and choice of antimicrobial would probably have an impact on cure rates, as these have been reported to vary for different antimicrobials used in the treatment of CNS mastitis (McDougall, 1998). However, variation in the cure rates would not actually have a major impact on the economic outcome of treatment.

Besides the direct financial loss likely to occur with lactational treatment with antimicrobials, other potential negative consequences need to be considered, namely selection for antimicrobial resistance. There is much debate around the effects of antimicrobial usage

on the emergence of antimicrobial resistance at farm level (Call et al., 2008), but there is some evidence of such relationship, mainly through the comparison of conventional and organic farms (Tikofsky et al., 2003). These authors compared antimicrobial susceptibility profiles for *S. aureus* from 22 organic and 16 conventional dairy herds and found differences for 7 of 9 antibiotics studied. Public perception and consumer choices may also be affected by higher antimicrobial usage. Even though feeding milk during antimicrobial withdrawal period to calves is not recommended from the point of view of emergence of antimicrobial resistance, it was decided to assume that farmers feed this milk to calves in the model, as this may occur frequently.

As other pathogens come under control on farms, there is currently much attention devoted to the impact of CNS on udder health. Judging from our deterministic approach, it would appear that lactational antimicrobial treatment for subclinical mastitis due to CNS should not be part of a control programme, as it would lead to a net financial loss in most situations.

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Table 1. Input variables into the economic model and their respective source.

Input variable	Value	Source
Extra revenue		
Increased milk production after cure	0	van den Borne et al, 2010b
Improvement of bulk tank SCC leading to premium payment/avoiding penalties	0	Calculated
Total	0	
Reduced costs		
Prevention of new mastitis cases	€212·26	Calculated
Prevention of culling	0	This paper
Total	+ €212·26	
Reduced revenue		
Milk discarded		
Treatment period	€1113·04	Calculated
Withdrawal period	€856·31	Calculated
Total	- €1969·35	
Extra costs		
Cost of antimicrobial therapy	€439·39	Calculated
Labour	€34·85	Calculated
Diagnostic testing	0	This paper
Antibiotic residues	0	This paper
Total	- €474·25	
Total for the herd	- €2231·33	
Total for an individual case	- €38·74	

Table 2. Sensitivity analysis considering possible scenarios for different input variables.

Input variable (current input value)	Level	Net profit (€/quarter)
Antimicrobial treatment cost (€7·63)	€9·30	- 40·38
	€3·23	- 34·42
Duration of antimicrobial treatment + withdrawal period (1·86 + 3·55 days)	2·0 + 5·0 days	- 46·26
	1·5 + 2·0 days	- 28·49
Cure rate for antimicrobial treatment (81·9%)	70%	- 39·28
	90%	- 38·38
Price of milk (€0·299)	€0·336	- 45·55
	€0·274	- 34·01
Prevalence of infection at quarter level (14·4%)	6·4%	- 38·74
	34·7%	- 38·74
Premium payment/avoiding penalties	€0·005/L	+71·25
R value (0·53)	1·63	- 31·00
	6·81	+5·22

Discussion

Mastitis is the most frequent disease of dairy cows. It is unavoidable to have mastitis episodes in any dairy farm, but their prevalence and overall financial impact can be greatly reduced if the appropriate control measures are implemented. When performing research on the relative prevalence of udder pathogens or when performing udder health investigations, the isolation of coagulase-negative staphylococci is a frequent finding. However, their importance as mastitis pathogens needs to be clarified. Aspects regarding their diagnosis, effects *in-vivo* in the field and the economics of treatment are discussed, which hopefully will help clarify their role, as well as that of *Corynebacterium* spp., another group of so-called minor mastitis pathogens, on udder health.

Identification of non-aureus staphylococci

Up to the present time, 48 staphylococcal species have been identified (NCBI taxonomy, 2010). It is difficult to say at this stage how many of these are actually involved in bovine mastitis because the acceptable standard for identification at research level has recently been changed. Whereas some years ago, phenotypic identification was acceptable in scientific publications, nowadays it is no longer viewed as having sufficient accuracy for valid information to be gained from it. This is based on the finding by several researchers that commercially available phenotypic identification systems are quite inaccurate (Zadoks & Watts, 2009). This implies that much of the previous research on individual CNS species regarding bovine mastitis, despite having historical value, should be carefully interpreted in light of current knowledge on this subject.

Despite the term coagulase-negative staphylococci still being currently accepted, it is not scientifically precise. In fact *S. hyicus* which can be coagulase-positive (Blaiotta et al., 2010) and coagulase-negative strains of *S. aureus* (Fox, Chester & Jackson, 1996), have been implicated in bovine mastitis (Sampimon et al., 2009), and the coagulase test is not often a part of the routine laboratory identification of staphylococci. Hence the more accurate term non-aureus staphylococci (NAS) will be used for the remainder of this text when referring to coagulase-negative staphylococci.

In study I, several methods for species-level identification of NAS were used: a phenotypic method (API ID 32 Staph system) and two genotypic methods including an internal transcribed spacer region PCR (ITS-PCR) and sequencing of two housekeeping genes. For strain typing, pulsed-field gel electrophoresis was used. The API ID 32 Staph system has been used in similar studies (Taponen et al., 2007) but its accuracy in the identification of bovine mastitis isolates is not very high (Sampimon et al., 2009). To overcome that, it was decided to perform a species genotypic identification of every PFGE profile, with more than one isolate being identified from each pulsotype, whenever available.

This way, every pulsotype would have a genotypic species identification. It can be questioned if the same PFGE profile can occur in different staphylococcal species, but there was no evidence of that in the identifications performed and no such evidence could be found in the literature.

Initially, genotypic identification was performed by ITS-PCR according to Couto et al. (2001). This method is based on the fact that, in prokaryotes, the rRNA genetic loci contain the genes for the several ribosome subunits separated by spacer regions that do not code for specific proteins, and hence have a high degree of variability in both sequence and size. This allows for amplification of these highly polymorphic areas to be targeted for genus and species level identification, by using as primers conserved sequences from the adjacent 16S and 23S genes. However, this technique presents some challenges. Namely, some staphylococcal species have very similar profiles, and some can present more than one profile with clinical isolates not always having the same profile as reference strains (Couto et al., 2001). This technique allowed for most of the isolates in the present study to be confidently identified to species level, being a high throughput genotypic method and fairly economical (if avoiding the use of a high resolution agarose). Unfortunately, not all the species could be identified with this method. Namely, several isolates that had been identified phenotypically as *S. intermedius* did not match any of the available reference strains, and several isolates identified previously as *S. chromogenes* or as *S. epidermidis* were difficult to distinguish with this methodology. A majority of isolates of animal origin identified phenotipically as *S. intermedius* have been shown to correspond to *S. pseudintermedius* in the past (Devriese, Hermans, Baele & Haesebrouck, 2009), so a field strain of this species was also used for comparison, but it still did not allow for an identification to be confidently performed.

It was decided to perform a second genotypic identification method to confirm the previous identifications, using sequencing of housekeeping genes, a technique that is considered the gold standard for NAS identification (Zadoks & Watts, 2009). Several candidate genes were available, so the choice was based on the genes that would allow for the problem species to be more confidently identified. Evaluating neighbour joining trees available for six such genes in Ghebremedhin, Layer, König and König (2008), the *sodA* and *rpoB* genes appeared to be the most discriminatory and it was decided to sequence them both in the name of accuracy. The *sodA* gene codes for manganese cofactored superoxide dismutase and the *rpoB* gene codes for the beta subunit of RNA polymerase.

