Eesti Maaülikool
Estonian University of Life Sciences

CLINICAL MASTITIS IN ESTONIA: DIAGNOSIS, TREATMENT EFFICACY AND ANTIMICROBIAL RESISTANCE OF PATHOGENS IN ESTONIA

KLIINILISTE MASTIITIDE DIAGNOOSIMINE,
RAVI TULEMUSLIKKUS JA PATOGEENIDE ANTIMIKROOBNE
RESISTENTSUS EESTIS

PIRET KALMUS

A thesis
for applying for the degree of Doctor of Philosophy in Veterinary Medicine and Food Sciences (clinical veterinary medicine)

Väitekiri
filosoofiadoktori kraadi taotlemiseks veterinaarmeditsiini ja toiduteaduse alal (kliiniline veterinaarmeditsiin)

Tartu 2013

EESTI MAAÜLIKOOL
ESTONIAN UNIVERSITY OF LIFE SCIENCES

Eesti Maaülikool
Estonian University of Life Sciences

CLINICAL MASTITIS IN ESTONIA: DIAGNOSIS, TREATMENT EFFICACY AND ANTIMICROBIAL RESISTANCE OF PATHOGENS IN ESTONIA

KLIINILISTE MASTIITIDE DIAGNOOSIMINE,
RAVI TULEMUSLIKKUS JA PATOGEENIDE
ANTIMIKROOBNE RESISTENTSUS EESTIS

PIRET KALMUS

A thesis
for applying for the degree of Doctor of Philosophy in Veterinary Medicine and Food Sciences (clinical veterinary medicine)

Väitekiri
filosoofiadoktori kraadi taotlemiseks veterinaarmeditsiini ja toiduteaduse alal
(kliiniline veterinaarmeditsiin)

Tartu 2013

Institute of Veterinary Medicine and Animal Sciences,
Estonian University of Life Sciences

According to verdict N° 1 of October 21, 2013 the Doctoral Commitee of Veterinary and Food Science of Estonian University of Life Sciences has accepted the thesis for the defence of the degree of Doctor Philosophy in Veterinary Medicine and Food Sciences.

Opponent: **Prof. Karin Persson Waller**
Department of Animal Health and Antimicrobial Strategies, National Veterinary Insitute, Sweden
Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences

Supervisors: **Prof. Toomas Orro**
Insitute of Veterinary Medicine and Animal Sciences
Estonian University of Life Sciences

Assoc. prof. Kalle Kask
Insitute of Veterinary Medicine and Animal Sciences
Estonian University of Life Sciences

Defence of the thesis:
Estonian University of Life Sciences, Tartu, Kreutzwaldi 62, room A-201, on January 23, 2014 at 10.00

The publication of this dissertation is granted by the Graduate School in Biomedicine and Biotechnology and by the Estonian University of Life Sciences

ISBN 978-9949-536-13-9 (trükis)
ISBN 978-9949-536-14-6 (pdf)

To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on four original publications (I-IV). The articles are referred to in the text using Roman numerical. The paper II is reproduced with kind permission from The Journal of Dairy Science (License Number: 3212481301190).

I Kalmus, P., Viltrop, A., Aasmäe, B., Kask, K., 2006. Occurrence of clinical mastitis in primiparous Estonian dairy cows in different housing conditions. Acta Veterinaria Scandinavica, 48, 21.

II Kalmus, P., Simojoki, H., Pyörälä, S., Taponen, S., Holopainen, J., Orro, T., 2013. Milk haptoglobin, milk amyloid A and NAGase activity in bovine naturally occurring clinical mastitis diagnosed with a quantitative PCR test. Journal of Dairy Science, 96, 3662 - 3670.

III Kalmus, P., Aasmäe, B., Kärssin, A., Orro, T., Kask, K., 2011. Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. Acta Veterinaria Scandinavica, 53, 4.

IV Kalmus, P., Simojoki, H., Orro, T., Taponen, S., Mustonen, K., Holopainen, J., Pyörälä, S., 2013. Efficacy of 5-day intramammary vs. systemic benzylpenicillin treatment of clinical mastitis caused by Gram-positive bacteria susceptible to penicillin *in vitro*. Journal of Dairy Science, accepted 18 December 2013.

The contribution of author's to the research papers

Paper	Original idea, study design	Data collection, sample analysis	Data analysis	Manuscript writing
I	**PK,** KK, AV	**PK**, BA, AV	**PK**, AV, KK	All
II	**PK**, SP, ST, HS, TO	**PK**, HS, ST, JP, TO, SP	**PK**, HS, TO, ST	All
III	**PK**, TO, KK	**PK**, BA, AK	**PK**, TO, KK, BA	All
IV	**PK**, SP, ST, HS, TO	**PK**, HS, ST, KM, JP, TO, SP	**PK**, HS, TO, ST	All

PK - Piret Kalmus, AV - Arvo Viltrop, AK - Age Kärssin, BA - Birgit Aasmäe, HS - Heli Simojoki, JP - Jani Holopainen, KK - Kalle Kask, KM - Katja Mustonen, SP - Satu Pyörälä, SV - Suvi Taponen, TO - Toomas Orro, All - all authors of the paper

ABBREVIATIONS

APP	Acute phase proteins
APR	Acute phase response
CI	Confidence interval
CMSCC	Composite milk somatic cell count
CNS	Coagulase-negative staphylococci
CV	Coefficients of variation
EARC	Estonian Animal Recording Centre
FAO	Food and Agricultural Organization
FVE	European Veterinary Federation
Hp	Haptoglobin
IDF	International Dairy Federation
IMI	Intramammary infection
IM	Intramuscular
IMM	Intramammary
MAA	Milk amyloid A
MIC	Minimum inhibitory concentration
NAGase	Milk N-acetyl-β-D-glucosaminidase
OIE	World Organization for Animal Health
OR	Odds ratio
PCR	Polymerase chain reaction
SAA	Serum amyloid A
SCC	Somatic cell count
WHO	World Health Organization

1. INTRODUCTION

Mastitis, an inflammation of the mammary gland, is the most common disease affecting dairy cattle worldwide. Despite long-term research and the implementation of preventive measures aimed at reducing the incidence of clinical mastitis, its occurrence ranges between zero and 200 cases per 100 cows per year (Schukken *et al.*, 2001). The economic losses to the dairy industry due to clinical and subclinical mastitis can reach millions of euros per year (Halasa *et al.,* 2007).

Mastitis is a multi-factorial disease, which is related to the dairy cow's immune status, mastitis-causing infectious agents and surrounding environment along with milking practices as the factors contributing to mammary gland inflammation. In excess of 150 different pathogens have been associated with bovine mastitis. Bacteria entering via the teat canal cause intramammary infection (IMI) and the host responses with the developing inflammation. Inflammatory reaction can be mild, causing only an increase in the inflammatory indicators of the milk, defined as subclinical mastitis. Clinical mastitis characterized by visible abnormalities in the milk, in the udder, or in both, can be classified as mild, moderate or severe (International Dairy Federation (IDF), 1999), whereas the severity of clinical mastitis is based on the inflammatory response. The exact reasons why in one cow subclinical and in another cow clinical mastitis with different severity will develop are not well understood. However, it is likely to be influenced by the pathogen involved as well as the immune status of the cow (Sordillo, 2005). As mastitis is a dynamic process, subclinical mastitis can develop into the clinical form, and then return to the subclinical.

To identify the mastitis causing agents, microbiological diagnostic methods have been available and routinely used all over the world. Molecular diagnostic methods have advanced rapidly over the last decade, and the DNA-based mastitis diagnostic system has been introduced for the routine use (Koskinen *et al.,* 2009). Due to the high sensitivity of polymerase chain reaction (PCR)-based methods, often more than one udder pathogen has been detected from the milk samples and the interpretation of laboratory results could be challenging (Koskinen *et al.,* 2010).

Clinical mastitis is a cow welfare issue and promt treatment of clinical mastitis is of great importance. The goal of mastitis therapy is rapid elimination of the infectious agent and prevention of extensive tissue damage. Approximately 70% of the antimicrobials used in dairy production are for treatment of clinical mastitis (Thomson *et al.,* 2008), but the cure rates for clinical mastitis are not always satisfactory. Moreover, several antimicrobials and active substances have been used for the treatment of clinical mastitis for more than fifty years, but consensus on the most efficient, safe and economical treatment is still lacking (Pyörälä, 2011). Widespread use of antibiotics may promote the development of antimicrobial resistance of all bacteria that are in contact with antimicrobial agents (Prescott, 2007). International agencies including the Food and Agricultural Organization (FAO), World Health Organization (WHO), and World Organization for Animal Health (OIE) have stressed the need for finding alternative approaches to address the issue of antimicrobial use in food animal production and its effect on the emergence of antimicrobial resistance in human pathogens (FAO/ OIE, 2007) and the guidelines for prudent use of antibiotics have been described by the European Veterinary Federation (FVE).

In recent decades only broad-spectrum antibiotics have been used for treatment of clinical mastitis in Estonia. For example, during the years 2006-2009 fifteen different combinations of antibiotics were available for use in 18 intramammary preparations that were authorized by the Estonian State Agency of Medicines, while according to the Finnish Medicines Agency only five products for lactating cows were available in 2011. There are around 96,000 dairy cows in Estonia, whereas more than half a million intramammary syringes for mastitis treatment are sold every year. Data on the distribution of mastitis pathogens and antimicrobial resistance are still lacking in Estonia.

The average herd size is increasing in Estonia, and three-fourths of the dairy cows are kept in herds with more than hundred cows (Estonian Animal Recording Centre, 2012). Tie-stall housing system had previously been prevalent, but in recent years extensive transition to loose-housing system has changed the calving conditions of cows and heifers. Although clinical mastitis has frequently been observed at parturition, no data are available on udder health in freshly calved heifers and multiparous cows in Estonia.

This dissertation focuses on different aspects of clinical mastitis. In Study I, clinical mastitis pathogens and some risk factors in primiparous and multiparous dairy cows were evaluated. In Study II, the associations between the quantified bacterial DNA and the local inflammatory response were studied. The distribution of udder pathogens and their antimicrobial resistance was evaluated in Study III, and treatment efficacy of penicillin-susceptible Gram-positive bacteria was evaluated in Study IV.

2. REVIEW OF THE LITERATURE

2.1. Definition of mastitis

Mastitis in dairy cattle is an inflammation of the mammary tissue of the udder. The inflammation of the udder is mainly caused by intramammary infection (IMI), where different bacterial species enter the udder quarter via the teat canal. A case of mastitis can be classified as either subclinical or clinical mastitis. Subclinical mastitis is characterized by changes in milk composition and inflammatory parameters in the milk, e.g. somatic cell count. In addition, milk N-acetyl-*β*-D-glucosaminidase (NAGase) activity, lactate dehydrogenase content and electrical conductivity have been found as promising parameters for monitoring subclinical mastitis (Pyörälä, 2003). A threshold value of 100000 cells/ml has been suggested to determine whether a quarter is healthy or not (Hillerton, 1999; Krömker *et al.,* 2001). Clinical mastitis is characterized by visual changes in the milk, udder, or even a cow. Clinical mastitis can be mild, moderate, or severe. Cows with mild clinical mastitis typically have abnormalities in the milk such as clots and flakes with little or no swelling of the gland or systemic illness. Cows with severe clinical mastitis typically have a sudden onset of udder inflammation, abnormal milk, and systemic signs such as fever, increased heart rate, dehydration, weakness and depression (IDF, 1999).

2.2. Clinical mastitis in primiparous and multiparous dairy cows

The incidence of clinical mastitis in dairy cows has been investigated in numerous studies all over the world. The occurrence of clinical mastitis on dairy farms in England, Czech Republic, and Ireland was 47, 53, and 54 cases per 100 cows per year, respectively (Wolfova *et al.,* 2006; Bradley *et al.,* 2007; More *et al.,* 2012). The mean clinical mastitis incidence in Canada was 23 cases per 100 cows per year (Riekerink *et al.,* 2008). Although replacement heifers are generally expected to have good udder health, the IMI has been detected at least as early as breeding age (8-19 months), and the prevalence of IMI of heifers was the highest during the periparturient period (Pankey *et al.,* 1991; Matthews *et al.,* 1992; Fox *et al.,* 1995, Nickerson *et al.,* 1995). A higher risk of developing clinical mastitis occurs also during early lactation, where three-fourths of the

cases of clinical mastitis in primiparous dairy cows occur within the first 30 days postpartum, most commonly chronologically closer to parturition (Jonsson *et al.,* 1991; Barnoiun and Chassagne, 2001; Persson Waller *et al*., 2009). A nested case-control study in Norway showed that 5% (6,410 out of 128,027) of cases of clinical mastitis were treated in first-calving heifers (Waage *et al*., 1999). In Finland, the frequency of treatments for heifer mastitis from one week before to one week after calving was 3.9% for Ayrshires and 5.6% for Friesians (Myllys and Rautala, 1995). One year prevalence of clinical mastitis was 9.1% among the heifers from ten German dairy herds (Tenhagen *et al.,* 2009), and 8.1% in a Dutch study (van den Borne *et al.,* 2007). In a study performed in the Netherlands, the rate of clinical mastitis around parturition was found to be higher in heifers (>30%) compared to older cows (13%) (Barkema *et al.,* 1998).

The distribution of clinical mastitis pathogens varies between regions, countries, and even between farms. For example, 60.2 % of the cases of clinical mastitis in Israel have been caused by *Escherichia coli* (*E. coli)*, whereas in Norway *Staphylococcus aureus* (*S. aureus)* is the most common clinical mastitis pathogen (Shpigel *et al.,* 1998; Osterås *et al.,* 2006). Observational studies from the United States, Canada, and the United Kingdom have reported coliforms, *Streptococcus uberis* (*Str. uberis*) and *S. aureus* to be the most frequently isolated bacteria from clinical mastitis (Wilson *et al*., 1997; Reksen *et al.,* 2006; Riekerink *et al.,* 2008). Coagulase-negative staphylococci (CNS) are showing increase in Finland (Pitkälä *et al.,* 2004). The most common CNS in bovine mastitis are *S. chromogenes*, *S. simulans*, *S. xylosus* and *S. epidermidis*. *S. hyicus* was described as the most pathogenic CNS (Myllys, 1995), and more common finding among the clinical mastitis, while *S. epidermidis* was the major pathogen isolated from subclinical mastitis cases (Persson Waller *et al.,* 2011). S*treptococcus agalactiae (Str. agalactiae)* has been largely eradicated from herds in Europe, while in the studies from the United States, 7.7% and 13.1% of samples were reported to contain *Str. agalactiae* (Wilson *et al.,* 1997; Makovec and Ruegg, 2003). The main subclinical mastitis pathogens in Germany, the Netherlands and France are CNS, *Corynebacterium bovis* (*C. bovis*), and *S. aureus* (Tenhagen *et al.,* 2006; Piepers *et al.,* 2007; Botrel *et al.,* 2009).

Despite the fact that primiparous cows may benefit from separate grouping and management conditions prior to calving, the causative

pathogens of clinical mastitis are the same that cause clinical mastitis in multiparous dairy cows (Oliver and Mitchell, 1983; Jonsson *et al.,* 1991, Tenhagen *et al.,* 2009; Persson Waller *et al.,* 2009). Among the primiparous dairy cows CNS, *S. aureus*, *Str. dysgalactiae,* and coliforms have been found the major pathogens causing clinical mastitis (Trinidad *et al.,* 1990; Myllys, 1995; Waage *et al.,* 1999). From CNS, *S. chromogenes* predominates in heifers around calving, whereas *S. simulans* predominates in the subsequent lactations (Taponen *et al.,* 2006; Thorberg *et al.,* 2009).

2.3. Risk factors for clinical mastitis in primiparous dairy cows

The transition phase, typically defined as the period from three weeks before to three weeks after parturition, is viewed as a critical time in the lactation cycle of a dairy cow. During this period, the cow experiences a series of nutritional, physiological and social changes which render the cow more susceptible to infectious and metabolic diseases (Goff and Horts, 1997). In general, management factors at the herd level, including housing, feeding and milking systems as well as pathogens surrounding the cows, increase the incidence of clinical mastitis in both primiparous and multiparous cows (Schukken *et al.,* 1990; Valde *et al.,* 1997; Barkema *et al.,* 1999; Aland, 2003). The presence of udder odema at calving, blood in the milk, and milk leakage were associated with increased risk of postpartum clinical mastitis of primiparous dairy cows (Waage, 2001). A recent study by German researchers showed that loose-housing systems during pregnancy (as opposed to tie-stalls), juvenile intersucking, clinical mastitis during the first week after calving, teat diameters <18 mm, and employing organic bedding material in the stables before calving were associated with subclinical mastitis measured at 41days in milk (DIM) (Krömker *et al.,* 2012). An open teat canal before parturition has been found to be an important risk factor in the etiology of heifer mastitis and the incidence of clinical mastitis during the first lactation has been influenced by the duration of IMI prior to parturition (Krömker and Friedrich, 2009). Despite the region, Holstein dairy heifers seem to be more susceptible to clinical mastitis than other breeds (Parker *et al.,* 2007; Nyman *et al.,* 2009; Persson Waller *et al.,* 2009).