Sequencing of these two particular genes proved to be quite accurate and useful, with identifications coinciding between the two genes and with high homology generally being obtained between the isolates' sequences and the ones present in available databases. For the *rpoB* gene, the homology was always higher than the 97% proposed by Silvadon et al. (2004) and Heikens, Fleer, Paauw, Florijn and Fluit (2005) as a homology cut-off. For the

sodA gene, out of the 24 isolates that were sequenced, four had values lower than 97%, with three *S. simulans* at 96% and one *S. hyicus* at 88%. However, these identifications were confirmed by sequencing of the *rpoB* gene.

As for strain typing, a PFGE technique was used. This has been extensively used for strain typing of staphylococci and other genus with good results (Goering, 2010). Briefly, whole bacterial DNA is split by a low frequency cleavage restriction enzyme. The DNA fragments are then separated by an electrophoresis in which the voltage periodically shifts direction allowing for a better resolution to be obtained. The end result is a profile with a certain banding pattern, with each band corresponding to a DNA fragment of different size, which can be compared with the banding pattern of other isolates. The interpretation criteria to define different banding patterns or pulsotypes is however, still open to debate (Goering, 2010). A "one size fits all" approach is not possible because single genetic events can result in a diverse number of band differences and because different species have a diverse genomic shift (Van Belkum et al., 2007; Goering, 2010). Computer assisted cluster analysis is necessary for high numbers of profiles to be compared and this is dependent on the definition of similarity cut-off values. Definition of these cut-off levels is dependent on the technique used to estimate the positional correspondence of the bands, with the Dice coefficient being the most widely used for this purpose (Van Belkum et al., 2007). A certain level of input from the user is necessary, to define the parameters used in the analysis (band position tolerance), to check for gel imperfections and to account for differences due to imperfect reproducibility of the technique, which may lead to quantitatively different results for the same isolates. To avoid variability as much as possible, criteria that had previously been used in other studies on staphylococci (McDougal, 2003; Faria, Carriço, Oliveira, Ramirez & Lencastre, 2008) were used, including use of a Dice coefficient with a band position tolerance of 1.25%, optimization of 0.5% and a cut-off of 80%. A cut-off of 70% was also used in the analysis because the time period under consideration was close to one year and because four farms with different geographic locations participated in the study, both facts potentially leading to increased variability (Tenover et al., 1995).

Other methodologies have the potential to help characterise which strains of a particular species are involved in IMI episodes. Single nucleotide polymorphism (SNP) genotyping or multilocus sequence typing (MLST), a particular type of SNP genotyping, may allow for a closer scrutiny of the differences between isolates since these techniques focus on single nucleotide differences in particular genomic sequences, thus also having the potential to gain information on phylogeny (Van Belkum et al., 2007). Despite the high reproducibility and standardization attained with these techniques and of being more easily interpreted than results obtained with PFGE, these techniques are also comparatively expensive due to the relatively high number of targets that need to be sequenced (Aguiar-Alves et al., 2006).

The end product of the identification process allowed most of the IMI due to NAS to be characterised in terms of aetiology and duration. It was assumed that whenever the same PFGE profile was found in a given mammary gland in successive months, it was implicated in the same infection. It is acknowledged that it is possible for the same strain to cause two independent IMI cases within the four week interval between sampling visits, but this was considered less likely than a continuing infection with the same strain.

Impact of different non-aureus staphylococci species on udder health

As stated in study I, differences in terms of impact on udder health between NAS species were apparent but generally not significant.

The most frequently isolated NAS was *S. epidermidis*, followed by *S. simulans*, *S. chromogenes* and *S. haemolyticus*. Not all the species had the same distribution per farm: despite being the most frequently isolated species, *S. epidermidis* and *S. simulans* were only found on two farms (two isolates were identified as *S. epidermidis* on farm C, but these isolates were unfortunately not genotyped), whereas *S. chromogenes* and *S. haemolyticus* were found on all farms. The relative distribution of the different NAS species in each farm raises questions about the presence of specific risk factors for the prevalence of each individual NAS species and most likely form of infection. Previous studies on individual NAS species have generally looked at prevalence on individual research farms (Taponen et al., 2006) or have addressed prevalence for several commercial farms grouped together (Waage et al. 1999), without addressing differences between farms. Piessens et al. (2010b) also observed herd-to-herd differences in the prevalence of individual NAS species. In the current study, no risk factors were analysed because the number of farms was too small for relevant conclusions to be drawn. A larger scale study, evaluating the relative prevalence of individual species and potential risk factors across several farms could bring some light on to this subject.

The impact of individual species on quarter SCC was not significantly different between species, with geometric means varying between 95,000 cells/ml for *S. epidermidis* and 202,000 cells/ml for *S. chromogenes*. Overall, a geometric mean of 132,000 cells/ml was obtained for all the NAS considered together. This value is very close to the 138,000 cells/ml reported for NAS by Djabri et al. (2002) in a meta-analysis of quarter SCC for different pathogens.

There was a relationship between the logarithmic transformation of the somatic cell count and both sampling occasion and cow, that was influenced by species. In other words, certain cows had a higher SCC than others, and certain sampling occasions had a higher cell count than others, but these observations were not independent of which NAS species was considered. This was to be expected for cows since it has been observed that individual cows have different responses to infection with the same pathogens (Burvenich, Grondin,

Shkreta, Lacasse & Talbot, 2003) and in fact this can be improved through sire selection (Heringstad, Klemetsdal & Steine, 2003). It is difficult to ascertain the impact of individual NAS species on the observed effect of sampling occasion on $\log_{10}$ SCC because of the small number of observations. It is not impossible for different NAS species to have slightly different pathogenic effects and/or rate of pathogenic effects, hence leading to differences in inflammatory response over time.

Somatic cell count is generally used as a measure of mammary gland inflammation, almost always linked to the presence of infection. A high somatic cell count is associated with a decrease in both quality and quantity of milk produced, both leading to an economic loss for the farmer. Somatic cells include mostly leukocytes (neutrophils, lymphocites, macrophages) and also some mammary gland epithelial cells (Riollet, Rainard & Poutrel, 2000). Milk produced in infected mammary glands has less lactose, less casein (the main protein in milk) and altered levels of electrolytes. It has increased levels of plasmin that degrades casein in stored milk, even after pasteurization. It also has increased levels of lipases which degrade milk fat into fatty acids, leading to a rancid flavour and to the inhibition of starter cultures for cheese and yoghurt production (Barbano, Ma & Santos, 2006). It is easy to understand the importance that milk produced by non-infected glands has for the dairy industry. This determines its active search for better quality milk through a system of premiums and penalties according to the level of somatic cells in the bulk tank milk. A European Union Directive (92/46) also determines that milk cannot be sold if it has a SCC above 400,000 cells/ml.

Milk yield is also affected by the presence of infection in the mammary gland – it is estimated that for every two-fold increase in SCC above 50,000 cells/ml, there is a 0.5 kg decrease in individual cow milk yield per day (Seegers, Fourichon & Beaudeau, 2003). Considering these values, milk production losses would be associated with the NAS species considered in our study since all present geometric mean SCC recorded were above 50,000 cells/ml.

In order to estimate the duration of infection, we made the assumption that finding the same PFGE pulsotype in successive samplings corresponded to the same infection that was active in the four week interval between samplings. We acknowledge that this is not necessarily true for two reasons. One infection can be cured and a new infection acquired with the same pulsotype in the time frame considered. This would be more likely when the same pulsotypes are responsible for most infections. The other reason is that the PFGE technique may not be discriminatory enough to differentiate between different strains.

Duration of infection was also not significantly different for the analysed NAS species. The survival analysis revealed that *S. simulans* had the longest mean duration of infection with 213 days, and *S. haemolyticus* had the shortest with 123 days. Mean duration of infection for all the NAS was 188 days. The impact of duration of infection on udder health is

two-fold: through the quality and quantity of milk being affected for a longer or shorter period of time, and through the increased possibility of transmission of infection to other mammary glands for longer lasting infections. The probability of transmission of disease can be expressed as the transmission parameter ß, the probability per unit of time that an infectious quarter will infect a non-infected quarter (Zadoks et al., 2001). The longer the infection lasts, the more likely it is for transmission to occur. The product of ß and duration of infection is the reproductive ratio (R), the total number of new infections caused by an existing infection (Zadoks, Allore, Hagenaars, Barkema & Schukken, 2002). As ß measures the probability of transmission of disease, it will vary for different pathogens and for different farms or on the same farm according to the implementation of control measures (Swinkels, Hogeveen & Zadoks, 2005a; Swinkels, Rooijrndijk, Zadoks & Hogeveen, 2005b). Estimation of this parameter makes sense for pathogens that lead to IMI mainly in a contagious way. The classification of mastitis pathogens into contagious or environmental is not as clear-cut as before, with evidence that some "environmental" pathogens can also behave in a contagious way (Bradley & Green, 2001; Zadoks et al., 2003; Rato et al., 2008) and vice-versa (Joo, Fox, Davis, Bohach & Park, 2001).

The most likely route of transmission for the different NAS species was tentatively addressed in the present work by calculation of the Simpson diversity index. Diversity can be measured by species richness (the number of species in the unit of study), by its evenness (the variability in species abundances) and by a proportional statistic that combines both measures (diversity index). The two most widely used diversity indices are (Hill, Walsh, Harris & Moffett, 2003; Magurran, 2005):

- the Shannon index - measures the amount of information (entropy) in the system and hence is a measure of the difficulty in predicting the identity of the next individual sampled;
- Simpson index - estimates the probability of any two individuals drawn at random from an infinitely large community belonging to the same species.

The Simpson diversity index (SDI) has been used in studies on bovine mastitis in the past, to evaluate strain diversity within herd and between herds for *Klebsiella pneumoniae* (Paulin-Curlee et al., 2008), to study strain diversity during an outbreak caused by the same agent (Munoz, Welcome, Schukken & Zadoks, 2007) and to compare the discriminatory power of different *S. aureus* strain typing methods (Ikawaty et al., 2009). The objective of its use in the current study was to understand how often the same strain was responsible for mastitis in a particular farm.

As stated previously there has been some debate as to the most likely source of infection for NAS. Non-aureus staphylococci are frequently isolated from the mammary glands of pre-calving heifers (Aarestrup & Jansen, 1997), in which case an environmental source of

infection is the most likely culprit since the animals have not been submitted to milking. Strictly speaking, contagious mastitis can still occur pre-calving when animals are infected through contaminated milk early in life, such as might occur for mastitis due to *Mycoplasma* spp. (Fox, Muller, Wedam, Schneider & Biddle, 2008; Roy, Francoz & Labrecque, 2008). As for infections acquired after the first calving, there is still no consensus as to the most likely source of infection for the different NAS species. The most frequently isolated NAS species in the environment are not the same as the most frequently isolated species from milk samples (Taponen et al. 2008, Piessens et al., 2010b), which could be due to different capabilities of colonising the bovine mammary gland. Namely, *S. equorum* and *S. sciuri* seem to be the most frequently isolated species from environmental samples, whereas in milk samples the most frequently isolated species are *S. chromogenes* and *S. simulans*. According to the same authors, there is also evidence that for some NAS species, common strains are shared between environmental and milk samples.

The approach to understanding routes of infection in the current study was not based on culturing of environmental samples, but based on how often the same PFGE pulsotypes were shared between IMI within the same herd. A similar approach has been used by other authors to assess how often a strain or a small number of strains are responsible for *S. aureus* mastitis within each herd (Joo et al., 2001). The hypothesis behind this approach is that if contagious transmission is occurring, a small number of pulsotypes will likely be responsible for most mastitis cases, whereas if infection has an environmental source, a multitude of pulsotypes is more likely (Phuektes et al, 2001b). However likely this might seem, it still lacks scientific confirmation which will be difficult to obtain because of the many factors involved in disease transmission. Certain pathogen species or strains may be more or less contagious, certain hosts more or less susceptible and the environment and husbandry of a certain farm more likely to contribute to transmission of infection between animals.

Estimation of the SDI for different species showed a significant difference between *S. epidermidis* and *S. simulans*, and *S. haemolyticus* when considering all the farms, but this could be influenced by farm factors as the distribution of species per farm was not uniform. Considering the diversity in both species and strains per farm, significant differences were observed between farms C and D, and between farms B and D, depending if a cut-off value of 80 or 70% was considered. There was a significant difference in the SDI of *S. epidermidis* on farms A and D when considering the 80% similarity cut-off value, which indicates this species' diversity is different between these farms. This could be due to differences in management leading to the more common source of infection being other cows or the environment. The SDI for *S. simulans* was not different between the same farms. It can be hypothesised that transmission of this species is less affected by herd related factors. There were no significant differences between these two species on farms D and A, although that difference approached significance for farm A.

The observed differences in SDI do not allow for the clear establishment of a likely transmission route. In fact, a single environmental point source of infection could still be responsible for the same strain of *S. simulans* being so often isolated from milk samples in farm A for example. There are not many accounts of what such source could be. Thorberg et al. (2006) observed that the *S. epidermidis* strain or strains that predominated in milk samples from two farms were also found on the milkers' hands and elbows. It is difficult to know if the source of those particular strains of *S. epidermidis* were the cows, in which case, the milkers would be acting as fomites and transmission from cow to cow would be contagious, or if the source were the milkers, in which case infection would originate from an environmental point source. Irrespective of considerations regarding contagious or environmental patterns of infection, knowledge about diversity of strains within species can be useful for control strategies to be implemented.

Considering the high likelihood of a scenario where diversity within NAS species is affected by pathogen factors, it would appear that IMI due to *S. epidermidis* and *S. simulans* are more often acquired from other animals than *S. chromogenes* and *S. haemolyticus*, which would explain why the former show less diversity. This is consistent with what Taponen et al. (2008) observed in their study, with *S. chromogenes* being often isolated from extramammary sites and from mammary glands, but with *S. simulans* not being encountered on extramammary sites despite often being present in milk samples. White et al. (1989) have also isolated *S. chromogenes* much more often on extramammary sites than *S. simulans*.