Poor hygiene in farm facilities during the dry and the calving period is one of the risk factors for mastitis, especially for pathogens like *E. coli*, *Str. uberis,* and *T. pyogenes* originating from the environment. In addition,

there are several possible sources of *S. aureus* that may cause clinical mastitis before and after parturition of heifers. The risk of *S. aureus* IMI depends on the length of time the heifers are housed together with older cows and the proportion of *S. aureus* infected cows in the herd (Bassel *et al.*, 2003). In addition, early spread of *S. aureus* from older cows to first lactating cows may happen during the first milking (Tenhagen *et al.,* 2009), when older cows with infected udder are kept together with freshly calved heifers. Colonization of *S. aureus* in the teat skin or in the inguinal area, accompanied by transmission with flies has been found to be another source of *S. aureus* infection (Sears and McCarthy, 2003).

2.4. Mastitis diagnostics

2.4.1. Microbiological diagnosis of mastitis

Early diagnosis of mastitis is of the utmost importance due to the high costs of disease. Diagnostic methods have been developed to check the quality of the milk through detection of mammary gland inflammation and diagnosis of the infection and its causative pathogens. Although more than 150 different bacterial species have been isolated from the milk of inflamed udder, mastitis is usually caused by one primary pathogen (Watts and Yancey, 1994), and approximately 10 bacterial species or species groups account for more than 95% of all clinical and subclinical infections (Makovec and Ruegg, 2003; Tenhagen *et al.,* 2006; Bradley *et al.,* 2007).

For a long time, the golden standard for identification of bacterial species in case of clinical and subclinical mastitis was conventional microbiology. Over the last decade, polymerase PCR-based methods have been introduced for detection of single mastitis pathogens (Phuektes *et al.,* 2001; Gillespie and Oliver, 2005; Graber *et al.,* 2007). Recently, very high analytical accuracy was reported for a real-time PCR-based reagent kit capable of detecting 11 important IMI species/species groups and the beta-lactamase gene (PathoProof ™ Mastitis PCR Assay, Thermo Fisher Scientific, Espoo, Finland) based on a large collection of culture isolates (Koskinen *et al.,* 2009).

Molecular methods have several advantages compared with conventional methods. PCR-based methods are more sensitive than conventional

bacteriology, and more species are detected in the sample (Koskinen *et al.,* 2010). Conventional bacterial culturing is relatively slow to perform, as incubation of primary cultures often requires 48 h (or up to 72 h) to complete, and additional confirmation tests are also time-consuming (National Mastitis Council, 2004). PCR-based methods provide good accuracy and speed for analysing milk samples (Koskinen *et al.,* 2009). According to the literature, no bacterial growth is detected in at least 20 to 30% of milk samples taken from udder quarters with clinical mastitis (Hogan *et al.,* 1989; Nevala *et al.,* 2004; Bradley *et al.,* 2007). PCR method provides a bacteriological diagnosis for almost half of the cases where conventional culture results are negative (Taponen *et al.*, 2009).

In Estonia, PCR-based mastitis diagnostics was implemented in 2011. In Finland already over 80 % of the milk samples are investigated using a commercial PCR test (PathoProof™, Thermo Fisher Scientific) and the same trend can be seen elsewhere.

2.4.2. Inflammatory reaction during clinical mastitis and an acute phase response

The incidence of mastitis increases when the defense mechanisms of the mammary gland are impaired. The mammary gland is protected by innate and specific immunity. The innate immunity is not pathogen-specific and inflammatory responses are either present or are activated quickly during early stages of infection (Rainard and Riollet, 2006). Those primary defence mechanisms of the mammary gland are mediated by the physical barrier of the teat end, macrophages, neutrophils, natural killer cells, and by certain soluble factors (Sordillo, 2005). The specific or acquired immune system recognizes specific determinants of a pathogen that activate selective elimination. Recognition of pathogenic factors is mediated by antibody molecules, macrophages, and several lymphoid populations.

The inflammatory reaction is a series of complex physiological events occurring in the host after tissue injury or infection. The severity of clinical mastitis depends on the number of bacteria entering via the teat canal, access of the microbe to the target tissue, virulence of the strain and immunity of the host (Burton and Erskine, 2003).

Tissue injury due to inflammation causes acute phase response (APR), which most commonly begins by the release of inflammatory mediators from tissue macrophages or blood monocytes that gather at the site of damage (Baumann and Gauldie, 1994; Koj, 1996). Also, the mammary epithelium has been suggested to be a site of synthesis of cytokines and other inflammatory mediators (Persson Waller *et al.,* 1997). A prominent event in the APR is the increase production of acute-phase proteins (APP) in liver. APP are heterogeneous group of proteins with different functions. In general, APP prevents spread of infectious agents, induce anti-inflammatory cytokines, promote healing by supplying nutrients to destroyed tissue, and restore homeostasis (Murata *et al.,* 2004). Two of these proteins, haptoglobin (Hp) and serum amyloid A (SAA), contribute to host defense through antibacterial activity and play a significant role in the early response to invasion of mammary tissues by pathogenic bacteria. Haptoglobin is diffused from blood into the milk, but it also originates from milk leukocytes and the mammary gland epithelium (Hiss *et al.,* 2004). Serum amyloid A is secreted by hepatocytes and, in addition, the mammary gland epithelium appears to secrete a mammary gland-specific isoform mammary-associated serum amyloid A 3 (MSAA3) milk amyloid A (MAA)(Eckersall *et al.,* 2001). MAA and Hp synthesized in udder have been suggested to be sensitive and potentially suitable markers for detection of mastitis and evaluation of udder inflammation severity during clinical mastitis (Eckersall *et al.,* 2001, Eckersall *et al.,* 2006; Pyörälä *et al.,* 2011).

NAGase is an intracellular, lysosomal enzyme that is released into milk from neutrophils during phagocytosis and cell lysis, but also from damaged epithelial cells, indicating udder tissue destruction (Kitchen *et al.,* 1984). Milk NAGase activity correlates very closely with SCC and can be analysed also from frozen milk samples (Kitchen *et al.,* 1984).

Local APR in the udder has been studied using experimental models, where *E. coli* (Hyvönen *et al.,* 2006; Suojala *et al.,* 2008; Larsen *et al.,* 2010) or staphylococci (Grönlund *et al.,* 2003; Simojoki *et al.,* 2009) have been inoculated into the udder quarter. Results from these studies indicated that coliform bacteria caused a larger increase in concentrations of APP in milk, compared to CNS and *S. aureus*. Studies on the innate immune response in naturally occurring clinical mastitis are less common. Wenz *et al.* (2010) found that the concentration of Hp in milk was the highest in

E. coli mastitis as compared with mastitis caused by either environmental streptococci or CNS. A larger study conducted by Pyörälä *et al.* (2011) concluded that the concentrations of APP and NAGase activity in milk varied according to isolated mastitis pathogens, where concentrations of APP were the highest in case of *E. coli* mastitis compared to other mastitis pathogens, streptococci and *S.aureus*. Inflammatory responses of MAA and Hp were very mild in CNS mastitis (Pyörälä *et al.,* 2011).

2.5. Treatment of clinical mastitis

Knowledge of clinical mastitis severity, culture-based therapy and treatment efficacy are essential in effectively and efficiently managing mastitis cases (Roberson, 2012). The primary objective of antibacterial treatment of clinical mastitis is rapid elimination of the infectious agent to prevent serious tissue damage and maintain further milk production (Constable *et al.,* 2008). In attempts to improve the response to treatment, various classes of antimicrobial compounds, drug combinations, application routes, and treatment durations have been investigated (Hillerton and Kliem, 2002; Serieys *et al.,* 2005; Bradley and Green, 2009). Therefore it is not always possible to differentiate between the effect of the active compound and the effect of the commercial product and its route or dose of administration (Barkema *et al.,* 2006).

In antimicrobial treatment of animal infections such as mastitis, targeting the treatment towards the causing agents is recommended in global prudent use guidelines (OIE, 2012). Moreover, the risk for emergence of resistance among bacteria is higher if blanket therapy is used. A 2008 study of 165 clinical mastitis cases found that the treatment of nearly 50% of the cases was unnecessary (no bacterial growth) or inappropriate (*in vitro* resistant isolates) (Roberson, 2008). If the causative agent of infection is susceptible to the so-called first-line antimicrobials, such as agents with a relatively narrow spectrum, including benzylpenicillin, they should be used for treatment (Constable *et al.,* 2008). However, in the majority of countries, the treatment of mastitis remains reliant on the routine use of combinations of several active substance or broad-spectrum antimicrobials (Ruegg, 2010). Selection pressure for the development of antimicrobial resistance among bacteria is greater when broad-spectrum agents are used (Hunter *et al.,* 2010).

2.5.1. Antibiotics used in treatment of clinical mastitis

According to Ziv (1980), the ideal antibacterial for the treatment of clinical mastitis would have a low minimum inhibitory concentration (MIC) against the udder pathogens and a high bioavailability from the intramuscular injection sites. The antibacterial agent would be weakly basic or non-ionized in serum, and sufficiently lipid-soluble. Also, a low degree of protein binding and long half-life to maintain activity in inflammatory secretions, are necessary characteristics of an effective antibiotic. For example, intramuscularly injected penicillin G, which is a weak acid, penetrates poorly into the mammary gland, but due to very low MIC values of susceptible organisms, therapeutic concentration can be achieved in milk. Pharmacokinetics and pharmacodynamics of different antibiotics greatly affect their suitability for mastitis treatment. The antimicrobial should preferably have bactericidal action, as phagocytosis is impaired in the mammary gland (Constable and Morin, 2003). Activity of broad-spectrum antibiotics like oxytetracycline and trimethoprim-sulfonamides has been shown to be reduced in milk (Louhi, 1992). Almost all active substances for mastitis treatment work as time-dependent antimicrobials. The efficacy is maximized by keeping the concentration of drug at the site of infection above the level necessary to inhibit microbial growth as long as possible between two administered doses of the drug. The concentration-dependent antibiotics, fluoroquinolones, have been used for the treatment of *E. coli* mastitis as the high concentration of the antibiotic increases the rate of killing rapidly proliferating bacteria (Prescott, 2007).

Broad-spectrum antibiotics like fluoroquinolones, ceftiofur, cefquinome and oxytetracycline have been used or recommended for the treatment of *E. coli* mastitis. At the same time, antibiotic treatment of *E. coli* mastitis is still controversial. Cows with severe clinical mastitis and in the stage of bacteremia would be suggested to be treated with antimicrobials for 3-5 days (Roberson, 2012). Some studies using enrofloxacin or cephalosporins have shown faster elimination of bacteria and increased survival (Erskine *et al.,* 2002b; Rantala *et al*., 2002; Poutrel *et al.,* 2008), but other studies did not support this hypothesis (Wenz *et al.,* 2005; Suojala *et al.,* 2010;). Cure rates for mastitis caused by penicillin-resistant *S. aureus* isolates seem to be inferior to those of mastitis due to penicillin-susceptible isolates (Sol *et al.,* 2000; Taponen *et al.,* 2003a).

Different antibacterial agents have been used in clinical trials for the evaluation of treatment efficacy. The bacteriological cure rates of clinical mastitis caused by Gram-positive bacteria have ranged between 15.4 and 91.6% (Table 1).

Table 1. Bacteriological cure rates of clinical mastitis caused by Gram-positive udder pathogens.

Pathogens (No. of treated quart.)	Treatment regimen[3]	Antimicrobials used	Bact. cure, %	Reference
S. aureus penS (n = 10)	IM every 24 h 5 days	Penicillin G	30	Taponen *et al.,* 2003a
S. aureus penS (n = 86)	IM+IMM every 24 h 5 days	Penicillin G; penicillin + neomycin	79.1	Taponen *et al.,* 2003b
S. aureus (n = 17)	IM every 24 h 3 days	Penethamate	24	Serieys *et al.,* 2005
S. aureus (n = 18)	IM every 24 h 3 days	Penethamate	33.3	McDougall *et al.,* 2007b
S. aureus penR[2] (n = 15)	IM every 24 h 5 days	Spiramycin	33.3	Taponen *et al.,* 2003b
S. aureus penR (n = 24)	IM+IMM every 24 h 5 days	Amoxicillin-clavulan acid	33.3	Taponen *et al.,* 2003b
S. aureus (n = 25)	IMM every 24 h 3 days	Amoxicillin + cloxacillin	24	Serieys *et al.,* 2005
S. aureus (n = 118)	IM every 24 h 3-5 days	PenicillinG	33.9	Pyörälä and Pyörälä, 1998
S. aureus (n = 38)	IMM every 24 h 2 days	Cephalexin + kanamycin	36.8	Bradley and Green, 2009
S. aureus (n = 9)	NA	Cephapirin sodium	78	Apparao *et al.,* 2009
S. aureus (n = 15)	IMM every 12 h 3 days	Cefquinome	15.4	Bradley and Green, 2009
S. aureus (n = 22)	IM every 24 h 3 days	Tylosin	31.8	McDougall *et al.,* 2007b
Streptococci (n = 36)	IM every 24 h 5 days	Penicillin G	83.3	Taponen *et al*., 2003a
Streptococci (n = 270)	IM every 24 h 3 days	Penethamate	88.5	McDougall *et al.,* 2007b
Streptococci (n = 109)	IM every 24 h 3-5 days	Penicillin G	65.1	Pyörälä and Pyörälä,1998
Streptococci (n = 37)	IMM every 24 h 4 days	Penicillin + neomycin	81.1	Taponen *et al.,* 2003a
Streptococci (n = 28)	IMM every 24 h 3 days	Amoxicillin + cloxacillin	71.4	Serieys *et al.,* 2005
Streptococci (n = 100)	IMM every 24 h 3 days	Cephalexin + kanamycin	66	Bradley and Green, 2009
Streptococci (n = 19)	NA	Cephapirin sodium	74	Apparao *et al.,* 2009
Streptococci (n = 250)	IM every 24 h 3 days	Tylosin	91.6	McDougall *et al.,* 2007b
Streptococci (n=60)	IMM every 12 h 3 days	Cefquinome	73.3	Bradley and Green, 2009
Str. uberis[4] (n = 37)	IM every 24 h 5 days	Ceftiofur	88	Oliver *et al.,* 2004
Str. uberis (n = 55)	IMM every 12 h 5 days	Lincomycin + neomycin	95	Krömker *et al.,* 2010

*Str. uberis**exp (n = 40)	IM every 24 h 8 days	Pirlimycin	80	Oliver *et al.,* 2003
Streptococci and staphylococci (n = 404)	IMM every 12 h 3 days	Lincomycin + neomycin vs. penicillin G + streptomycin	76.7 76.7	McDougall, 2003
Mixed (n = 43)	IMM every 12 h 3 days	Sulfamycin	69.2	Vasil, 1994

[1] Penicillin susceptible bacteria

[2] Penicillin resistant bacteria

[3] IM - intramuscular treatment; IMM - intramammary treatment

[4]- experimental study

2.5.2. Treatment route

The pharmacological goal of antimicrobial therapy is to attain effective concentration of the drug at the site of infection. The most common route of administration of antimicrobials in mastitis is the intramammary route (Gruet *et al.,* 2001). Systemic (parenteral) treatment has been suggested to be more efficient than intramammary (IMM) treatment, due to better distribution of the drug throughout the mammary gland (Ziv, 1980; Franklin *et al.,* 1984). The parenteral antibacterial treatment of mastitis is widely used in the Nordic countries (Grave *et al.,* 1999; Thomson *et al.,* 2008).

There are three potential therapeutic targets or pharmacological compartments for mastitis: the milk and the mammary gland epithelial layer, the mammary gland parenchyma, and the cow. The important question is whether the antibiotic should accumulate in the milk or in the udder tissue. Streptococci, CNS and corynebacteria are known to remain in the milk compartment, therefore IMM treatment could be a preferable choice (Erskine *et al.,* 2003). The advantage of the IMM route would be high concentrations of the substance achieved in the milk and low consumption of the antimicrobial, as the drug is directly infused into the quarter (Moretain *et al.,* 1989). The disadvantage is distribution of the antimicrobial throughout the udder and the risk of infecting the quarter when infusing the product via the teat canal (Ehinger and Kietzmann, 2000). Parenteral treatment strategies, either used alone or in combination with IMM treatment, have been suggested in case of *S. aureus* mastitis (Erskine *et al.,* 2003).