If, as hypothesised before, pre-calving heifers acquire infection strictly from the environment and adult cows may acquire from both the environment and other infected cows, one would expect the prevalence of different species to be different for pre-calving heifers and for multiparous cows. Comparisons between these two groups have not been made, but Thorberg et al. (2009) observed differences between the frequencies of isolation of three NAS species in first and other lactations. *Staphylococcus epidermidis* was significantly more often found on second and later lactation cows than in primiparous heifers. *Staphylococcus chromogenes* was more often found in primiparous and *S. simulans* was equally distributed between primiparous and multiparous. These differences may also be due to changes to the animals' environment.

In summary, across the studied farms there were differences between NAS species in terms of duration of infection and impact on quarter SCC, albeit not significant ones. Intramammary infections were long-lasting, but quarter SCC was relatively low. There were differences between NAS species in terms of strain diversity, however the number of strains available for each species was insufficient for a complete inter-species analysis within farm. Put together these conclusions do not justify performing a complete species level identification for routine purposes.

Economic impact of lactational therapy for non-aureus staphylococci intramammary infection

Despite the thousands of scientific publications on bovine mastitis, performing udder health investigations can be somewhat demoralising because of the huge gap between what is addressed in scientific journals and what goes on in the real world of dairy farms. This probably occurs for two reasons. On one hand it is difficult to lead farmers to implement measures with known positive effects on udder health (Green, Leach, Breen, Green & Bradley, 2007). Recent publications have tentatively addressed communication issues for the successful implementation of mastitis control measures (Jansen, Steuten, Renes, Aarts & Lam, 2010). On the other hand, there are many instances in which research is not trying to answer questions that have immediate impact on mastitis control (it can be argued that effective communication with researchers is just as hard as with farmers).

One of the objectives of the current work was to generate useful information that could be directly used by practitioners in the field. Currently there is little evidence on the effectiveness of control measures issued specifically for NAS. It is well established that antimicrobial therapy is effective in solving NAS IMI (Taponen et al., 2006) and at least some disinfectant formulations applied to the teats after milking, are capable of reducing the number of new infections by these agents (Foret et al., 2005). A vaccine against staphylococci and coliforms has recently been introduced in the European market after approval by the European Medicines Agency, for which approval its efficacy in field trials had to be demonstrated. The three control measures mentioned above, antimicrobial therapy, use of post milking teat disinfection and vaccination may all have a certain degree of efficacy in the control of NAS IMI, but they are not all equally efficient. In fact, they may even result in a net financial loss. A recent publication evaluated the efficacy and cost efficiency of management measures to improve udder health on Dutch dairy farms (Huijps, Hogeveen, Lam & Oude Lansink, 2010). The authors concluded that the most effective method in the prevention of clinical mastitis and in the reduction of bulk tank somatic cell count - use of postmilking teat disinfection - was not the most cost-effective, with measures such as milkers wearing gloves being more efficient because of the low associated costs.

As stated before, there are several studies published on the cure rates of NAS IMI after antimicrobial therapy (McDougall, 1998; Wilson et al., 1999; Taponen et al., 2006). Using data from those publications and from our own studies, it was possible to estimate the financial outcome of treating NAS IMI during the lactation. In the conditions considered, there would be a net loss of €38.74 on average per treated quarter, with a possible loss of €46.26 when the treatment and withdrawal periods were the longest. There would be a net financial benefit to treating NAS IMI during the lactation if the bulk tank somatic cell count suffered a reduction that would lead to a premium payment or to avoidance of penalties, an unlikely scenario because NAS mastitis is often associated with minor increases on quarter level

SCC. The other situation in which lactational treatment would have a positive financial outcome would result from the prevention of transmission of NAS in the milking parlour if the possibility of transmission were at its highest level.

This was a likely outcome. Literature available on the economics of treating subclinical mastitis caused by other, more deleterious, udder pathogens has generally reached the same conclusion. Over 20 years ago, studies performed on the economics of treating subclinical mastitis due to *Streptococcus agalactiae* concluded that there would generally be a net benefit (Yamagata, Goodger, Weaver & Franti, 1987; Edmonson, 1989). Considering today's prices, this might not be the case anymore. A study performed on lactational treatment of *S. aureus* subclinical mastitis (Swinkels et al., 2005a) found that there would be a gain in a scenario in which transmission of this agent between quarters was high, clearly a situation that would need additional control measures for an effective control. If transmission was low, there would be a net loss resulting from treatment. The same authors also studied the effects of lactational treatment of *S. uberis* subclinical mastitis and depending on which mathematical method was used, obtained different results: a net profit of €11.62 when using a deterministic approach (Swinkels et al., 2005b) and a net loss of €11 when using a stochastic approach (Steeneveld, Swinkels & Hogeveen, 2007).

There is of course a certain degree of uncertainty associated with these economic models. They represent the most likely outcome taking into account the most likely scenario and are therefore not applicable to a number of situations. One of the input parameters for these models is the likelihood of transmission of the considered pathogens. As we have seen above, there might be differences between individual NAS species in terms of transmission in the milking parlour. Our economic model was not species specific, rather grouped all the NAS together, disregarding these differences. It is still informative enough for practitioners not to advise treatment of subclinical mastitis due to NAS during the lactation. Despite what has been said, there is no reason why dry cow antimicrobial therapy should not be performed.

Are we dealing with true udder pathogens?

From what has been written in the literature review and in previous headings of this discussion, it is apparent that the impact of NAS on udder health is not high. Despite causing infections that can be long-lasting, this group leads to mean quarter SCC that are not very high; it might have a protective effect against other more damaging udder pathogens and its impact on milk production is still open for debate. We can even ask the question: are NAS truly udder pathogens or are they desirable microbiota?

There are some conflicting notions surrounding the potential pathogenic effect that probably warrant further research. On one hand, NAS lead to increased SCC, which has been shown to lead to milk production losses (Seegers et al., 2003); however, several

authors have found that NAS infected cows produce more milk. Schukken et al. (2009) studied records of 4200 herds and found that NAS infected cows produced slightly but significantly more milk (0.45 kg/day) than culture-negative cows, whereas cows infected with a major mastitis pathogen produced significantly less milk (between 1.6 and 3.6 kg/day depending on the agent). Piepers et al. (2010) studied milk production in 191 heifers across 20 farms and also found a significantly higher milk production for NAS infected heifers than for culture-negative heifers. The difference in milk production was partially, but not totally, explained by a significantly lower incidence of clinical mastitis in the NAS infected animals than in their culture-negative counterparts. Yet unidentified confounding factors may explain the rest of the observed difference in milk production, if not all. One hypothesis would be that higher producing cows are more susceptible to NAS IMI, which is quite plausible since the correlation between mastitis occurrence and production traits has been found to be unfavourable (Negussie, Strandén & Mäntysaari, 2008). Another possible explanation for this observation has to do with the fact that the studies that report an increased milk yield for NAS infected animals report results at cow level, not at quarter level. Schukken et al. (2009) report results for composite milk samples which are known to have a lower sensitivity for the detection of pathogens (Lam et al., 1996) and do not reflect true milk production or SCC at affected quarter level. The problems with considering cow-level infections are illustrated by the fact that in the study by Schukken et al. (2009) mean cow SCC for culture negative cows was 304,000 cells/ml, which is well above the accepted 200,000 cells/ml threshold to detect infection at cow level (Dohoo & Leslie, 1991).

Other studies addressing the issue of milk production for NAS infected cows have found a decrease in milk production. Timms and Schultz (1987) found a mean milk production loss of 821 kg of milk at 305 days for NAS infected animals, when compared with uninfected animals in two herds. Borm et al. (2006) studied the effect of prepartum antibiotic treatment of heifers on nine herds and observed no significant difference in milk yield between animals cured and not cured of NAS IMI, even though the latter showed a lower yield.

Milk production for NAS infected cows was not addressed in the current study as the sampling procedure for study I aimed at following NAS IMI through time and would not warrant a valid comparison with cows infected with other pathogens.

The question in this heading can also be asked for *C. bovis*. During sampling, despite not being the aim of study I, other microorganisms were unavoidably isolated from milk samples, the most frequent of which was *C. bovis*. Figure 1 shows quarter SCC for NAS affected quarters throughout the study and Figure 2 shows quarter SCC for *C. bovis* infected quarters, both only when single isolates were present in milk samples. Also displayed in Figures 1 and 2 are the geometric mean quarter SCC recorded in a meta-analysis by Djabri et al. (2002) for different pathogens. As can be seen from these figures, a great number of samples from which NAS and *C. bovis* were isolated had SCC that were lower than the

average reported by Djabri et al. (2002) for culture-negative quarter samples. With such a low inflammatory response, it is doubtful that we are truly in the presence of an IMI, and it is likely that the isolates are rather contaminating flora from the teat canal. In the opposite situation, when the SCC recorded for samples, in which NAS or *C. bovis* were isolated, is higher than the threshold normally associated with major pathogens, we can ask if we are we truly in the presence of IMI by these minor pathogens. These organisms may be simply easier to isolate than for example a *S. aureus* with an intermittent excreting pattern or that is intracellularly located.

These questions were the basis for studies II and III. In study II, the effects of freezing milk samples prior to conventional culturing and of performing a multiplex RT-PCR, were evaluated for the detection of major pathogens. In study III, the effect of using a teat cannula, surpassing the teat canal, on the reduction of sample contamination with teat canal flora was evaluated.

To summarise, it is well established that NAS have the potential to cause clinical mastitis (Simojoki et al., 2009), with all the implications associated with this: antimicrobial treatment, milk production loss and clinical signs. Despite the negative aspects of any clinical mastitis, NAS seem to be minor offenders. Cure rates with antimicrobial treatment are high (Waage et al., 2000), intensity of clinical signs is mild (Taponen et al., 2006), including milk production loss (Simojoki et al., 2009). Likewise, it is also well established that NAS can cause subclinical mastitis (Simojoki et al., 2009), but there is no definitive answer as to whether their presence has negative consequences or actually has a positive effect, potentially through protection against infection by other, more pathogenic agents.

Therefore, it would appear that the term minor mastitis pathogen for NAS is still appropriately descriptive.

Figure 1. Quarter SCC for NAS infected quarters in the current study, and mean SCC for the meta-analysis by Djabri et al. (2002): culture-negative (-), NAS (-) and minimum mean SCC for major pathogens (-).

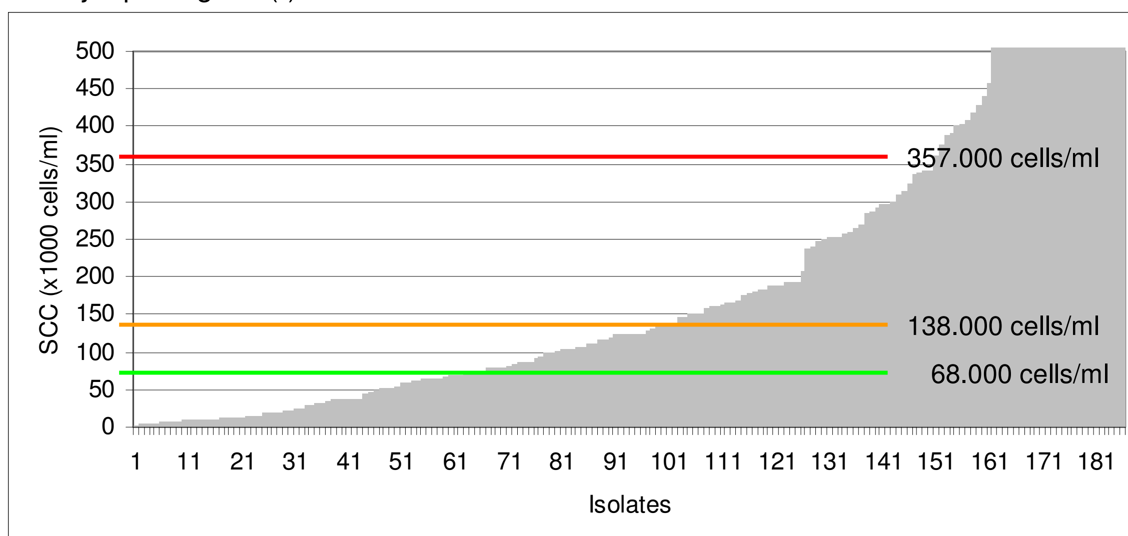
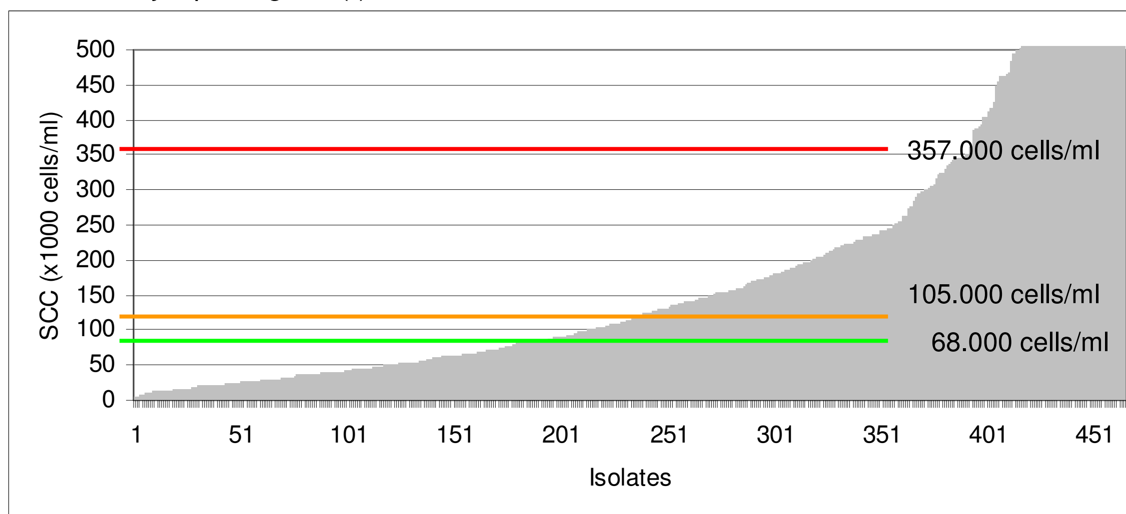


Figure 2. Quarter SCC for *C. bovis* infected quarters in the current study, and mean SCC for the meta-analysis by Djabri et al. (2002): culture-negative (-), *C. bovis* (-) and minimum mean SCC for major pathogens (-).