2.6. Antimicrobial resistance of udder pathogens causing clinical mastitis

Resistance of bacteria to antibacterial drugs was first reported soon after antibacterial drugs were accepted for use in both human and veterinary medicine. The two main factors involved in the development of antibiotic resistance in bacteria are the selective pressure by the use of antibiotics and the presence of resistance genes (Witte, 2000). Resistance that is acquired by horizontal transfer of resistance genes can become rapidly and widely disseminated either by clonal spread of the resistant strain itself or by further genetic exchanges between the resistant strain and other susceptible strains (Chambers, 2001).

National studies of mastitis prevalence provide important information through the monitoring of national udder health status. For instance, national guidelines for the prudent use of antibiotics have been available in Finland (EVIRA, 2009). In recent decades, only broad-spectrum antibiotics have been used for the treatment of clinical mastitis in Estonia. According to the Estonian State Medical Agency, 15 different combinations of antibiotics are available for use in 18 intramammary preparations that have been authorized.
Approximately 80% of all antimicrobials used in dairy production are used to treat clinical or subclinical mastitis (Pol and Ruegg, 2007). Myllys *et al.* (1998) have reported upward trend over time in resistance among mastitis pathogens, whereas others do not agree with that statement (Erskine *et al.,* 2002a). A large-scale study was carried out by Makovec and Ruegg (2003) to investigate the trends of development of antimicrobial resistance over a 7-year period. In all Gram-positive pathogens, the percentage of isolates resistant to beta-lactam antimicrobials decreased significantly over time, whereas the percentage of *Staphylococcus* spp. isolates and resistance to tetracycline and macrolides increased significantly.

For *in vitro* antimicrobial susceptibility testing several different methods are available, among which agar diffusion and broth microdilution are most frequently used. In both methods bacteria are classified as resistant, intermediate or susceptible to target antibiotics. Although there is a certain correlation between the diameter of zone of growth of inhibition and susceptibility of the bacteria, it is not appropriate to convert diameter values into the MICs (Schwarz *et al.,* 2009).

The main feature of staphylococci is production of β-lactamase, which causes hydrolysis and destruction of the β-lactam ring of penicillin rendering it ineffective. The number β-lactamase positive *S. aureus* strains have been varied a lot in different studies carried out in many countries. Studies from France, Finland, Argetina and the UK reported high prevalence of penicillin-resistant *S. aureus* (36.2%, 52.1%, 40,3% and 56%, respectively) (Guerin-Faublee *et al.,* 2003; Pitkälä *et al.,* 2004; Gentilini *et al.*, 2000; Bradley *et al.,* 2007), whereas low numbers of resistant strains (4 to 9%) were found in Norway and in Canada (NORM/NORMVET 2003; Saini *et al.,* 2012). De Oliveira *et al.* (2000) compared the data from 11 countries, where β -lactamase resistance varied from 4% in Norway to 76% in Ireland. Compared with *S. aureus*, CNS are more often resistant to several antibiotics (Taponen and Pyörälä, 2009). For example, in Finland, reported prevalence of β-lactamase positive *S. aureus* was 13% and β-lactamase positive CNS 23% (Nevala *et al.,* 2004). In Sweden, among the cases of clinical mastitis 7.1% of *S. aureus* and 12.5% of CNS produced β-lactamase (Bengtsson *et al.,* 2009), despite several decades of using penicillin as the antibiotic of first choice.

Other classes of antibiotics used in the treatment of staphylococcal clinical mastitis are macrolides and lincosamides. Macrolide and lincosamide antibiotics have common targets in the bacterial ribosome, and organisms that are resistant to one class can be resistant to the other class (Berger-Bächi, 2002). Resistance against those antimicrobials generally ranges between 0 and 17% (Erskine *et al.,* 2002a; SVARM 2004; Bengtsson *et al.,* 2009), however, high levels of resistance have been described in China, being 93% for erythromycin and 36.1% for clindamycin (Wang *et al.,* 2008).

Mastitis caused by streptococci has remained susceptible to β –lactams (Myllys *et al.,* 1998; Pitkälä *et al.,* 2004). Studies from France and Germany revealed that all *Str. dysgalactiae* and *Str. agalactiae* strains were susceptible to β-lactams, whereas *Str. uberis* strains showed an elevated penicillin G MIC (Haenni *et al.,* 2010; Minst *et al.,* 2012). Resistance to macrolides and lincosamides ranged between 0 and 20% in different studies (FINRES-Vet, 2005-2006; SVARM, 2007; MARAN, 2008).

Cephalosporins are commonly used in the treatment of clinical *E. coli* mastitis due to high antibacterial activity against coliforms. Resistance against cephalosporins has been highlighted in recent years, and

monitoring of extended-spectrum β-lactamases (ESBL) producing *E.coli* was started in the national antimicrobial resistance programs. In case of clinical mastitis ESBL producing *E. coli* was not detected in Sweden, Finland, Norway and Canada, whereas low prevalence (0.4%) has been found among *E. coli* strains in France (Dahmen *et al.,* 2013; SVARM, 2011; EVIRA, 2012; Saini *et al.,* 2012). Data from the U.S. showed higher resistance to cephalosporins, but the disk-diffusion method was used in this report. The highest resistance against tetracycline, from 14.8 to 37.3%, has been reported (Makovec and Ruegg, 2003; MARAN, 2008; Saini *et al.,* 2012). *E. coli* resistance against ampicillin has ranged between 7 and 45.5% (Hendriksen *et al.*, 2008)

3. AIMS OF THE STUDY

1. Identify common udder pathogens of clinical mastitis in primiparous and multiparous cows on the day of calving in Estonia (I).

2. Evaluate mastitis occurrence in primiparous cows in different housing systems, and investigate whether it is affected by the time interval between movement of heifers to their calving facility and their day of calving (I).

3. Investigate associations between different quantities of bacterial DNA detected using a PCR based method, and concentrations of APP and NAGase activity in the milk in naturally occurring clinical mastitis (II).

4. Compare APP concentrations and NAGase activity in the milk from cows with clinical mastitis caused by different udder pathogens (II).

5. Estimate the distribution of udder pathogens and their antibiotic resistance in Estonia over the period 2007–2009 (III).

6. Compare the efficacy of intramammary and parenteral treatment with benzylpenicillin of bovine clinical mastitis caused by penicillin-susceptible Gram-positive bacteria (IV).

4. MATERIALS AND METHODS

4.1. Study design

To evaluate the occurrence of clinical mastitis in heifers on the day of calving (Study I), a case-control study was designed. Eleven dairy herds with more than 100 cows and 50 replacement heifers calving per year were included. All heifers (n = 1,063) that calved during the one-year period were eligible for inclusion. Heifers with clinical mastitis on the day of calving were included as cases (n = 68), and the remaining freshly calved heifers (n = 995) were controls. Heifers on each farm were moved from their rearing facility to the milking farm according to the availability of space. The stall-length and type of management system (free-stall or tie-stall), the number of days between the day of transfer of the heifer to the cowshed and the day of calving, were recorded.

In Studies II and IV, milk samples from cows diagnosed as having clinical mastitis were collected from four herds, in the practice area of the large animal clinic of the Estonian University of Life Sciences. All farms were with loose-housing system and with parallel milking parlour. All herds were milked three times a day. The mean herd bulk milk somatic cell count per month ranged between 198,000-408,000 cells/ml during the study period.

A retrospective study (Study III) was based on an analysis of milk samples submitted to the Estonian National Veterinary Laboratory over a three-year period from 2007 to 2009. Quarter milk samples were collected from cows on Estonian dairy farms by local veterinarians or farmers.

Table 2. Description of study populations.

Study	Study period	No. of farms	Herd size Mean (min-max)	305-day milk yield Mean (min-max)	No. of milk samples
I	2004-2005	11	259 (200; 350)	7625 (5822; 9130)	3418
III	2007-2009	274	86.7 (2; 1800)		8204
II; IV	2007-2009	4	300-1000	8387 (6900; 9850)	281

4.2. Definition of clinical mastitis (I-IV)

According to the definition of clinical mastitis in the Bulletin of the IDF No 32 (1999) at least some visible signs are present in the udder or in the milk. If the milk from a quarter had abnormal viscosity (watery, thicker than normal), color (yellow, blood-tinged), or consistency (flakes, clots), clinical mastitis was diagnosed, and samples from diseased udder quarters were collected for bacteriological examination. Clinical mastitis was diagnosed and milk samples were collected by local trained farm personnel or veterinarians. Normal milk appearance, together with a positive California Mastitis Test result (score greater than 1), were used to make a diagnosis of subclinical mastitis.

Additionally, in Studies II and IV the severity classification was used. Systemic and local signs were recorded and categorized using a 3-point scale as follows: (1) mild clinical mastitis: milk from a quarter had abnormal viscosity (watery or thicker than normal), color (yellow or blood-tinged), or consistency (flakes or clots), but no udder swelling or systemic signs; (2) moderate clinical mastitis: similar to mild clinical mastitis, but with the addition of visible or palpable changes in the udder (swelling or pain) without systemic signs; and (3) severe clinical mastitis: both local and systemic signs (fever above 39.2°C).

4.3. Collection of milk samples (I-IV)

Once clinical mastitis was diagnosed, aseptic quarter milk samples were taken. Before sampling, the teat end was cleaned with 70%-ethanol swabs and allowed to dry. After discarding a few streams of milk, 2-7 ml of milk samples were collected in sterile 10 ml plastic tubes. All collected milk samples were cooled in the refrigerator and frozen at -20°C until further investigation (Study I). In Studies II and IV, all collected milk samples were frozen after preliminary on-farm bacteriology. In Study III, cooled milk samples were sent to the Estonian Food and Veterinary Laboratory.

4.4. Analytical methods

4.4.1. Conventional bacteriology (I, III, IV)

In Studies I and III, bacterial species were cultured and identified using accredited methodology based on the National Mastitis Council standards (2004) in the Estonian Veterinary and Food Laboratory. From each sample, 0.01 ml of milk was cultured on blood-esculin agar and incubated for 48 h at 37°C. The plates were examined after 24 and 48 h of incubation. A minimum of five colonies of the same type of bacterium was recorded as bacteriologically positive, and growth of more than two types of bacterial colonies was categorised as mixed growth. No bacterial growth was recorded when fewer than five colony-forming units were detected during 48h of incubation.

In Study IV, on–farm the preliminary on-farm bacteriology was used to differentiate between Gram-positive and Gram-negative bacteria. Ten µl of milk was streaked onto each section and plates were cultured for 12-24h. Bacterial growth was evaluated at first on the blood-esculin agar and then on the McConkey agar (the media for detection of Gram-negative bacteria) or on the salt-mannitol agar (the media for detection of staphylococci). After detection of staphylococci, penicillin resistance indicated by β-lactamase production was determined using a chromogenic nitrocefin test (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) (NCCLS 2002).

4.4.2. Real-time PCR assay (II, IV)

In Studies II and IV, a commercial real-time PCR test kit (Patho Proof Mastitis PCR Assay, Thermo Fisher Scientific, Espoo, Finland) was used for direct analysis of all milk samples. The kit protocol involved 4 separate multiplex real-time PCR reactions, which targeted 11 bacterial species and groups (covering more than 25 mastitis-causing species in total): *Staphylococcus* spp. (including *S. aureus* and all relevant CNS species), *Enterococcus* spp. (including *Enterococcus faecalis* and *Enterococcus faecium*), *C. bovis*, *E. coli*, *Str. dysgalactiae*, *Str. agalactiae*, *Str. uberis*, *Trueperella (*formerly *Arcanobacterium) pyogenes* / *Peptoniphilus indolicus* (Yassin et al., 2011), *Klebsiella* spp. (including *K. oxytoca* and *K. pneumoniae*), and *Serratia marcescens*. The PCR assay also detects the staphylococcal beta-lactamase gene *blaZ* coding for penicillin resistance. The tests were performed in

accordance with the user manual, and described by Koskinen *et al.* (2010). Based on the cycle threshold (Ct) values, the bacterial DNA quantity in each targeted bacterial species was grouped into three classes: +, ++, or +++, according to the manufacturer's manual.

4.4.3. Analytical methods for determination of inflammatory response in milk

4.4.3.1. Determination of milk amyloid A and haptoglobin (II)

All analyses for determination of acute phase protein were performed at the Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences. The concentration of MAA in milk was determined using a commercial ELISA kit **(**Phase MAA Assay Kit, Tridelta Development Ltd, Ireland). Milk samples were initially diluted to 1:500. If the concentration was above the range of a standard curve, they were further diluted as necessary. For very high MAA values, milk samples were diluted up to 1:10000 (maximum concentration 1500 mg/l). A 1:100 dilution was used (minimum concentration 0.94 mg/l) for very low values. Intra- and inter-assay coefficients of variation (CV) were <13% and <12%, respectively.

Milk Hp concentrations (mg/l) were determined by a method based on the ability of Hp to bind to haemoglobin (Makimura and Suzuki, 1982) and using tetramethylbenzidine as a substrate (Almsegeest *et al.,* 1994). The assay is originally aimed to determine concentrations of Hp in serum, but was here adapted to be used for milk, as described by Hyvönen *et al.* (2006). Optical densities of the formed complex were measured at 450 nm using a spectrophotometer. Lyophilised bovine acute phase serum was used as a standard, and calibration was carried out according to the European Union concerted action on standardization of animal APP (number QLK5-CT-1999-0153). The working range of the assay was 60 to 1900 mg/l. The inter-assay and intra-assay CV values for Hp analysis were <8% and <13%, respectively.

4.4.3.2. Milk N-acetyl-β-D-glucosaminidase (NAGase) activity determination (II, IV)

Milk NAGase activity was measured by a fluoro-optical method using an in-house microplate modification developed by Mattila and Sandholm

(1986) and further modified by Hovinen *et al.* (2010) in the laboratory of the Department of Production Animal Medicine, University of Helsinki. The calibrated milk sample was replaced with a control milk sample with a known 4-methyl-umbelliferon (4-MU) concentration, and NAGase activity was expressed as picomoles of 4-MU/min/µl of milk at 25°C. The upper detection limit for NAGase activity was 24.5 pmol 4-MU/min/µl. Inter-assay and intra-assay CVs for the NAGase activity were 5% and 4%, respectively.

4.4.4. Antimicrobial susceptibility testing (III)

Pure cultures of clinical mastitis pathogens were tested for antibacterial susceptibility with the disc diffusion assay on Mueller–Hinton agar. Testing was performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) document M31-A2 in the years 2007–2008 and M31-A3 in 2009 (CLSI 2002; 2008). Quality control strains, *S. aureus* ATCC® 25923, *E. coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853 and *Str. pneumoniae* ATCC® 49619, were included with each batch of isolates tested. The antimicrobial susceptibility of Gram-positive bacteria was tested with penicillin, ampicillin, cephalothin, clindamycin, erythromycin, gentamycin, trimethoprim/sulfa and tetracycline. The antimicrobial susceptibility of Gram-negative bacteria was tested with ampicillin, gentamycin, trimethoprim/sulfa, tetracycline, enrofloxacin, streptomycin, neomycin and cefaperazone. The list of antibiotics in susceptibility testing may vary, as different veterinarians preferred a different set of antibiotics in order to find the best solution for treatment after having received the laboratory test results. The criteria for the interpretation of zone diameter used in this study are described in Table 3.

Table 3. Zone diameter (mm) interpretive criteria of susceptible (S), intermediate (I) and resistant (R) bacteria.

Disc content, μg	*Staphylococcus* spp.			*Streptococcus* spp.			*Enterococcus* spp.			*Enterobacteriaceae* spp.		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin 10	≥ 29	-	≤28	≥ 26	19-25	≤18	≥ 17	-	≤16	≥ 17	15-16	≤14
Penicillin 10	≥ 29	-	≤ 29	≥24	-	-	≥15	-	≤14	-	-	-
Cephalothin 30				≥		≤	-	-	-			
Cefaperazone 75	-	-	-	-	-	-	-	-	-	≥21	16-20	≤15
Clindamycin 2	≥ 21	15-20	≤14	≥19	16-18	≤15	-	-	-	-	-	-
Erythromycin 15	≥ 23	14-22	≤14	≥21	16-20	≤15	-	-	-	-	-	-
Gentamycin 10	≥ 12	13-14	≤15	≥12	13-14	≤15	≥10	7-9	≤6	≥ 12	13-14	≤15
Tetracycline 30	≥ 19	15-18	≤14	≥23	19-22	≤18	≥19	15-18	≤14	≥ 19	15-18	≤14
Enrofloxacin 5										≥ 20	15-19	≤14
Trimet/sulfa 1.25/23.75	≥ 16	11-15	≤10	≥16	11-15	≤10	≥16	11-15	≤10	≥ 16	11-15	≤10

4.5. Treatments (IV)

In the study IV, any lactating dairy cow with clinical mastitis was considered for enrollment in the trial. Initial exclusion criteria were cows with more than one quarter affected and cows with known chronic mastitis, defined as cows with known high somatic SCC or cows having had more than three mastitis episodes before the beginning of the study.

All cows with clinical mastitis were allocated into treatment groups A and B using cow ID (A: even numbers and B: odd numbers). The following two treatments were used: parenteral treatment with benzylpenicillin procaine (Penovet® vet 300 mg/mL, Boehringer Ingelheim Vetmedica, Denmark) in group A or IMM treatment with benzylpenicillin procaine (Carepen® 600 mg, Vetcare Oy, Finland) in group B. One IMM tube was infused into the affected quarter once a day. The dose of benzylpenicillin used for parenteral treatment was 20 mg (20, 000 IU) per kg intramuscularly once a day. The duration of treatment was 5 days in both groups. The use of supportive treatment with non-steroidal anti-inflammatory agents (NSAID) was possible, but treatment with corticosteroids was not allowed. Treatment with penicillin according to the defined treatment groups began on the day of diagnosis. Treatment was stopped on the next day if Gram-negative bacteria were detected on the selective media or if the isolated staphylococci were resistant to penicillin (a positive nitrocefin test). Cases of clinical mastitis caused by Gram-negative bacteria (n=41) were treated with NSAID, fluid therapy and if the case was severe, with fluoroquinolones. Udder quarters infected with penicillin-resistant staphylococci were treated with IMM cloxacillin (Wedeclox mastitis® 1,000 mg cloxacillin, WDT, Garbsen, Germany) once a day for 5 days.

4.5.1. Assessment of treatment outcome

The outcome of the treatment was assessed 3-4 weeks after the onset of treatment by using clinical, inflammatory and bacteriological criteria. The milk samples were collected as described above and frozen at -20°C. The cow was defined as clinically cured if the affected quarter was free from clinical signs. A quarter was defined as bacteriologically cured if the DNA of the same bacterial species detected in the pre-treatment milk sample was not present in the follow-up milk sample. A quarter with the DNA from the same bacterial species detected before the treatment was defined as not cured.

Milk NAGase activity was determined in the pre-treatment and post-treatment milk samples, and used as an additional parameter to compare outcomes in the treatment groups (Hovinen et al., 2010).

Composite milk somatic cell counts (CMSCC) from the cows included in the study were collected from the study herds once each month during a 3 month period after the treatment; the mean number of recordings per cow was 2.6. The culling data were analyzed during a six month period after the treatment. These data originated from the routine herd health recording system.

4.5.2. The final enrollment criteria

Only cows with one affected udder quarter (n=140) with penicillin-susceptible Gram-positive bacteria were included into the study based on the following criteria regarding the species detected by the PCR assay: 1) DNA of one bacterial species only; or 2) DNA of one bacterial species in proportion over 99% from DNA of all target bacterial species detected 3) >90% DNA of a major pathogen combined with a low quantity (+) of DNA of a minor pathogen (CNS or *C. bovis*).

PCR-negative samples (n = 25), contaminated samples (more than three different species detected) (n = 27), and samples containing DNA from Gram-negative bacteria (n = 11) were removed from the study. Cows (n=44) treated with IMM cloxacillin, and cows (n = 26) with *bla*Z gene positive staphylococcal species, but treated unintendedly with benzylpenicillin were excluded from the main material, but analyzed separately.

4.6. Statistical analysis (I-IV)

Stata 9.2 software and Stata 10.0 (StataCorp, Texas, USA) were used for statistical analysis in Study I and in Studies II-IV, respectively. Statistical significance was assumed at $p \leq 0.005$.
In Study I, the logistic regression with a random herd effect for controlling clustering was used to analyse the effect of the housing system (free-stall, tie-stall with short stall-length or tie-stall with long stall-length) and the time span between moving heifers to the calving facility and the day of calving on the occurrence of clinical mastitis.

To simplify the modelling, the continuous variable, number of days from moving heifers to the calving facility and expected parturition, was transformed to a dichotomous variable (≤14 days vs. >14 day- classes) in the model. Odds ratios (OR) with a 95% confidence interval (95% CI) were calculated.

A two-sample proportion test was used to estimate statistical significance of differences in occurrence of udder pathogens between first-calving heifers and multiparous cows. These analyses were conducted using statistical software Statistix for Windows 2.0.

In Study II, generalized linear mixed models (GLMM) were used to investigate associations between milk APPs and PCR results. Only milk samples with PCR negative result or ≤3 pathogen species were analysed (n=253). The outcome variable MAA was logarithmically transformed, and the inverse square root transformation of Hp was used. As Hp is evaluated using a model in inverse scale, negative model estimates represent higher Hp concentrations. The full models included lactation number as a 4-level categorical variable (1, 2, 3 and ≥4 lactations), days in milk was categorized as a 4-level variable according to quartiles (1-19, 20-59, 60-118, and 119-412 days in milk), farm as a 3-level variable, and affected quarter as a 2-level variable (fore and hind quarters). These variables were kept in all models to control possible confounding effects. As these variables were not significant in any of the models (except affected quarters in the MAA model), they are not shown in results. PCR results by pathogen were included as categorical variables (negative, +; ++; or +++). If PCR results were under 6 cases per level, they were consolidated with the previous factor level as follows: *C. bovis* ++ with *C. bovis* +; *Str. agalactiae* +++ with *Str. agalactiae* ++; *Str. dysgalactiae* +++ with *Str. dysgalactiae* ++; *T. pyogenes* +++ with *T. pyogenes* ++; and CNS +++ with CNS ++. To account for clustering of data (13 cows had two samples from different quarters), cow was included as a random factor.

The Wald test was used to evaluate the overall significance of the categorical variables with more than two levels. Non-significant PCR result variables were removed using the stepwise backward elimination procedure. Both final models were tested for interactions between minor pathogens (*C. bovis* and CNS) and major pathogens (*E. coli*, *T. pyogenes*, *Str. uberis*, and *Str. dysgalactiae*).

The random effects Tobit regression model for censored data was used to investigate associations between milk NAGase activity and PCR results. The Tobit regression model was chosen because >40% of the NAGase results were over the maximum working limit of the assay (24.5 pmol 4-MU/min/µl), which would violate the regression model's assumptions. In the Tobit regression, all cases falling above (or below) a specified threshold value are censored, although these cases remain in the analysis (Long, 1997). A more detailed explanation using Tobit regression for analysing milk NAGase activity data is given by Pyörälä *et al.* (2011). Square root transformation of milk NAGase activities was used to achieve a normal distribution of uncensored data; 104 samples were censored at the level of 24.5 pmol 4-MU/min/µl. All other model building strategies and variables in each model were as described for the APP models above.

A linear regression model for APPs and a Tobit regression model for NAGase were employed to investigate the association between milk APP concentrations and milk NAGase activities with the severity of clinical mastitis (mild, moderate, and severe signs). Assumptions of all models were controlled using normality and scatter plots of model residuals.

In Study III, the logistic regression model with a random herd effect for the control of clustering was used for all the analyses in this study. OR with 95% confidence intervals (95% CI) were calculated.

The influence of milk samples with mixed growth or no bacterial growth on the occurrence of clinical or subclinical mastitis was assessed. Potential interactions (no growth or mixed growth × year) were assessed in the logistic regression model. The effects of herd size and year on the pathogens that caused clinical and subclinical mastitis were analysed.

Prior to the beginning of the study IV, the sample size necessary for statistical evaluation was calculated as 106 in both treatment groups. The calculations were based on the hypothesis that differences in the cure rates of the parenteral vs. IMM treatment are less than 20% (bacteriological cure rates of 65% and 45%, respectively; two-sided p-level at 0.05 and a study power of 80%). This hypothesis was based on the assumption that a large proportion of cases would be caused by *S. aureus*. However, after collection of the data a large proportion of the cows were lost, due to

missing data or reasons for post-inclusion exclusions and the power of the study to detect at least a 20% difference in the bacteriological cure was 59% (sample size of 61 in the parenteral group and 79 in the IMM group).

Logistic regression models were used to evaluate the associations between clinical and bacteriological cures, with treatment route. Bacteriological and clinical cures were the outcome variables. The treatment route (IMM, parenteral), bacteriological diagnosis as a 7-level categorical variable (*S. aureus*, CNS, *Str. agalactiae, Str. dysgalactiae*, *Str. uberis*, *C. bovis*, *T. pyogenes),* and a continuous variable milk NAGase activity in the pre-treatment milk samples (as a marker of the severity of the inflammation) were included as independent variables. Additionally, the lactation number was used as a 4-level categorical variable (1, 2, 3 and ≥ 4 lactations), the days in milk was used as a 4-level categorized variable (1-30, 31-69, 70-140 and > 140 days in milk), and the farm and affected quarter were used as a 4-level variable. Non-significant variables were removed using a stepwise backward elimination procedure. The Wald test was used to evaluate the overall significance of the categorical variables with more than two levels. No significant interactions were detected and as no included variables were associated with any outcome variables both final models included only treatment route as independent variable.

Differences in the number of culled cows between the treatment groups during the 6 months after treatment were analyzed with logistic model in which the treatment, farm, days in milk and lactation number were included. Variables were categorized similarly to the previous models.
A linear regression model was used to investigate the associations between milk NAGase activity in the post-treatment milk samples and the route of treatment. Before analysis, the outcome variable milk NAGase activity was logarithmically transformed. The full models included bacteriological recovery (yes/no), clinical recovery (yes/no), treatment route, diagnosed pathogens and milk NAGase activity in clinical mastitis in the pre-treatment milk samples, lactation number, days in milk, farm and affected quarter as fixed variables. The variables categorized similarly to the previous logistic regression models. The Wald test was used to evaluate the overall significance of the categorical variables with more than two levels. Non-significant variables were removed using a stepwise backward elimination procedure. Possible

interaction effects of the treatment with diagnosed clinical mastitis pathogens, bacteriological cure, clinical cure and farm were verified. No significant interactions were detected. Assumptions of the model were controlled using normality and scatter plots of the model residuals. Stata 11.0 (Stata Corp, Texas, USA) software was used for logistic regression models and linear regression model.

For analyzing associations between the treatment and low CMSCC (<200,000 cells/ml) occurrence during the 21-110 days after the mastitis cases, generalized linear mixed model was used. For this model, the GLIMMIX procedure in the SAS/STAT 9.1 software (SAS Institute Inc., Cary, NC, USA) was used. An auto-regressive correlation structure was used for modeling serial correlations of repeated measurements within cows. Treatment, time group after mastitis (21-50, 51-80 and 81-110 days) and their interaction, sample time in relation to mastitis, days in milk at the time of mastitis, and farm were included as fixed factors.

5. RESULTS

5.1. Risk factors for clinical mastitis in primiparous cows at calving (I)

Approximately 40% (423) of the first-calving heifers were housed on tie-stall farms and approximately 60% (640) were kept on free-stall farms. The overall occurrence of clinical mastitis at calving of the heifers was 6.4% (n = 68), being 9.7% (n = 41) on tie-stall farms compared with 4.1 % (n = 27) on free-stall farms.

Housing system was not a significant risk factor for clinical mastitis of freshly calved heifers (Table 4). The number of days from moving heifers to the calving facility and expected parturition was from 0 to76, whereas the average number of days was 26. On tie-stall farms, the heifers moved to the calving facility less than two weeks prior to the expected date of parturition had a higher risk (OR = 5.9; p = 0.001) to develop clinical mastitis at calving than the heifers moved more than 14 days before calving.

Table 4. Summary of logistic modeling of risk factors for clinical mastitis in heifers on the day of calving in eleven Estonian dairy herds.

Risk factor	Cases (n = 68)	Controls (n = 995)	OR[1]	95% CI[2]	p-value
Model 1					
Tie-stall, short stall length (≤ 175 cm) vs.	27	214	1		
Tie-stall, long stall length (> 175 cm)	14	168	2.12	0.32-14.2	0.43
Free-stall vs.	27	613	1		
Tie-stall long stall length (> 175 cm)	14	168	0.60	0.09-3.75	0.58
Model 2					
Tie-stall vs.	41	382	1		
Free-stall	27	613	0.39	0.85-1.83	0.237
>14 days between moving to calving facility and the day of calving vs.	36	576	1		
≤14 days between moving to calving facility and the day of calving	32	419	3.39	1.42-8.07	0.006
Tie-stall and >14 days moving vs.	16	260	1		
Tie-stall and ≤14 days,	25	122	5.91	1.98-17.7	0.001
Free-stall and >14 days,	20	284	0.78	0.13-4.57	0.790
Free-stall and ≤14 days,	7	329	1.08	0.16-7.05	0.940

[1] Odds ratio
[2] 95% confidence interval of odds ratio

The most common udder pathogens in both housing systems were *Str. uberis, E. coli* and CNS (Figure 1). Occurrence of *E. coli* mastitis was higher in free-stall farms compared to tie-stall farms, while *Str. uberis* was more frequent on tie-stall farms. The differences were not statistically significant (Figure 1).

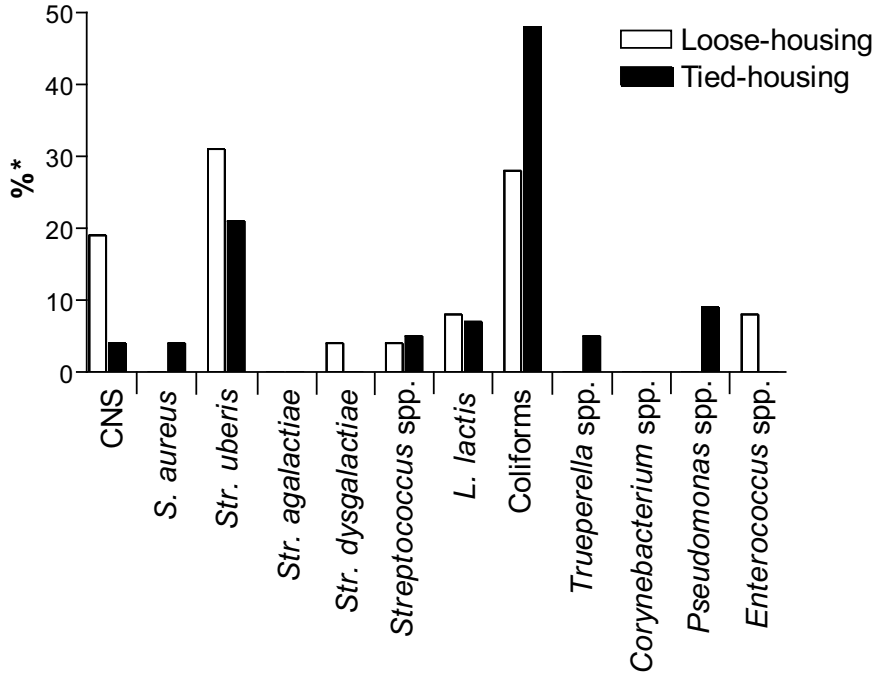

Figure 1. Distribution of udder pathogens in freshly calved heifers in two housing systems (I).
*calculated against the total number of isolates from heifers from both housing systems

5.2. Udder pathogens isolated from clinical and subclinical mastitis in Estonia

5.2.1. Clinical mastitis pathogens in primiparous and multiparous dairy cows at calving (I)

In total, 303 cases of clinical mastitis were identified in 2355 multiparous cows (12.8%) on the day of parturition. Udder pathogens were isolated from 49 (72%) out of 68 cases of clinical mastitis in freshly calved heifers and from 185 (61%) out of 303 cases in multiparous cows.

The most frequently isolated bacteria from milk samples of freshly calved heifers were *E. coli* and *Str. uberis*. No clinical mastitis caused by *Str. agalactiae* or *Corynebacterium spp*. was discovered, and only one case of *S. aureus* mastitis was found in heifers. In contrast, *S. aureus* was the most common bacterium isolated from the milk of affected multiparous cows, followed by *Str. uberis,* and *E. coli*. Differences between heifers and cows regarding the occurrence of clinical mastitis were statistically significant for *Str. uberis* ($p = 0.037$), coliforms ($p = 0.002$), and *S. aureus* ($p = 0.019$). Bacteriological findings are presented in Table 5.