Other aspects of pathogenicity should, however, be considered in this discussion, including the potential impact on Public Health. Non-aureus staphylococci have been responsible for many different conditions occurring in humans, but mostly NAS have been implicated in nosocomial infections (Piette & Vershraegen, 2009). To the author's knowledge, thus far there are no reported cases linking human disease with NAS of bovine origin. That does not completely exclude the potential negative effect of such organisms on human health. As stated previously, antimicrobial resistance can be transferred between NAS and *S. aureus*, thus potentially contributing to its pathogenic effect (Muhammad et al., 1993).

Sampling to reduce contamination from teat canal flora

Corynebacterium bovis are very often isolated from milk samples (Pitkälä et al., 2004), but do not always cause true mastitis (Honkanen-Buzalski & Bramley, 1984). Thus, how often is their presence in milk samples not associated with an inflammatory response? How often do they truly cause mastitis? Could this occur with other microorganisms? Could the same phenomenon occur with NAS, offering an explanation as to why these agents have low quarter SCC averages?

As mentioned previously in the literature review, isolation of *C. bovis* from milk samples collected by surpassing the teat canal is less frequent than when milk samples are drawn in the usual way. Two papers published decades ago (Black et al., 1972; Honkanen-Buzalski & Bramley, 1984) show this, although the technique they resort to, with samples being collected by way of a transdermic needle, is not animal or operator friendly.

The technique used in study III allowed for samples to be taken beyond the teat canal, therefore avoiding contamination with its microbiota, in a much safer and animal welfare

friendly way than the aforementioned studies. A significant difference in the number of *Corynebacterium* spp. isolated was observed between sampling with the standard technique and sampling with the cannula. A difference was also observed for NAS, albeit not significant, so the role of NAS as contaminants of milk samples through teat canal colonisation is not completely clear yet.

Using a teat cannula to collect milk samples has the potential of introducing microorganisms beyond the teat canal and before its use can be recommended, larger scale studies should be performed to demonstrate its safety. Results of study III in this respect were not completely straightforward. Despite the overall decrease in quarter SCC between sampling day and seven days later, for quarters with a SCC below 200,000 cells/ml, there was actually an increase in the mean SCC, suggesting that some quarters without inflammation, showed signs of it following collection with a cannula, even though the observed difference was not significant.

Recommendations for milk sampling include eliminating the first milk squirts to reduce contamination (NMC, 1999). However, the flushing action provided by milking a few times is probably not enough to reduce contamination to an avoidable level. Collecting milk samples after milking would increase the amount of flushing, but as reported by Sears, Smith, English, Herer and Gonzalez (1991) it could also reduce the sensitivity of detection, thus not offering a likely alternative.

Black et al. (1972) suggest that the presence of keratin in the teat canal is related to *C. bovis*' ability to colonise the teat canal. The lipid requirements of *C. bovis* (Watts et al., 2000a) and the lipid composition of keratin (Bitman, Wood, Bright &Miller et al., 1988), would therefore offer a likely explanation for its efficient colonisation of the teat canal.

The impact of sample contamination with *C. bovis* colonising the teat canal is further discussed under the heading "Improving mastitis diagnosis".

Improving detection of true pathogens

It is well established that detection of major pathogens in milk samples through conventional bacteriological techniques is not very sensitive. Sears et al. (1990) experimentally infected 21 quarters of seven cows with a *S. aureus* strain to study its shedding pattern. The mean sensitivity of a single milk sample to detect infection with this pathogen was 74.5%, but was as low as 41% for some quarters.

Sensitivity for detection depends on the minimum number of cfu/ml necessary to consider presence of a microorganism in a sample relevant. Several authors resort to different cfu/ml values to define infection. Østerås et al. (2006) grouped samples positive for growth of *S. aureus* into three classes according to the number of cfu per 0.01 ml of milk: 1 to 3, 4 to 15 or more than 15 cfu. Some international standards consider infection when ≥ 5 cfu/0.01 ml are present (NMC, 1999), whereas some authors only consider an infection when ≥ 50 cfu/0.01

ml are present in a sample (Dingwell et al., 2004), a 10-fold increase from the former. In the current study, the more widely accepted ≥ 5 cfu/0.01 ml was used.

Several techniques have been tentatively used to improve detection of *S. aureus* and other major mastitis pathogens. Freezing milk samples prior to culture, increased inoculation volume, pre-culture incubation, sedimentation, use of Petrifilm (Schukken et al., 1989; Dinsmore, English, Gonzalez & Sears, 1992; Zecconi, Piccinini, Zepponi & Ruffo, 1997; Sol, Sampimon, Hartman & Barkema, 2002; Silva, Caraviello, Rodrigues & Ruegg, 2005; Artursson, Nilsson-Ost & Persson Waller, 2010) all have been used to improve detection rates, with varying results. The effect of freezing on the recovery of major pathogens seems be dependent on the duration of freezing (Schukken et al., 1989). Freezing milk samples has been used because it has been demonstrated that several udder pathogens are capable of invading epithelial or immune cells in the udder. Brouillete, Grondin, Shkreta, Lacasse and Talbot (2003) observed that *S. aureus* internalised both mammary epithelial cells and neutrophils, and Tamilselvam, Almeida, Dunlap and Oliver (2006) observed that *S. uberis* internalised mammary epithelial cells. *Escherichia coli* has also been demonstrated to invade mammary epithelial cells (Döpfer, Nedebragt, Almeida & Gaastra, 2001), and so have NAS (Almeida & Oliver, 2001; Hyvönen, Käyhkö, Taponen, von Wright & Pyörälä, 2009). Freezing milk samples could lead to cell lysis, resulting in release of internalised bacteria and their subsequent detection (Dinsmore et al., 1992). However, internalising epithelial cells and leukocytes is not the only potential reason why detection of major pathogens is troublesome. Another potential mechanism is formation of microabscesses in the mammary gland, such as occurs with *S. aureus* (Sordillo, Nickerson & Akers, 1989b).

In the current study we evaluated if freezing milk samples overnight at -20ºC allowed for an increase in the detection of udder pathogens. We observed that after freezing, milk samples yielded significantly less Gram-positive catalase-positive cocci (group that includes staphylococci), Gram-negative bacilli, Gram-positive bacilli (group that includes *Corynebacterium* spp.) and significantly more samples leading to no growth, than samples before freezing, while the number of samples yielding Gram-positive catalase-negative cocci (group that includes streptococci) was not significantly affected by freezing. Judging from our results, simply freezing milk samples prior to standard bacteriology does not allow for the recovery of extra udder pathogens and should not therefore be recommended. In fact, freezing milk samples before submitting them to a laboratory might be detrimental for the isolation of several groups of pathogens.

Recent advances have allowed the development of a RT-PCR test that is proving to be a useful tool in the detection of udder pathogens truly responsible for mastitis episodes. Despite previous reports on the use of a RT-PCR in the identification of bacteria directly from milk (Phuektes, Mansell & Browning, 2001a; Gillespie & Oliver, 2005), only in the last few years has a kit become commercially available for the simultaneous, accurate, fast and

affordable detection of several mastitis pathogens directly form milk samples (Koskinen et al., 2010). This kit can therefore be used in routine mastitis diagnosis.