Table 5. Bacterial species isolated from milk samples of heifers and multiparous cows having clinical mastitis at parturition.

	Heifers		Cows	
Pathogens	%	n	%	n
E. coli [1]	22.1	15	6.6	20
Str. uberis [1]	19.1	13	9.9	30
CNS	8.8	6	7.3	22
Lactococcus lactis	4.4	3	5.0	15
Klebsiella spp.	4.4	3	2.3	7
Str. spp.	2.9	2	3	9
Enterococcus spp.	2.9	2	2.3	7
Pseudomonas spp.	2.9	2	0.7	2
S. aureus [1]	1.5	1	11.2	34
Arcanobacterium spp.	1.5	1	2.6	8
Str. dysgalactiae	1.5	1	3.6	11
Corynebacterium spp.	0	0	2.0	6
Str. agalactiae	0	0	3.3	10
Candida spp.	0	0	1.3	4
No growth	25	17	29.4	89
Mixed culture	2.9	2	9.6	29
Total	100	68	100	303

[1] The difference between heifers and multiparous cows is statistically significant ($p < 0.005$)

5.2.2. Isolation of mastitis pathogens from milk samples submitted to the Veterinary and Food Laboratory in 2007-2009 (III)

Over the three-year period, 3058 clinical mastitis samples from 190 farms and 5146 subclinical mastitis samples from 274 farms were investigated. Positive results were found in 57% of the samples (4680 out of 8204),

and this proportion did not change over the three years ($p > 0.05$). The proportion of bacteriologically negative samples was 22.3% and that of mixed growth 20.6%. There was a significantly higher chance (OR = 1.15, 95% CI = 1.01, 1.33, $p = 0.042$) of finding bacteriologically negative samples in the presence of subclinical mastitis (n = 1,317; 25.6%) in comparison with clinical mastitis (n = 554; 16.8%). The probability of obtaining mixed growth from milk samples was also significantly higher (OR = 2.2, 95% CI = 1.9, 2.6, $p < 0.001$) in case subclinical mastitis was found. The distribution of bacterial species isolated from milk samples of cows with clinical and subclinical mastitis is shown in Table 6.

Table 6. Distribution of bacterial species isolated from clinical and subclinical mastitis samples in 2007–2009.

Bacteria	Clinical mastitis			Subclinical mastitis		
	2007 (n = 598)	2008 (n = 692)	2009 (n = 726)	2007 (n = 939)	2008 (n = 1063)	2009 (n = 661)
S. aureus	11.7	11.7	11.7	19.2	22.8	16.6
CNS	4.8	7.1	8.5	16.1	13.6	17.4
CPS[1]	3.8	3.3	1.6	4.6	2.8	5.1
Str. agalactiae	9.0	11.3	14.7	13.6	9.0	10.7
Str. dysgalactiae	8.0	7.8	7.2	3.6	4.0	5.6
Str. uberis	16.1	21.8	17.1	10.2	12.3	12.9
Str. spp	3.2	3.3	1.9	1.2	2.0	2.7
Lactococcus lactis	10.9	3.9	5.7	8.9	8.2	3.9
E. coli	14.4	16.6	16.5	1.6	2.0	3.8
Klebsiella spp.	7.0	1.3	2.3	0.7	0.6	0.9
Enterococcus spp.	1.3	2.3	1.1	1.5	2.8	4.2
Corynebacterium spp.	2.2	2.6	5.0	16.5	17.3	8.5
T. pyogenes	2.2	3.8	3.6	0.1	0.6	0.6
Pseudomonas spp.	1	0.3	0.3	0	0	0.6
Proteus spp.	0.2	0	0.2	0.4	0.1	0.6
Yeast	2.3	2	1.6	1.5	1.6	5.6
Other	1.8	0.9	1	0.3	0.3	0.3
Total	100%	100%	100%	100%	100%	100%

[1] Coagulase-positive staphylococci

Among the bacteriologically positive (n = 2016) clinical mastitis samples, *Str. uberis* was the bacterium isolated most frequently (n = 371; 18.4%

of the positive samples), followed by *E. coli* (n = 321; 15.9%), and *Str. agalactiae* (n = 293; 11.9%). *S. aureus* (n = 532; 20%) and CNS (n = 411; 15.4%) were the bacteria isolated most commonly from milk in cases of subclinical mastitis, followed by *Corynebacterium* spp. (n = 395; 14.8%).

The probability of isolating *S. aureus* from milk samples was significantly higher on farms having fewer than 30 cows, compared with the farms with more than 100 cows (OR = 0.2, 95% CI = 0.11, 0.53, $p < 0.005$). Also, there was a significantly higher risk of diagnosing *Str. agalactiae* on farms with more than 600 cows (OR = 17.6, 95% CI = 1.2, 259.1, p = 0.034), compared with smaller farms.

5.2.3. Bacteriological findings using PCR-based analysis (II)

PCR-based mastitis diagnostics was used and ten different species of udder pathogens were detected in 281 quarter milk samples from cows with clinical mastitis. A total of 27 milk samples (9.6%) were PCR-negative, and 254 (90.4%) samples contained DNA from at least one target species. Milk samples containing DNA from four or more bacterial species (n = 28) were considered possibly contaminated and excluded from further analysis. In total, 443 bacterial identifications were made from the remaining 226 milk samples. A single bacterial species was found in 68 (30.1%), 2 species were found in 99 (40.8%), and 3 species were found in 59 (26.1%) of the DNA-positive milk samples.

The most prevalent bacterial species in the milk samples containing a single pathogen were *Str. uberis* (n = 20; 29.4%), *S. aureus* (n = 14; 20.5%), and *E. coli* (n = 13; 19.1%). *Str. uberis* was detected in 45 (45.4%) and CNS in 40 (40.4%) of the milk samples with two bacterial species. Different bacterial DNA quantities and proportions of mastitis-causing bacteria were detected in the milk samples (Table 7.).

Table 7. Bacterial DNA quantities of udder pathogens detected in 226 milk samples from cases of naturally occurring clinical mastitis.

Identified mastitis pathogens (n = 443)	% of all DNA-positive milk samples (n = 226)	n (%) mean Ct-values[2]		
		+[1]	++	+++
S. aureus (n = 49)	21.6%	12 (24.4) 33.9	19 (38.7) 27.1	18 (36.7) 21.9
CNS (n = 91)	40.2%	78 (85.7) 34.6	12 (13.1) 28.3	1 (1.1) 19.3
Str. agalactiae (n = 12)	5.3%	2 (16.6) 36.3	6 (50.0) 29.4	4 (33.3) 17.1
Str. dysgalactiae (n = 33)	14.6%	14 (42.4) 34.1	17 (51.5) 25.7	2 (6.1) 16.3
Str. uberis (n = 98)	43.3%	33 (33.6) 35.0	29 (29.6) 27.3	36 (36.7) 18.1
T. pyogenes (n = 34)	15.0%	24 (70.5) 35.1	5 (14.7) 25.1	5 (14.7) 19.4
C. bovis (n = 53)	23.4%	50 (94.3) 33.7	3 (5.7) 24.6	0
Enterococcus spp. (n = 4)	1.7%	4 (100) 35.6	0	0
E. coli (n = 67)	29.6%	19 (28.3) 35.6	30 (44.8) 30.2	18 (26.8) 18.5
Klebsiella spp. (n = 2)	0.9%	2 (100) 32.5	0	0

[1] + - low quantities of bacterial DNA (range of Ct-values 28.3-37)
++ - intermediate quantities of bacterial DNA (range of Ct-values 22.1-33.7)
+++ - high quantities of bacterial DNA (range of Ct-values 13.4-27.4)
[2]Ct-values of each pathogen based on quantity of bacterial DNA

None of the milk samples contained two or three bacterial species in high quantities simultaneously. The quantity was low (+) in 78 (85.7%) out of the 98 milk samples containing DNA from CNS. Bacterial DNA from *Enterococcus* spp. and *Klebsiella* spp. was detected rarely and only in low quantities.

5.3. The local inflammatory response in clinical mastitis (II)

5.3.1. Associations between the severity of clinical mastitis and acute phase proteins

An association between the severity of clinical signs and the concentrations of APP and NAGase activity in the milk was found. In cases of severe

clinical mastitis, MAA, Hp and NAGase activity values were significantly higher, compared with cases of clinical mastitis with mild or moderate signs ($p < 0.001$, $p = 0.006$, and $p = 0.021$, respectively). Concentrations of MAA and Hp and NAGase activity in milk from cows with moderate clinical mastitis were significantly higher than the values measured in milk from cows with mild clinical signs ($p < 0.001$, $p = 0.007$, and $p < 0.001$, respectively).

5.3.2. Udder pathogens of clinical mastitis and the concentration of APP and NAGase activity

The concentration of MAA in milk ranged between 0.94 and 1500 mg/l (median 43.3 mg/l, IQR 16.9-183.3 mg/l), and Hp varied from 59 mg/l to 1890 mg/l (median 214.1 mg/l, IQR 105.7-398.6). Two our of 253 milk samples had an MAA concentration below the working limits of the assay, while in 13 samples it was above the working limits of the assay (0.94 and 1500 mg/l, respectively). Twenty one samples had Hp concentrations under the working limit of the assay (59 mg/l). The activity of NAGase in milk ranged between 0.53 and 24.5 pmol 4-MU/min/µl (median 19.5 pmol 4-MU/min/µl, IQR 7.9-24.5 pmol 4-MU/min/µl), and in 104 milk samples the NAGase activity was above the working range of the assay (24.5 pmol 4-MU/min/µl). The median concentrations of APP and NAGase activity in different udder pathogens are presented in Table 8.

Table 8. Concentrations of milk amyloid A (MAA), haptoglobin (Hp) and N-acetyl-β-D-glucosaminidase (NAGase) activity in milk samples from clinical mastitis (n = 147)[1].

Pathogens	MAA mg/l median (min; max)	Hp mg/l median (min; max)	NAGase activity pmol 4-MU/min/µl median (min; max)
S. aureus (n = 18)	35.8 (6.7; 1500)	201.1 (59.0; 756.2)	22.5 (2.7; 24.5)
CNS (n = 11)	29.2 (8.5; 1500)	123 (59.0; 775.5)	11.86 (6.6; 24.5)
Str. agalactiae (n = 4)	53.5 (28.9; 735.7)	295.5 (172.1; 506.1)	20.6 (6.6; 24.5)
Str. dysgalactiae (n = 19)	147.6 (7.2; 905.2)	248.9 (74.8; 1118.2)	20.8 (3.28; 24.5)
Str. uberis (n = 36)	53.1 (4.5; 1500)	385.6 (59.0; 970.3)	24.5 (3.36; 24.5)
T. pyogenes (n = 10)	25.6 (0.95; 348.9)	618.5 (5.0; 1155.8)	24.5 (1.73; 24.5)
C. bovis (n = 4)	12.4 (0.09; 107.3)	95.8 (59.0; 108.6)	9.9 (0.5; 14.7)
E. coli (n = 18)	394.1 (1.75; 1500)	575.4 (59.0; 1288.1)	24.5 (2.5; 24.5)
PCR-negative (n = 27)	23.2 (7.4; 152.2)	164.1 (59.0; 548.3)	9.2 (1.3; 24.5)

[1] Only milk samples containing 1 bacterial species only or 1 bacterial species in large quantity (+++) are included in the table.

Concentrations of Hp and MAA and NAGase activity in the milk were not affected by farm, lactation number, or days in milk ($p > 0.05$). The affected quarter (fore or hind) did not affect the Hp concentrations or the NAGase activities in milk ($p > 0.05$; data not shown). Hp and MAA concentrations and NAGase activity in the milk were significantly higher when *E. coli* ++ and +++ or *Str. dysgalactiae* ++/+++ were detected, compared with all other milk samples where these species were not detected.

Furthermore, a low quantity (+) of *E. coli* in milk samples was associated with an elevated NAGase activity and MAA concentration (Tables 9, 10 and 11). The presence of *T. pyogenes* at high levels in milk samples caused a significant elevation of Hp, as well as an increased NAGase activity in the milk ($p < 0.001$; $p < 0.001$), compared with milk samples without *T. pyogenes*. However, no association between the MAA concentration and the presence of *T. pyogenes* was found ($p > 0.05$; data not shown). Milk samples containing DNA from *C. bovis* or a low quantity of DNA from CNS had a significantly lower concentration of APP and a lower

NAGase activity, compared with all other milk samples where other species were detected.

No interaction was detected in the models between minor (*C. bovis* and CNS) and major (*E. coli, Str. dysgalactiae, T. pyogenes,* and *Str. uberis*) pathogens. This means that none of the associations between the major pathogen DNA and the concentrations of the inflammatory markers were influenced by the presence of the minor pathogen DNA in the milk samples.

Table 9. Final generalized linear mixed model (GLMM) of associations between the concentration of milk amyloid A (MAA) in the milk and the pathogens detected with PCR (n = 253) from naturally occurring clinical mastitis.

Variable[1]	Estimate[2]	95% CI	p-value	Wald test p-value
Quarters				
fore quarters (n = 102)	0			
hind quarters (n = 179)	0.539	0.176; 1.011	0.005	
E. coli				<0.001
E. coli negative[3] (n = 186)	0			
+[c] (n = 19)	0.857	0.104; 1.610	0.026	
++ (n = 30)	0.636	-0.025; 1.299	0.059	
+++ (n = 18)	1.68	0.843; 2.525	0.000	
Str. dysgalactiae				0.001
Str. dysgalactiae negative (n = 220)	0			
+ (n = 14)	0.124	-0.737; 0.985	0.788	
++/+++ (n = 19)	1.386	0.635; 2.136	0.000	
C. bovis				
C. bovis negative (n = 200)	0			
+/++ (n = 53)	-0.664	-1.151; -0.140	0.012	
Intercept	3.116	2.192; 4.040	0.000	

[1] + - low quantities of bacterial DNA
++ - intermediate quantities of bacterial DNA
+++ - high quantities of bacterial DNA

[2] Estimates are on the logarithmic scale

[3] Number of milk samples not containing DNA from detected bacteria

Table 10. Final generalized linear mixed model (GLMM) of associations between concentration of haptoglobin (Hp) in the milk and the pathogens detected with PCR (n = 253) from naturally occurring clinical mastitis.

Variable[1]	Estimate[2]	95% CI	p-value	Wald test p-value
T. pyogenes				<0.001
T. pyogenes negative[3] (n = 219)	0			
+ (n = 24)	-0.005	-0.017; 0.007	0.415	
++/+++ (n = 10)	-0.037	-0.055; -0.019	0.000	
E. coli				<0.001
E. coli negative (n = 186)	0			
+ (n = 19)	-0.004	-0.018; 0.009	0.532	
++ (n = 30)	-0.021	-0.033; -0.008	0.001	
+++ (n = 18)	-0.029	-0.047; -0.014	0.000	
Str. uberis				<0.001
Str. uberis negative (n = 164)	0			
+ (n = 33)	0.002	-0.009;0.012	0.784	
++ (n = 29)	-0.009	-0.021; 0.003	0.150	
+++ (n = 36)	-0.023	-0.034; -0.012	0.000	
Str. dysgalactiae				0.008
Str. dysgalactiae negative (n = 220)	0			
+ (n = 14)	0.006	-0.010; 0.021	0.465	
++/+++ (n = 19)	-0.020	-0.034; -0.007	0.003	
CNS				0.103
CNS negative(n = 162)	0			
+ (n = 78)	0.008	0.0003; 0.016	0.040	
++/+++ (n = 13)	0.008	-0.009; 0.024	0.358	
C. bovis				
C. bovis negative (n = 200)	0			
+/++(n = 53)	0.013	0.005; 0.023	0.002	
Intercept	0.089	0.072; 0.107	0.000	

[1] + - low quantities of bacterial DNA
++ - intermediate quantities of bacterial DNA
+++ - high quantities of bacterial DNA

[2] Estimates are on the inverse square root scale (negative estimate means higher concentration of Hp)

[3] Number of milk samples not containing DNA from detected bacteria

Table 11. Random effects Tobit regression model of associations between N-acetyl-β-D-glucosaminidase (NAGase) activity in the milk and the pathogens detected with PCR (n = 253) from naturally occurring clinical mastitis.

Variable[1]	Estimate[2]	95% CI	p-value	Wald test p-value
T. pyogenes				0.006
T. pyogenes negative[3] (n = 219)	0			
+ (n = 24)	0.041	-0.721; 0.804	0.914	
++/+++(n = 10)	2.173	0.847; 3.499	0.001	
E. coli				0.002
E. coli negative (n = 186)	0			
+ (n = 19)	0.806	-0.025; 1.638	0.057	
++ (n = 30)	1.247	0.486; 2.007	0.001	
+++ (n = 18)	1.464	0.500; 2.428	0.003	
Str. uberis				<0.001
Str. uberis negative (n = 164)	0			
+ (n = 18)	-0.276	-0.934; 0.382	0.411	
++ (n = 17)	0.159	-0.544; 0.861	0.658	
+++ (n = 30)	2.416	1.555; 3.276	0.000	
Str. dysgalactiae				0.003
Str. dysgalactiae negative (n = 220)	0			
+ (n = 14)	-0.966	-1.882; -0.049	0.039	
++/+++ (n = 19)	1.100	0.274; 1.926	0.009	
CNS				<0.001
CNS negative (n = 162)	0			
+ (n = 78)	-0.908	-1.390; -0.425	0.000	
++/+++ (n = 13)	-1.239	-2.162; -0.316	0.009	
C. bovis				
C. bovis negative (n = 200)	0			
+/++ (n = 53)	-1.323	-1.855; -0.791	0.000	
PCR-positive (n = 226)	0			
PCR-negative (n = 27)	-0.918	-1.687; -0.149	0.019	
Intercept	3.116	2.018; 4.214	0.000	

[1] + - low quantities of bacterial DNA
++ - intermediate quantities of bacterial DNA
+++ - high quantities of bacterial DNA
[2] Estimates are shown on a square root scale
[3] Number of milk samples not containing DNA from detected bacteria

5.4. Treatment efficacy of clinical mastitis of Gram-positive mastitis pathogens using penicillin G (IV)

5.4.1. Outcome of benzylpenicillin treatment

In total, 140 quarters with clinical mastitis were included in the study. Clinical signs were defined as mild in 83 cows (59.2%) and moderate in 55 cows (39.2%). Mastitis was defined as severe in two cows (1.4%). Of 140 quarter cases with clinical mastitis, 61 (43.6%) were treated with benzylpenicillin via the parenteral route and 79 (56.4%) with benzylpenicillin via the IMM route Distribution of the bacteria detected in the milk samples did not significantly differ between the treatment groups (Table 1). *Str. uberis* was the most common bacteriological finding, followed by other streptococcal species. No significant associations between the clinical cure (OR = 1.38; 95% CI 0.62, 3.12; p= 0.431) or bacteriological cure (OR = 0.94; 95% CI 0.48, 1.83; p= 0.851) and the route of treatment were observed. The cure rates for the 140 quarters with clinical mastitis infected by Gram-positive bacteria susceptible to benzylpenicillin *in vitro* are shown in Table 12.

Table 12. The outcome of parenteral and intramammary 5-day treatment with benzylpenicillin of bovine clinical mastitis (n = 140 quarters) caused by Gram-positive bacteria susceptible to benzylpenicillin *in vitro*.

Pathogen	Clinical cure		Bacteriological cure	
	IM[1] n	IMM[2] n	IM[1] n	IMM[2] n
S. aureus (n = 8)	1/2	5/6	1/2	5/6
CNS (n = 13)	4/6	6/7	2/6	4/7
Str. uberis (n = 66)	29/34	22/32	20/34	16/32
Str. agalactiae (n = 14)	6/6	6/8	4/6	6/8
Str. dysgalactiae (n = 19)	5/8	9/11	5/8	8/11
C. bovis (n = 6)	1/1	3/5	0/1	3/5
T. pyogenes / *P.indolicus* (n = 14)	3/4	8/10	1/4	2/10
Total (n = 139) (%)[3]	49/61 (80.3)	59/79 (74.7)	33/61 (54.1)	44/79 (55.7)

[1] Intramuscular (parenteral) treatment
[2] Intramammary treatment
[3] The propotion of cured udder quarters

Milk NAGase activities in the post-treatment samples did not differ between the two treatment groups (p= 0.688; Table 13). Milk NAGase

activity was significantly lower (p = 0.003) in the quarters with a clinical cure than the quarters with no clinical cure and in the bacteriologically cured quarters compared with those without bacteriological cure (p = 0.002; Table 13)

Table 13. Linear regression model of associations between milk NAGase activity in the post- treatment milk sample (n = 140) and route (intramammary or parenteral) of treatment in clinical mastitis caused by Gram-positive bacteria.

Variable	Estimate[1]	95% CI	p-value	Wald test p-value
Treatment				
IMM (n = 79)	0			
IM (n = 61)	-0.08	-0.44; 0.26	0.688	
Bacteriological cure				
No (n =63)	0			
Yes (n = 77)	-0.58	-0.95; -0.21	0.002	
Clinical cure				
No (n = 32)	0			
Yes (n = 108)	-0.67	-1.11; -0.23	0.003	
Farm				0.000
Farm 1. (n = 17)	0			
Farm 2. (n = 11)	-0.28	-1.12; 0.55	0.507	
Farm 3. (n = 66)	-1.01	-1.67; -0.47	0.001	
Farm 4. (n = 46)	-0.13	-0.81; 0.43	0.544	
Intercept	2.532	1.851; 3.213	0.000	

[1] Estimates are in logarithmic scale

The median NAGase activities in the milk before treatment and in the post-treatment samples are presented in Table 14.

Table 14. Milk NAGase activity in milk samples from quarters with clinical or bacteriological cure or no cure (n = 140) before and after 5-day parenteral or intramammary penicillin treatment of clinical mastitis caused by Gram-positive bacteria.

	Median milk NAGase activity (min; max) (pmol 4-MU/min/µl	
	Before treatment	After treatment
Clinical cure		
Yes (n = 108)	24.18 (0.53; 24.49)	2.73 (0.75; 24.29)
No (n = 32)	17.17 (1.49; 24.49)	5.84 (0.59; 24.49)
Bacteriological cure		
Yes (n = 77)	17.58 (1.49; 24.49)	2.44 (0.15; 24.49)
No (n = 63)	24.49 (0.53; 24.49)	3.41 (0.16; 24.49)
Treatment		
IM (n = 61)	24.49 (1.22; 24.49)	2.32 (0.15; 24.49)
IMM (n =79)	20.53 (0.53; 24.49)	3.12 (0.16; 24.49)

In total, the number of culled cows was 18 (13.1%) by the end of the 6 month follow-up period after treatment. No data were available for 3 cows. No significant differences between the treatment groups (OR = 0.91, 95% 0.33, 2.46, p = 0.507) were found.

5.4.2. Composite milk somatic cell count after treatment

Individual cow CMSCC data from three test milkings during the 3-month follow-up period after treatment (21-110 days) were available for 126 cows. The summary of data and the proportion of cows with CMSCCs less than 200,000 cells/ml in the two treatment groups at different time points after treatment is shown in table 15. No association (p= 0.787) between the route of penicillin treatment and the proportion of cows with CMSCC <200,000/ml was seen.

Table 15. Individual cow composite milk somatic cell counts (CMSCCs) and proportions of cows with CMSCCs <200,000 cells/mL collected during a 3-month period (21-110 days) after parenteral or intramammary penicillin treatment of clinical mastitis caused by Gram-positive bacteria.

Period (days) after clinical mastitis	Individual cow CMSCC (cells/ml)		Proportion of samples with CMSCC below 200,000 cells/ml	p-values
	Mean (±SD)	Median (min; max)	%	
21-50 days				
IM[1] (n = 59)	456,400 (± 649,800)	194,000 (17,000; 3,287,000)	50.8	0.137
IMM[2] (n = 70)	851,246 (± 1,332,200)	260,000 (5,000; 7,073,000)	39.6	
51-80 days				
IM[1] (n = 49)	408,604 (± 526,540)	210,000 (11,000; 2,384,000)	47.4	0.312
IMM[2] (n = 62)	678,700 (± 1,392,500)	1,818,000 (9,000; 6,565,000)	56.8	
81-110 days				
IM[1] (n = 46)	670,100 (± 1,175,300)	256,500 (8,000; 6,062,000)	43.2	0.456
IMM[2] (n = 53)	648,900 (± 1,251,200)	195,000 (10,000; 8,272,000)	50.6	

[1] Intramuscular (parenteral) treatment

[2] Intramammary treatment

5.5. Antimicrobial resistance of clinical mastitis pathogens (III)

The proportion of *S. aureus* isolates resistant to penicillin and ampicillin accounted for 61.4% and 59.5%, respectively. In addition, CNS showed resistance to penicillin and ampicillin (38.5% and 34.4%, respectively), while resistance to erythromycin and lincomycin was also common (14.9% and 17.6%, respectively). Six isolates (3.8%) of *S. aureus* and three isolates (3.6%) of CNS were resistant to cephalothin. All streptococci (Table 16) were susceptible to penicillin, ampicillin and cephalothin, except for one isolate of *Str. uberis*. Of the 90 isolates of *Str. dysgalactiae*, 32.2% were classified as resistant to tetracycline. Of the 151 isolates of *Str. uberis* 14.3% isolates resistant to tetracycline were recorded.

Table 16. Antimicrobial resistance of staphylococci and streptococci isolated from bovine clinical mastitis cases.

	S. aureus		CNS		*Str. agalactiae*		*Str. dysgalactiae*		*Str. uberis*	
Disc content (μg)	n	R[1] (%)	n	R (%)	n	R (%)	n	R (%)	n	R (%)
Ampicillin 10	173	59.5	91	38.5	162	0	111	0	265	0.4
Penicillin 10	174	61.4	93	34.4	168	0	111	0	267	0.4
Cephalothin 30	160	3.8	84	3.6	143	0	101	0	254	0.4
Clindamycin 2	169	18.1	91	17.6	161	6.2	115	7.8	273	6.6
Erythromycin 15	83	4.8	47	14.9	77	1.3	60	6.7	134	8.2
Tetracycline 30	147	4.1	86	11.6	151	14.6	90	32.2	234	19.7
Trimethoprim /sulfa 1.25/23.75	162	3.4	76	2.6	140	6.4	103	1	223	3.2
Gentamycin 10	146	6.8	69	1.4	143	24.5	88	11.4	210	18.6

[1] Proportion of resistant (R) isolates.

Among the *E. coli* isolates (Table 17), the highest percentage showing intermediate susceptibility and resistance was observed for ampicillin, neomycin, streptomycin, and tetracycline. *E. coli* was 98.4 % susceptible to enrofloxacin and 100% to cefaperazone.

Table 17. Antimicrobial resistance of *E. coli* and *Klebsiella* spp. isolated from bovine clinical mastitis cases.

	E. coli				*Klebsiella* spp.			
Disc content (µg)	n	S1 (%)	I (%)	R (%)	n	S (%)	I (%)	R (%)
Ampicillin 10	201	68.7	7.0	24.3	39	15.4	7.7	76.9
Cefaperazone 75	137	100	0	0	32	100	0	0
Tetracycline 30	184	77.8	8.7	13.5	39	79.6	10.2	10.2
Trimethoprim /sulfa 1.25/23.75	191	84.3	3.7	12.0	40	97.5	0	2.5
Gentamycin 10	161	94.3	2.5	2.2	40	95.0	0	5.0
Streptomycin 30	154	78.6	5.8	15.6	37	73.0	8.1	18.9
Neomycin 30	155	72.9	20.6	6.5	37	83.8	13.5	2.7
Enrofloxacin 5	185	98.4	0	1.6	37	100	0	0

[1] Propotion of susceptible (S), intermediate (I) and resistant (R) isolates.

6. DISCUSSION

6.1. Risk factors for clinical mastitis in heifers at parturition (I)

The clinical mastitis incidence rate in our study population of freshly calved heifers was quite modest (6.4%). In 11 large herds using the traditional Estonian dairy management system, the occurrence of clinical mastitis of first-calving heifers did not differ significantly between the two housing systems. However, as the number of herds in this study was limited and sample sizes were small in some herds, the results should be interpreted with caution.

In some trials, a higher incidence of clinical mastitis has been found in tie-stall compared to free-stall housing (Ekesbo, 1966; Bakken *et al.,* 1988; Matzke *et al.,* 1992). The incidence of clinical mastitis decreased over an 18-month period after the management system was changed from the tie-stall to the free-stall system (Hultgren, 2002). On tie-stall farms the main risk factors for clinical mastitis are teat injuries, short stalls, and shortage of bedding material (Koskiniemi, 1982; Bendixen *et al.,* 1988), especially during the periparturient period (Elbers *et al.,* 1997). Also, in the tie-stall systems, a higher frequency of lying down and rising may increase the risk of teat tramping, leading to an increased incidence of clinical mastitis (Oltenacu *et al.,* 1990). In contrast, in loose-housing systems cows have sufficient space for lying down and standing up in a more natural way during parturition. At the same time, poor hygiene in calving areas is associated with a new IMI around calving and an increased clinical mastitis rate (Barellie *et al.,* 2007; Green *et al.,* 2007). Mucking out the calving area less often than once a month is a significant risk factor for clinical mastitis (Peeler *et al.,* 2000). We found an association between the time of moving close-to-term heifers to the milking farm, and the occurrence of clinical mastitis on tie-stall farms. It has been shown that moving heifers to a confined area on the day of calving instead of doing it earlier, moving heifers out from the calving pen too late, or milking heifers in the calving pens, leads to an increased incidence of clinical mastitis (Svensson *et al.,* 2006; Nyman *et al.,* 2009). Stress and sudden changes in environmental and management conditions during the periparturient period may weaken natural defense mechanisms in animals, making them more susceptible to clinical mastitis.

6.2. Clinical mastitis pathogens in primiparous and multiparous dairy cows at calving (I)

Comparing tie-stall and free-stall farms, the bacterial findings on the day of parturition were generally the same. Mainly bacteria present in the surrounding environment were isolated in cases of clinical mastitis of the freshly calved heifers. These results agree fairly well with those from similar studies, reported by other researchers, in which the bacteria commonly isolated after parturition were CNS, coliforms and streptococci (Gröhn *et al.,* 2004; Tenhagen *et al.,* 2009). In Danish and Swedish studies, the most frequently isolated organism was also *S. aureus* followed by *Str. dysgalactiae* and *E. coli* (Waage *et al.,* 1998; Persson Waller *et al.,* 2009). Our investigation did not show clinical *S. aureus* mastitis in freshly calved heifers, although *S. aureus* was the predominant pathogen in multiparous cows. Predisposing factors for mastitis around calving are likely to be similar in heifers and older cows, where the primary source of infection is bovine feces and where the secondary multiplication of bacteria to high numbers in bedding and manure is often a risk factor (McCarthy and Sears, 2003).

6.3. Distribution of udder pathogens in Estonia in 2007-2009 (III)

The distribution of subclinical and clinical mastitis pathogens was analysed in the study based on the analysis of milk samples submitted to the Estonian Veterinary and Food Laboratory over a three-year period. The laboratory protocols remained unchanged during the study period. In total, 22.3% of the samples investigated were bacteriologically negative. Several other studies have also demonstrated bacteriologically negative findings in 17.7 to 26.5% cases of clinical mastitis, and as many as in 28.7 to 38.6% cases of subclinical mastitis (Sargeant *et al.,* 1998; Bradley *et al.,* 2007; Roesch *et al.,* 2007), which are in line with our results. The possible reasons for bacteriologically negative findings in milk samples might be the presence of antibacterial substances in the milk that lead to a decrease in the viability of bacteria in the culture (Rainard and Riollet, 2006), or failures in the conventional culture compared with identification of bacteria using PCR-test (Taponen *et al.,* 2008).

E. coli and *Str. uberis* were the pathogens most frequently isolated from the milk samples of the cows with clinical mastitis, while *S. aureus*, CNS

and *Corynebacterium* spp. were mostly associated with subclinical mastitis. Similar results were obtained in a study carried out in Estonia ten years ago, where *C. bovis* (47.5%), *S. aureus* (21%) and CNS (15.8%) were the pathogens isolated most commonly from subclinical cases of mastitis (Haltia *et al.,* 2006). The proportion of *Str. agalactiae* positive milk samples was surprisingly high in our study. We found a strong association between the isolation of *Str. agalactiae* and large-scale farms. According to the data provided by the Estonian Animal Recording Centre, the number of dairy cows was approximately 98000 and the mean herd size was 88 cows in Estonia in 2009. Rapid changes in the management system (from tie-stall to free-stall) during the last eight years may explain the coexistence of environmental pathogens with *Str. agalactiae*. Although teat disinfection and dry cow therapy are routine mastitis control measures used on Estonian dairy farms, proper eradication programmes for *Str. agalactiae* have not been implemented. In contrast, an increased probability of finding *S. aureus* was correlated with farms with fewer than 30 cows. The average age of cows on small farms was 5.3 years vs. 4.3 years on farms with more than 300 cows (EARC, 2009). The culling policy may be different, and the owners of smaller farms may keep (possibly chronically infected) cows in the herd for a longer period of time.