The RT-PCR is based on the detection and amplification of bacterial DNA specific to the pathogens that are being searched. This implies that the test can detect even a small number of DNA copies, with no need for viable bacteria to be present in the samples. This way, a high sensitivity for detection of the pathogens considered can be achieved.

In our study, we resorted to this RT-PCR test to tentatively detect major mastitis pathogens in three groups of subclinical mastitis samples (quarter SCC>500,000 cells/ml) that had previously been submitted to conventional bacteriological culturing: a group that was culture-negative, a group that yielded *C. bovis* and a group that yielded NAS. A mastitis pathogen, beyond the result obtained with standard bacteriology, was detected on 47% of the no-growth samples, on 35% of the samples from which *C. bovis* had been isolated and on 25% of the samples from which NAS had been isolated. These results are similar to the ones reported by Taponen, Salmikivi, Simojoki, Koskinen and Pyörälä (2009) for clinical mastitis samples. These authors detected the presence of one or two udder pathogens in 43% of clinical mastitis samples that were culture negative with conventional bacteriology. Therefore there is potential for the use of this RT-PCR in milk samples from clinical mastitis cases or from subclinical cases with a high cell count. This diagnostic test can be performed with milk samples that have been kept frozen or that have a preservative, allowing for retrospective sample analysis. It is a genotypic rather than phenotypic identification, developed specifically for bovine mastitis pathogens, unlike the available biochemical identification systems which are mainly based on human pathogens. Another problem in mastitis diagnosis is that, despite standardization of laboratory techniques there is a high level of operator input in the diagnosis, which can lead to heterogeneous results. A Finnish study comparing 40 laboratories found that correct identification of mastitis pathogens could be as low as 63% for some laboratories (Pitkälä, Gindonis, Wallin & Honkanen-Buzalski, 2005). The RT-PCR discussed here, can be automated and is less dependent on operator input, which contributes to make its results more accurate. This particular RT-PCR also detects a gene that is responsible for resistance to penicillin in staphylococci (*blaZ*), which can positively influence treatment decisions at the same time that the diagnosis is made.

Despite these benefits, there are also some pitfalls to this methodology. It allows for the simultaneous identification of a limited number of pathogens, far from the approximately 140 microbial species, subspecies and serovars that have been isolated from the bovine mammary gland (Radostits, Gay, Hinchcliff & Constable, 2007). This constitutes a relative disadvantage because isolating this plethora of microbial species from cases of bovine mastitis, does not equate to causality for all of them. Nor do conventional laboratory techniques allow for the detection of all these pathogens anyway. This RT-PCR detects the most frequent and well established udder pathogens, including *Mycoplasma bovis*

(introduced after our study was completed), a microorganism that not every laboratory has diagnostic capacity to detect. Detection of *Prototheca* spp. would further complete the panel of microorganisms detected by this diagnostic test.

Because the RT-PCR can detect small numbers of viable as well as non-viable bacteria, it might have lower specificity, leading to a higher number of false positives. Considering the environment in which milk samples are taken, environmental contaminations are highly likely and need to be differentiated from true intramammary infection. In our study, the most frequently detected udder pathogens beyond those that had been found with conventional bacteriology, were *E. coli*, followed by *S. uberis*, *Arcanobacterium pyogenes*/*Peptoniphilus indolicus* and *S. dysgalactiae.* It is highly likely that the *E. coli* that was detected originated at least on some occasions from environmental contamination. Koskinen et al. (2010) addressed this issue in a large trial in which 1,000 quarter milk samples were analysed with RT-PCR and with conventional bacteriology. Of these 1,000 samples, 132 came from clinically healthy quarters with a low cell count. Seventeen per cent of these samples were positive for mastitis bacteria in culturing and 28% gave RT-PCR positive results, indicating that specificity issues exist with both methodologies but are more serious with the RT-PCR. It is possible that there is an advantage in detecting only viable bacteria in cases of bovine mastitis, in which case technology to inactivate free DNA from dead cells is available (Nocher, Cheung & Camper, 2006) and could be used. This of course does not address the issue of the number of DNA sequence copies necessary to establish causality.

The application of the RT-PCR to mastitis diagnosis is a relatively new development. It already constitutes a great tool in udder health investigations, but the aforementioned issues still need to be resolved.

Improving mastitis diagnosis

The distinction between subclinical mastitis and intramammary infection is not always clear. Strictly speaking, mastitis refers to the inflammation of the mammary gland, therefore a measure of inflammatory response needs to be associated with such cases, including SCC or NAGase levels for example (Urech, Puhan & Schällibaum, 1999). An intramammary infection on the other hand, indicates colonisation of one or more parts of the mammary gland with a microorganism. According to the IDF (1999) an IMI is defined as an infection occurring in the secretory tissue and/or the ducts and tubules of the mammary gland, which is diagnosed by microbiological culture of aseptically obtained milk samples.

Different SCC cut-off points have been used to define mastitis presence, including 100,000 (Bansal, Hamann, Grabowskit & Singh, 2005), 200,000 cells/ml (Schukken, Wilson, Welcome, Garrison-Tikofsky & Gonzalez, 2003) and 300,000 cells/ml (Deluyker, Van Oye & Boucher, 2005). The choice of these cut-off values is generally based on the desired sensitivity and specificity for the detection of intramammary infection, with 200,000 cells/ml

representing the best combination between these (Scheppers, Lam, Schukken, Wilmink & Hanekamp, 1997). This rapidly becomes a circular discussion: one is using a SCC cut-off to detect infection, but also using infection to define presence of inflammation. Mastitis presence (inflammation) is defined by the detection of microorganisms (infection) and vice-versa. Green et al. (2006) discuss whether high somatic cell counts lead to lower milk yield or high milk yield leads to lower somatic cell count, and also give examples of other circular issues in the scientific literature regarding dairy cattle.

Presence of mastitis should take into account a measure of inflammatory response. A single cut-off level to define presence of mastitis is probably not enough, since the impact of mastitis can be measured at several levels. The SCC for a quarter without inflammation is probably around 50,000 cells/ml (Merle, Schröder & Hamann, 2007), a value which is mildly influenced by several factors including stage of lactation, parity, season (Harmon, 1994) and presence of infection in other quarters (Merle et al., 2007). This is also the level beyond which milk production begins to be impaired as Djabri et al. (2002) describe in their meta-analysis on the impact of elevated SCC on milk production. Therefore 50,000 cells/ml could be used as one of the cut-off levels to define mastitis. Other candidate cut-off values could be defined for detection of pathogens at quarter-level or for negative economic impact, for example.