6.4. A local acute phase response in clinical mastitis diagnosed using a quantitative PCR test (II)

The amount of bacterial DNA detected in the samples from cases of mastitis by certain species was associated with MAA and Hp concentrations and NAGase activity in the milk. The highest concentrations of MAA and the highest NAGase activities in the milk were found in cows with large quantities of *E. coli* in their milk. This is in accordance with experimental studies showing a strong inflammatory response to *E. coli* (Hyvönen *et al.,* 2006; Suojala *et al.,* 2008). Wenz *et al.* (2010) found that the concentration of Hp was the highest in *E. coli*-induced mastitis, compared with the mastitis caused by environmental streptococci or CNS. However, even low quantities of *E. coli* resulted in elevated concentrations of MAA and an increased NAGase activity in the milk. Experimental studies have revealed that even a small quantity of *E. coli* can induce an acute inflammatory reaction in the udder (Frost *et al.,* 1982). *E. coli* bacteria are generally rapidly eliminated from the

udder, but trigger a strong inflammatory reaction which is mainly due to endotoxin (Burvenich *et al.,* 2003). In practice, the time of sampling after the onset of clinical mastitis could also influence the quantity of DNA of *E. coli* in the milk. Delayed sampling during the course of infection could explain the low quantity of *E. coli* detected in bacteriological examination, despite a strong inflammatory response.

Our findings support the results reported by Pyörälä et al. (2011), who found that higher concentrations of Hp and NAGase corresponded to the detection of *T. pyogenes* in mastitic milk samples but could establish no association between *T. pyogenes* and MAA. This may indicate that intramammary infection due to *T. pyogenes* does not induce significant local production of MAA. Release of different acute phase proteins may depend on the pathogens present. The major producers of Hp and NAGase in the milk are neutrophils and epithelial cells, whereas only mammary gland epithelial cells appear to secrete MAA in cows with mastitis (Kitchen *et al.,* 1984; Eckersall *et al.,* 2006; Lai *et al.,* 2009). Epithelial damage may manifest differently in different infections which, in turn, could affect MAA concentrations in the milk.

The presence of *S. aureus* in the udder increased the concentrations of APP and the NAGase activity in the milk less than other major pathogens, indicating a mild inflammatory response in this infection. In experimentally induced *S. aureus* mastitis, the concentrations of Hp and MAA ranged between 52 and 323 mg/dl and between 34 and 286 mg/dl, respectively (Grönlund *et al.,* 2003), and were lower than those found in experimentally induced *Str. uberis* or *E. coli* mastitis (Pedersen *et al.,* 2003; Suojala *et al.,* 2008). The concentrations of Hp and MAA and NAGase activity in naturally acquired *S. aureus* mastitis were also lower than in streptococcal or *E. coli* mastitis (Pyörälä *et al.,* 2011). In the current study, mastitis caused by *S. aureus* may have been very mild, which could explain the weak inflammatory response detected in the udder quarters. A low quantity of *S. aureus* DNA in the milk samples could also indicate that the bacteria were just skin contaminants and not the actual cause of mastitis (Haveri *et al.,* 2008).

In our study, CNS and *C. bovis* were common bacterial species detected, mainly in low quantities, in milk samples positive for several species. *C. bovis* and CNS were the main pathogens detected using PCR from

milk samples without growth (Taponen *et al.,* 2009) and in the study by Koskinen *et al.* (2010) comparing conventional bacterial culturing and PCR in mastitis milk diagnostics. The frequent detection of these bacteria may be due to their extramammary origin. *C. bovis* and CNS are generally considered to be opportunistic bacteria inhabiting teat skin and canals (Taponen *et al.,* 2008). Nevertheless, the presence of CNS and *C. bovis* in the milk samples alone could increase concentrations of APP and NAGase activity in the milk, indicating that these bacteria are able to invade the udder and induce an inflammatory reaction. The PCR method allows the quantitative detection of udder pathogens, and is especially useful when bacteria are present in low quantities and may be undetectable using conventional methods. On the other hand, the high sensitivity of the PCR analysis and the methods used to collect milk samples can cause false-positive results, especially in large herds when many staff members are involved in the sampling. Presence of microbes in a milk sample does not necessarily prove that those microbes caused the intramammary infection. Interpretation of PCR results can be challenging and would need more guidance, even though PCR-based diagnostics is already in routine use in some countries. In the interpretation of PCR results, detection of a single species, preferably in moderate or large quantities, or detection of one dominating species with some other species provides a likely bacteriological diagnosis. The final diagnosis of mastitis always requires a full complement of supporting information, such as knowledge of the clinical signs and inflammation in the quarter (Pyörälä, 2012).

In this study, Hp performed better than MAA in describing bovine inflammatory response. A constant increase in concentrations of Hp in the milk along with increasing quantities of DNA (except CNS and *C. bovis*) was observed. Hp could thus be a better marker than MAA for indicating the local inflammatory response in clinical mastitis caused by different pathogens.

6.5. Efficacy of treatment of clinical mastitis caused by Gram-positive bacteria (IV)

The outcome of benzylpenicillin treatment of clinical mastitis caused by Gram-positive bacteria susceptible to penicillin in vitro was not affected by the route of administration of the drug. Clinical

studies comparing the efficacy of parenteral and IMM treatment for clinical mastitis are in general rare. To the authors' knowledge, field trials comparing the efficacy of parenteral and IMM benzylpenicillin treatment of bovine clinical mastitis have not been published. Parenteral penethamate hydroiodide treatment was compared to IMM penicillin-dihydrostreptomycin treatment in a study performed in New Zealand, and no significant differences were observed (McDougall, 1998). The majority of mastitis cases in that study were caused by *Str. uberis*, a species susceptible to benzylpenicillin. Sérieys *et al.* (2005) compared treatment with parenteral penethamate to IMM ampicillin-cloxacillin, with no significant differences between the two treatment regimens. Specific information regarding the *in vitro* susceptibility of the causative agents was not available, and no real comparison can be made. In clinical mastitis experimentally induced by *Str. uberis* and treated with penicillin, bacteriological cure did not differ between IMM, parenteral or combined treatment groups; however, the groups were so small that no conclusions could be made (Hillerton and Kliem, 2002). The dose of benzylpenicillin procaine used in that study was half of that used in our study, which could affect the parenteral cure rates. No differences between parenteral benzylpenicillin and IMM penethamate were found for the treatment of subclinical mastitis caused by penicillin-sensitive *S. aureus* or streptococci (Hallen-Sandgren *et al.,* 2008). In an old U.S. study, the efficacy of IMM amoxicillin alone or combined with intramuscular benzylpenicillin was compared for the treatment of subclinical *S. aureus* mastitis (Owens *et al.,* 1988). Bacteriological cure rates were approximately 50% and did not differ between the treatments; however, because no information regarding penicillin susceptibility was available, drawing any conclusions is difficult.

Overall, the bacteriological cure rates of clinical mastitis caused by staphylococci and streptococci treated with different antimicrobials and routes of administration have ranged from 56% to 84% (Jarp *et al.,* 1989; Taponen *et al.,* 2003a; Serieys *et al.*, 2005; McDougall *et al.,* 2007; Apparao *et al.*, 2009; Bradley and Green, 2009, Ruegg, 2010). Taponen *et al.* (2003a) used a 4-day treatment with IMM benzylpenicillin for mastitis caused by penicillin-susceptible Gram-positive bacteria, and reported a clinical cure rate of 75% and a bacteriological cure rate of 73%. The clinical cure rate was similar to our study, but the bacteriological cure rate was approximately 20% higher than in the present study, which may

be due to the different clinical severity of mastitis or different methods used for the bacteriological follow-up. Jarp *et al.* (1989) reported a total bacteriological cure rate of 68% for clinical mastitis due to Gram-positive, penicillin susceptible bacteria, treated for 5 days with benzylpenicillin, using the same dose as here. The cure rate of that study was also higher than reported in our study, possibly due to the same reasons as mentioned earlier. Bacteriological cure rates of mastitis depend on the causative agent. McDougall *et al.* (2007) compared treatment of clinical mastitis with three different IMM products, one of them containing procaine penicillin alone. More than half of the cases were caused by *Str. uberis*, and treatment with penicillin IMM for 1.5 d resulted in a cure rate as high as 91%, which is much higher than found here. Different conditions in the New Zealand such as much lower average milk production and less severe clinical signs may at least partly explain the difference.

Mastitis causing streptococcal species have remained susceptible to benzylpenicillin (Pitkälä *et al.,* 2004; Hendriksen *et al.,* 2008; Bengtsson *et al.,* 2009). *S. aureus* and CNS isolated from bovine mastitis have developed resistance to penicillin (Hendriksen *et al.,* 2008; Bagcigil *et al.,* 2012), which may significantly influence the efficacy of treatment (Pyörälä and Pyörälä 1998; Sol *et al.;* 2000; Taponen *et al.,* 2003b). In our study, 6 of 8 cases of mastitis caused by penicillin-susceptible *S. aureus* were cured using either intramuscular or IMM penicillin treatment. The bacteriological cure of 20 quarters with mastitis caused by penicillin-resistant *S. aureus* treated for 5 days with cloxacillin was in the present study zero (data not shown). It is known that mastitis caused by penicillin-resistant *S. aureus* is difficult to cure (Taponen *et al.*, 2003b; Barkema *et al.,* 2006). The poor treatment response of these cases is mainly not derived from antibiotic resistance. The ability of penicillin-resistant *S. aureus* isolates to cause persistent infections may be due to several virulence factors, possibly linked to the β-lactamase gene of the resistant isolates (Haveri *et al.,* 2005; Van den Borne *et al.,* 2010). In the treatment of mastitis, tested or assumed in vitro susceptibility of the causing bacteria is considered a prerequisite for the use of a particular antibiotic, but pre-treatment susceptibility is not always predictive of treatment response *in vivo* (Barlow, 2011).

Benzylpenicillin is a weak acid, which after parenteral administration penetrates poorly into the mammary gland. However, because the MIC

values for susceptible organisms are generally very low (≤0.12 μg/ml for staphylococci and ≤0.06 μg/ml for streptococci (Prescott *et al.,* 2007; Bengtsson *et al.,* 2009), it is possible to achieve and maintain therapeutic concentrations in the milk using parenteral administration of 20 mg/kg benzylpenicillin procaine once a day as used in this study (Franklin *et al.,* 1984; Ziv and Storper, 1985).

IMM infusion results in concentrations as high as 100-1000-fold of those obtained with parenteral administration, which is advantageous for infections of the milk compartment, such as streptococcal mastitis (Moretain *et al*., 1989; Erskine *et al.,* 2003). The total dose of antimicrobials administered via the IMM route is considerably lower than that in parenteral treatment. Furthermore, painful injections can be avoided. When infusing IMMs containing narrow-spectrum antimicrobials antibiotics such as benzylpenicillin, strict hygienic measures should be used to avoid inducing mastitis (Middleton and Luby, 2008). IMM administration is the route of choice for mastitis caused by streptococcal species, which reside in the milk compartment (Erskine *et al*., 2003; Guardabassi *et al.,* 2008). Parenteral or combined treatment has been suggested for mastitis caused by *S*. *aureus* (Erskine *et al*., 2003; Constable *et al*., 2008). Taponen *et al.* (2003b) reported a bacteriological cure of 72% for mastitis caused by penicillin-susceptible *S*. *aureus* treated with 5-day combined parenteral and IMM treatment with penicillin. In our study, no difference was observed between the two routes of treatment, but the *S*. *aureus* group was too small to draw any conclusions. Our group infected with CNS was also small, but based on the literature, IMM is the route of choice in the treatment of CNS mastitis (Erskine *et al.,* 2003; Pyörälä and Taponen, 2009).

In this study, bacteriological diagnosis was based on a PCR assay. For the evaluation of the bacteriological cure, strict criteria were used. If DNA of the same species detected before treatment was found alone or together with the DNA of other species in the post-treatment sample, the case was classified as not cured. It is known that the PCR-based assay is more sensitive than a conventional culture (Koskinen *et al.,* 2010). This may be reflected as lower percentages of cure than in previous studies in which conventional culturing was used for assessment. Excluding all samples with more than one species from the analysis would result in the discarding of a considerable number of cases, because the PCR test often detects more than one species (Koskinen *et al.,* 2010).

Higher cure rates may have also been expected here because our 5-day treatment is longer than standard treatments used for mastitis in many countries. Longer treatments have been reported to result in higher cure rates, at least for mastitis caused by *S. aureus* and *Str. uberis* (Jarp *et al.,* 1998; Oliver *et al.,* 2004; Deluyker *et al.*, 2005; Krömker *et al.,* 2010). Recently, 5-day treatment with cefquinome did not increase cure rates in clinical *S. aureus* mastitis compared with 1.5-day treatment (Swinkels *et al.,* 2013). This discrepant result may be due to the drug used or differences in the virulence of the bacterial strains causing IMIs. In assessing cure rates, the possibility of contamination of the sample with the same species as detected in the pre-treatment sample should also to be taken into account. This could lead to a false positive sample and false classification of the case as not cured. However, this affects both conventional and PCR-based tests. If PCR assays are used to assess the outcome in treatment trials of mastitis, some adjustments to the tests may be necessary for the interpretation of results.

Combining bacteriology with some indicator of inflammation in the milk would be useful for confirming the assessment (Green and Bradley, 2010). The most common indicator used to monitor the inflammatory status of the udder is milk SCC. Milk NAGase activity is another good choice for this purpose (Pyörälä, 2003). NAGase originates from somatic cells but also from damaged epithelial cells (Mattila and Sandholm, 1986; Pyörälä, 2003). It correlates well with milk SCC and has the advantage that freezing the milk samples does not interfere with the analysis (Pyörälä, 2003). The threshold values of these parameters should perhaps be adjusted for this purpose, because the inflammatory reaction of the quarter may last longer than elimination of the infection. The threshold levels of the markers used for screening of mastitis may be too high for monitoring the recovery of the quarter (Pyörälä and Pyörälä, 1997).

Generally, two post-treatment samples are recommended for the bacteriological evaluation of cure (Schukken and Deluyker, 1995). Here, only one sample was collected for practical reasons, but we used a sensitive PCR assay for bacteriology, which could somewhat compensate the lack of the second sampling. Including the cow survival data and cow composite milk SCCs follow-up provides information regarding the long-term effects of the treatments and can be recommended for field trials of mastitis. In the present study, the CMSCCs remained higher

and the proportion of low CMSCC cows was numerically smaller in the IMM-treated group, even though no significant differences between the groups were found. A possible explanation for this result is that the cows had other quarters with subclinical IMI, which were also treated when the treatment was administered parenterally and this may have affected cow CMSCCs.

6.6. Antimicrobial resistance of clinical mastitis pathogens (II)

The disc diffusion method for *in vitro* antimicrobial susceptibility testing was used to determine antimicrobial resistance of clinical mastitis pathogens in Study II. This technique is the most widely used method for determination of the susceptibility of animal pathogens, especially in clinical work when it is necessary to determine the correct treatment. The primary disadvantage of using this method when monitoring development of resistance is that outcomes are reported on a qualitative basis (sensitive, intermediate, or resistant), and subtle changes in susceptibility may not be apparent. Therefore any comparison with studies that use other methods of susceptibility testing is not acceptable (Schwarz *et al.,* 2009). Generally, in our study, the *in vitro* antimicrobial resistance of the isolates examined from samples of clinical mastitis was high. Isolates of *S. aureus* had an alarming level of resistance to penicillin (61.4%) and ampicillin (59.5%), whereas CNS exhibited a lower degree of resistance (38.5% and 34.4%, respectively). The reported percentages for penicillin-resistant *S. aureus* in cases of clinical mastitis, detected by the disc diffusion method, were 50.4% and 35.4% in the two US studies (Erskine *et al.,* 2003; Makovec and Ruegg, 2003), 63.3% in Turkey (Güler *et al.,* 2005), and 12% in Northern Germany (Schröder *et al.,* 2005). In addition, cephalotin resistance among staphylococci was found in our study. As there is little published information on methicillin-resistant staphylococci causing bovine mastitis, the relevant samples found in our study need further investigation to prove or exclude the presence of the *mec*A gene. In this study, both staphylococci and streptococci showed resistance to erythromycin and lincomycin, but the figures for resistance in annual reports from some other countries show a low prevalence of lincomycin and erythromycin resistance in *S. aureus* and CNS (SVARM, 2004; NORM/NORMVET, 2003; MARAN, 2008). Given that *S. aureus* and CNS were the pathogens isolated most frequently from cases of subclinical mastitis, one possible explanation for resistance to several

antibiotics may be the collection and submission to the laboratory of milk samples from chronic clinical mastitis (which demonstrate poor treatment efficacy). Therefore, random sampling strategies should be used to provide a good evaluation of antimicrobial susceptibility.