This issue becomes further complicated by some of our studies' findings. The standard milk sampling technique may lead to samples being contaminated by teat canal microbiota, namely by *C. bovis* and other species of the genus *Corynebacterium*. Conversely, many of the culture negative samples may actually harbour mastitis pathogens that are not detected with conventional bacteriology techniques as demonstrated by detection of their DNA through RT-PCR. Therefore, isolation of a mastitis pathogen does not necessarily equate to presence of mastitis. If detection of mastitis pathogens is to be used in the determination of a SCC cut-off value that defines mastitis, much improved techniques should be used to sample and detect pathogens. Considering results of large scale surveys across the globe, over 50% of milk samples are culture-negative or result in the isolation of NAS or *C. bovis*. Makovec and Ruegg (2003) reporting on results of 83,650 samples submitted from cases of clinical and subclinical mastitis as well as samples obtained for mastitis surveillance programmes, found 46.6% of samples that were culture-negative or resulted in the isolation of *C. bovis* or NAS. Wilson et al. (1997), reporting on bacteriology results of 105,083 composite milk samples submitted from every animal on most farms of the study, found 66.0% of samples with one of the three aforementioned results and Pitkälä et al. (2004), reporting on 12,661 quarter samples representing the totality of the animals on each farm, found that 90.5% of samples were culture-negative or yielded *C. bovis* or NAS. None of these three results will be very informative, whenever major mastitis pathogens are truly causing the SCC problem at farm level. The other way in which these results can have a negative impact on mastitis

diagnosis is by multiple microorganisms being present in milk samples, rendering it difficult to determine the actual culprit. If normal teat canal microbiota contaminates milk sampled in a standard way, it is impossible to know its origin. Intramammary infections with multiple mastitis microorganisms will occur, but how often this is actually the case and what is each pathogen's responsibility share is very difficult to understand. The presence of multiple microorganisms in samples also has the potential to cause relevant pathogens to be discarded due to sample contamination. In fact, NMC recommendations (1999) state that relevant colonies of similar morphology should be selected for isolation and identification if there is evidence of growth of ≥500 cfu/ml, with samples yielding more than two morphologically different colonies being considered contaminated and thus not relevant. How often then does the presence of a teat contaminant lead to a relevant pathogen being discarded as a mastitis agent?

Therefore, enormous amounts of resources have been spent and will continue to be spent on bacteriology that is inconsequential because of an inefficient sampling technique. For our proposed new sampling technique to be widespread, further larger scale studies need to be performed to evaluate safety issues and recovery rates for true pathogens. As for the use of a RT-PCR on milk samples, several issues are being studied and literature on the subject being produced to address some unanswered questions (Koskinen et al, 2009; Taponen et al., 2009; Koskinen et al., 2010). The widespread use of these techniques has the potential to modify the current paradigm of mastitis diagnosis, potentially decreasing the number of false positives and the number of false negatives, respectively.

Conclusions

The main findings of the current study were:

1. There were differences in mean quarter SCC and duration of intramammary infection for individual species of NAS, albeit none was significant.
2. Despite generally leading to long-lasting subclinical mastitis, the impact of NAS on quarter SCC was low and SCC generally stayed below 200,000 cells/ml.
3. Because of this low impact and minor differences between individual species, NAS species level identification is probably not necessary for diagnostic purposes.
4. Antimicrobial treatment of subclinical mastitis due to NAS during the lactation is also not advisable because it would lead to a net financial loss.
5. Using a teat cannula to collect milk samples reduced the contamination with teat canal microbiota, namely with *Corynebacterium* spp.
6. Freezing milk samples did not lead to increased detection rates of udder pathogens and in fact generally led to a loss in recovery of microorganisms.
7. Use of a RT-PCR allowed for detection of udder pathogens beyond those that were detected with conventional bacteriology in subclinical mastitis samples.

Further studies

The current study led to some interesting findings, which have the potential to be generalised if larger scale studies are performed, and for further questions to be asked.

One such area of study is mastitis diagnosis. As stated before, the routine use of a RT-PCR to diagnose mastitis pathogens directly from milk samples will reduce the number of samples from which no valid information is retrieved when performing conventional bacteriology. Currently there are standards issued by global organisations, such as the National Mastitis Council and the International Dairy Federation, on how to perform and interpret bacteriology results. The same type of standards is probably necessary for interpretation of RT-PCR results. At this stage, this technique is generating information that cannot be soundly interpreted. It is a semi-quantitative technique that generates information about the number of copies of DNA fragments that have been detected in a given sample. Currently we do not know if that can be related to the total number of bacteria in a sample, because there are probably different numbers of copies of the DNA fragments which are detected, on the genome of the different bacterial species considered. We also cannot assume that one bacterial species is more important in terms of the negative effects on a mammary gland simply because it is present in higher numbers. As for the use of a teat cannula to surpass the teat canal avoiding contamination, larger scale studies need to be performed before its use can be generalised.

Another area of interest that could potentially be researched with this collection of samples is the relationship between characteristics of infection, NAS virulence factors and host inflammatory response. As stated before, several *in vitro* studies have focused on NAS virulence factors, including biofilm production, production of particular enzymes or toxins (Zhang & Maddox, 2000; Oliveira et al., 2006). Expression of virulence factors could be tentatively correlated with duration of infection, quarter SCC and relative predominance in a herd. A field that has recently shown some promising results is the use of microarray tools to study gene patterns in *S. aureus* and its prevalence on farms (Piccinini, Borromeo & Zecconi, 2010). This allows for several candidate genes to be studied simultaneously, making comparison between large numbers of strains possible.

Despite the multitude of studies on virulence factors, there are very few studies on the correlation of these with clinical characteristics *in vivo*. Haveri et al. (2005), Fournier et al. (2008) and Graber et al. (2009) found that certain *S. aureus* genotypes were more often associated with certain clinical characteristics such as persistence or contagiousness. However, Haveri et al. (2007) in a follow up study, failed to correlate persistence or other clinical signs with presence of a particular gene or group of genes. Specifically for NAS, Simojoki et al. (2010) observed no correlation between biofilm production and persistence of CNS IMI.

Cow factors would need to be included in such research, through the study of their inflammatory response. Several techniques have shown promise in evaluating host inflammatory response including proteomics. Measurement of haptoglobin and mammary-associated serum amyloid A have been shown to increase with *S. aureus* subclinical mastitis following experimental infection (Eckersall et al., 2006). Another area that might prove fruitful in understanding the host-pathogen interaction is the metabolomic approach, which has showed promising results in other research areas (Vinayavekhin, Homan & Saghatelian, 2010). Such study could potentially clarify the relative importance of host and pathogen factors on clinical characteristics of infection with NAS.

An area of great interest included in this study and in other studies resorting to molecular biology techniques, is the area of molecular epidemiology. This has the potential to clarify most likely routes of transmission and most likely sources of infection, not just for research purposes, but if it becomes widely available and accessible at practitioner level, also in the design of herd specific control programmes. Milk quality advisors confronted with a predominance of pathogens that can behave in an environmental or contagious way (*S. uberis* being the most likely candidate for the position) would welcome an affordable molecular technique that could characterise the most likely form of transmission, hereby allowing for a more targeted approach to take place. Such a study could potentially be performed in several farms known for their environmental or contagious problems, focusing on a certain microorganism or small group of microorganisms, on environmental samples and on diversity index estimation. The study could also include herd level characterisation of SCC dynamics through milk recording analysis. Based on the author's observations and in discussions with other milk quality advisors, when a contagious pattern of infection is leading to most problems in a herd, mean SCC tends to increase with increasing days in milk and with increasing parity, whereas when an environmental pattern of infection predominates, mean SCC tends to be fairly uniform between days of lactation and parities. Even though this has not been scientifically demonstrated, it seems possible if we consider exposure to milking or to the environment as risk factors for mastitis.

These are only a few possibilities in terms of future research around this subject. Irrespective of the specific research performed, it is important that besides scientific communication, there is also effective communication of results to farm level and good communication of questions to laboratory level.

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