The level of resistance of *E. coli* and *Klebsiella* spp. was high against all tested antimicrobials, except cefaperazone and enrofloxacin. Coliforms are often resistant to more than one antimicrobial (Lehtolainen *et al.,* 2002; Bengtsson *et al.,* 2009), and the number of multiresistant strains may influence the resistance figures. Coliform bacteria isolated from cases of mastitis may reflect the general situation of resistance in the herd, and can be considered more as an indicator of the bacteria present than an indicator of specific pathogens from the udder (Lehtolainen *et al.,* 2002). All the bacterial species investigated in this study showed resistance to tetracycline. A possible explanation for this phenomenon could be the fact that tetracycline has been the class of antimicrobials most widely used for treatment of several infections for many years. Furthermore, tetracycline has been found in multiresistant patterns with penicillin and streptomycin (Lehtolainen *et al.,* 2002; Güler *et al.,* 2005).

According to the data provided by the Estonian State Agency of Medicines, a total of 209,880 single intramammary syringes for lactating cows and 205648 for dry cow therapy were sold in the year 2009. Ampicillin and cloxacillin combinations, cephalosporins with aminoglycosides, and lincomycin with neomycin were the most common choices for the treatment of mastitis in lactating cows. For example, 255 grams of intramammary lincomycin (pure antimicrobial) and 44.2 grams of intramammary cephalosporins per thousand dairy cows were sold for the treatment of clinical mastitis in 2009. However, only 73.4 grams of penicillin G was used per thousand dairy cows for intramammary treatment of clinical mastitis. The use of broad-spectrum antibiotics and antibiotic combinations may influence the resistance of mastitis pathogens. Moreover, bacteriological examination of milk samples before treatment of clinical mastitis is not a common practice in Estonia. According to the data available in Sweden, intramammary and intramuscular penicillin G (Landin, 2006) are used in over 80% of cases for treatment of clinical mastitis, but the prevalence of resistance of *S. aureus* to penicillins is only 7.1% (Bengtsson *et al.,* 2009). In Finland, penicillin G and some broad-spectrum β-lactam antibiotics are used

in the treatment of clinical mastitis, but the prevalence of resistance in *S. aureus* is around 13% (Nevala *et al.,* 2004; Thomson *et al.,* 2008). Bacteriological examination before treatment is common in both countries. Considering these results, we can assume that the main reason for the occurrence of a high number of resistant strains in Estonian herds is the wide use of broad-spectrum antimicrobials and long-term keeping of infected cows in the herd.

7. CONCLUSIONS

- The most frequently isolated udder pathogens in primiparous cows were *E. coli, Str. uberis* and coagulase-negative staphylococci, while *S. aureus* was the predominant pathogen observed in multiparous cows with clinical mastitis at parturition. The udder pathogens isolated from primiparous dairy cows on tie-stall farms did not differ significantly from those of free-stall farms, whereas differences were found between primiparous and multiparous cows at parturition.

- The incidence rate of clinical mastitis in freshly calved heifers was 6.4%. Housing system was not a significant risk factor for clinical mastitis in freshly calved heifers. As regards the tie-stall farms, moving heifers to the cowshed less than two weeks prior to calving increased the risk for clinical mastitis at parturition.

- The quantity of bacterial DNA in a milk sample was associated with the concentrations of APP and NAGase activity in the milk. These indicators reflect the extent of inflammatory reaction in the mammary gland, as APP concentrations and NAGase activity increase along with the increase in severity of mastitis.

- Concentrations of APP and NAGase activity in the milk differed significantly between different mastitis-causing bacterial species. The MAA and Hp concentrations in milk and NAGase activity were significantly higher in the samples containing large amounts of bacterial DNA from *E. coli* or *Str. dysgalactiae* compared with mastitic milk samples not containing these species. High bacterial DNA concentrations from *T. pyogenes* or *Str. uberis* were associated with elevated concentrations of Hp and a high NAGase activity, but not with increased MAA concentrations. The milk samples containing *C. bovis* and coagulase-negative staphylococci had significantly lower concentrations of MAA and Hp and lower NAGase activity than the samples in which these species were not detected.

- The most frequently isolated pathogens from the cases of clinical were *Str. uberis* followed by *E. coli* and *S. aureus*. mastitis in 2007-2009 in Estonia. Most cases of subclinical mastitis were caused by *S. aureus* and CNS.

- The probability of isolating *S. aureus* from milk samples was significantly higher on the farms with less than 30 cows, compared with those housing more than 100 cows. A significantly higher risk of infection *Str. agalactiae* was observed on the farms with herds over 600 cows, compare to smaller farms.

- The *in vitro* antimicrobial resistance of the isolates examined from the samples of clinical mastitis was high. Isolates of *S. aureus* had an alarming level of resistance to penicillin (61.4%) and ampicillin (59.5%), whereas CNS exhibited a lower degree of resistance (38.5% and 34.4%, respectively). The level of resistance of *E. coli* and *Klebsiella* spp. was high against all tested antimicrobials, except cefaperazone and enrofloxacin. A general decline in the antimicrobial resistance levels in cattle in Estonia should be highlighted. Appropriate guidelines for antibiotic usage should be developed and implemented, to help veterinary surgeons make pathogen-specific treatment decisions.

- The outcome of parenteral or intramammary penicillin treatment of mastitis caused by penicillin-susceptible bacteria was determined to be similar.

- Intramammary treatment could routinely be used for the treatment of clinical mastitis caused by streptococcal species. Streptococci reside in the milk compartment, and there are no pharmacokinetic grounds for the use of parenteral administration of the antimicrobial. Parenteral treatment is more invasive and significantly increases dose of the antimicrobial.

- With a more sensitive PCR method, bacteriological cure rates may be lower, which should be considered by researchers, the pharmaceutical industry and authorities in the future.

- If PCR tests are used to assess the outcome of the treatment trials, it may be necessary to adjust some of the criteria for interpreting the results. It might be worthwile to use some indicators of inflammation in the milk to completement bacteriology.

8. REFERENCES

1. Aland A., 2003. Lüpsikarja tervise seiremudel ning selle rakendamine loomade tervise hindamisel ja parandamisel. PhD thesis. Estonian Agricultural University, Tartu, Estonia, 108-112.
2. Almsegeest, S. P. M., Kalsbeek, H. C., Wensing, T., Koeman, J. P., van Ederen, A. M., Gruys, E., 1994. Concentration of serum amyloid A and haptoglobin as parameters of inflammatory diseases in cattle. Vet. Q. 16, 21-23.
3. Anonymous. 2003. Use of antimicrobial agents in animals. Report of the working group on antimicrobial agents. Ministry of Agriculture and Forestry in Finland. MAFF Publications 9, 2003.
4. Apparao, M. D., Ruegg, P. L., Lago, S., Godden, T., Bey, R., Leslie, K., 2009. Relationship between in vitro susceptibility test results and treatment outcomes for gram-positive mastitis pathogens following treatment with cephapirin sodium. J. Dairy Sci. 92, 2589–2597.
5. Bagcigil, A. F., Taponen, S., Koort, J., Bengtsson, B., Myllyniemi, A. L., Pyörälä, S. 2012. Genetic basis of penicillin resistance of *S. aureus* isolated in bovine mastitis. Acta Vet. Scand. 54, 69.
6. Bakken, G., Røn, I., Østerås, O., 1988. Clinical disease in dairy cows in relation to housing systems. In: Proceedings of the 6 th International Congress of Animal. Hygiene, June 14-17, Sweden, Skara, 18.
7. Barellie, N., Djabri, B., Beaudeau, F., Aurelie, R., Seegers, R., 2007. Aetiology and risk factors of new intramammary infection in dairy heifers around calving. In: Heifer mastitis conference. June 24-26, Ghent, Belgium, 118.
8. Barlow, J., 2011. Mastitis therapy and antimicrobial susceptibility: a multispecies review with a focus on antibiotic treatment of mastitis in dairy cattle. J. Mammary Gland Biol. Neoplasia, 16, 383-407.
9. Baumann, H., Gauldie, J., 1994. The acute phase response. Immunol. Today 15, 74-80.
10. Barkema, H. W., Schukken, Y. H., Lam, T. J., Beiober, G. M., Wilmink, M. L., Benedictus, G., Brand, A., 1998. Incidence of clinical mastitis in dairy herds grouped in three catergories by bulk milk somatic cell counts. J. Dairy Sci. 81, 411-419.

11. Barkema, H. W., Van der Ploeg, J. D., Schukken, Y. H., Lam, T. J., Benedictus, G., Brand A., 1999. Management style and its association with bulk milk somatic cell count and incidence rate of clinical mastitis. J. Dairy Sci., 82, 1655-1663.
12. Barkema, H., Schukken, Y. H., Zadoks, R. N., 2006. Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. 89, 1877-1895.
13. Barnoiun, J., Chassagne, M., 2001. Predictive variables for the occurence of early clinical mastitis in primiparous Holstein cows under field conditions in France. Can. Vet. J. 42, 47-53.
14. Bassel, L., Kelton, D., Godkin, A., Leslie, K., Lissemore, K., 2003: Risk factors for intramammary infection at first calving in Ontario dairy heifers. In: proceedings of the. 36th Annual Conference of. AABP, Columbus, Ohio, United States of America, 177-178.
15. Bendixen, P. H., Wilson, B., Ekesbo, I., 1988. Disease frequencies in dairy cows in Sweden. V. Tramped teat. Prev. Vet. Med. 6, 17-25.
16. Bengtsson, B., Unnerstad, H. E., Ekman, T., Artursson, K., Nilsson-Öst, M., Persson Waller, K., 2009. Antimicrobial susceptibility of udder pathogens from cases of acute clinical mastitis in dairy cows. Vet. Microbiol. 36, 142-149.
17. Berger-Bächi, B., 2002. Resistance mechanisms of gram-positive bacteria. J. Med. Microbiol. 292, 27-35.
18. Botrel, M. A., Haenni, M., Morignat, E., Sulpice, P., Madec, J. Y., Calavas, D., 2009. Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. Foodborne Pathog. Dis.17.
19. Bradley, A. J., Leach, K. A., Breen, J. E., Green, L. E., Green, M. J., 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. Vet. Rec. 160, 253-257.
20. Bradley A. J., Green, M. J., 2009. Factors affecting cure when treating bovine clinical mastitis with cephalosporin-based intramammary preparation. J. Dairy Sci. 92, 1941-1953.
21. Burton, J. L., Erskine, R. J., 2003. Immunity and mastitis. Some newideas for an old disease. Vet. Clin. North Am. Food Anim. Pract. 19, 1–45.

22. Burvenich, C., VanMerris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L. 2003. Severity of E. coli mastitis is mainly determined by cow factors. Vet. Res. 34, 521-564.

23. Chambers, H. F., 2001. The changing epidemiology of *Staphylococcus aureus*. Emerg. Infect. Dis. 7, 178–182.

24. Clinical and Laboratory Standard Institute (CLSI), 2002. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved standard. Second edition. NCCLS document M31-A2. Clinical and Laboratory Standard Institute, Wayne, PA, USA.

25. Clinical and Laboratory Standard Institute (CLSI), 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved standard. Third edition. CLSI document M31-A3., Clinical and Laboratory Standard Institute ,Wayne, PA, USA.

26. Constable, P. D, Morin D. E., 2003. Treatment of clinical mastitis using antimicrobil susceptibility profiles for treatment desicion. Vet. Clin. North Am. Food Animal Pract. 19, 139-155.

27. Constable, P. D.; Pyörälä, S., Smith, G., 2008. Guidelines for antimicrobial use in cattle. In: Antimicrobial Use in Animals. Blackwell Publishing, UK, 143-160.

28. Green, M. J., Bradley, A. J., Medley, G. F., Browne, W. J., 2007. Cow, farm, and management factors during the dry period that determine the rate of clinical mastitis after calving. J. Dairy Sci. 90, 3764-3776.

29. Green, M., Bradley, A., 2010. Practical methods to evaluate treatment outcomes. In: proceedings of the National Mastitis Council 49th Annual Meeting, New Mexico, Albuquerque, USA, 131-140.

30. Goff, J. P., Horts, R. L., 1997: Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80, 1260-1268.

31. Dahmen, S., Metayer, V., Gay, E., Madec J. Y., Haenni, M., 2013. Characterization of extended-spectrum beta-lactamase (ESBL)-carrying plasmids and clones of Enterobacteriaceae causing cattle mastitis in France. Vet. Microbiol. 162, 2-4.

32. DeOliveira, A. P., Watts, J. L., Salmon, S. A., Aarestrup, F. M., 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. J. Dairy Sci. 83, 855-862.

33. Deluyker, H. A., van Oye, S. N., Boucher, J. F., 2005. Factors affecting cure and somatic Cell count after pirlimycin treatment of subclinical mastitis in lactating cows. J. Dairy Sci. 88, 604–614.

34. Eckersall, P., Young, F. J., Mc Comb, C., Hogarth, C. J., Safi, S., Weber, A., Mc Donald, T., Nolan, A. M., Fitzpatrick, J. L., 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. Vet. Rec. 148, 35–41.

35. Eckersall, P., Young, F. J., Nolan, A. M., Knight,C. H., Mc Comb,C., Waterston, M. M., Hogarth,C. J., Scott,E. M., Fitzpatrick, J. L., 2006. Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. J. Dairy Sci. 89, 1488-1501.

36. Ehinger, A. M., Kietzmann, M., 2000. Tissue distribution of benzylpenicillin after intramammary administration in the isolated perfused bovine udder. J. Vet. Pharm. Therapy, 23, 303-310.

37. Ekesbo I: Disease incidence in tied and loose housed dairy cattle. Acta Agric. Scan. 1966, Suppl. 15.

38. Elbers, A. R. W., Miltenburg, J. D., De Lange, D., Crauwels, A. P. P., Barkema, H. W., Shukken, Y. H., 1997. Risk factors for clinical mastitis in a random sample of dairy herds from the southern part of the Netherlands. J. Dairy Sci. 87, 3358- 3374.

39. Erskine, R. J., Walker, R. D., Bolin, C. A., Bartlett, P. C., White, D. G., 2002 a. Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J. Dairy Sci. 85, 1111–1118.

40. Erskine, R. J., Bartlett, P. C., Van Lente, J. L., Phipps, C. R., 2002 b. Efficacy of systemic ceftiofur as a therapy for severe clinical mastitis in dairy cattle. J. Dairy Sci. 85, 2571-2575.

41. Erskine, R. J. 2003. Antibacterial therapy of clinical mastitis–part I. Drug selection. Part II Administration. In: Proceedings of the North American Veterinary Conference: Januray 18–22, January, Orlando, Florida, USA, 13-16.

42. Estonian Animal Recording Centre, 2009. Eesti Jõudluskontrolli aastaraamat. ISSN 1406-734X, 13-15.

43. Estonian Animal Recording Centre, 2012. Eesti Jõudluskontrolli aastaraamat. ISSN 1406-734X, 9-11.

44. EVIRA, 2009. Mikrobilääkkeiden käyttösuositukset eläinten yleisimpiin tulehdus- ja tartuntatauteihin ISBN 978-952-225-032-2, 3/2009, Elintarviketurvallisuusvirasto Evira, Finland.

45. EVIRA, 2012. Mikrobilääkkeiden käyttösuositukset eläinten yleisimpiin tulehdus- ja tartuntatauteihin ISBN 978-952-225-032-2, 3/2009, Elintarviketurvallisuusvirasto Evira, Finland.

46. FAO/WHO/OIE 2007. Report of the Joint FAO/WHO/OIE expert meeting on critically important antimicrobials. Rome, Italy. November 26–30, 2007. Availablehttp://www.who.int/foodborne_disease/resources/Report%20joint%20CIA%20Meeting.pdf. Accessed October 1, 2010.

47. FINRES-Vet 2005-2006. Finnish veterinary antimicrobial resistance monitoring and consumption of antimicrobial agents. Evira publications 22/2007 http:/evira.fi/uploads/WebshopFiles/1198141211941.pdf.

48. Fox. L. K., Chetser, S. T., Hallberg, J. W., Nickerson, S. C., Pankey, J. W., Weawe, L. D., 1995. Survey of Intramammary infections in dairy heifers at breeding age and first parturition. J. Dairy Sci. 78, 1619-1628.

49. Franklin A., Holmberg, O., Horn, M., Rantzie, M., Åström, G., 1984. Effect of procaine benzylpenicillin alone or in combination with dihydrostreptomycin on udder pathogens in vitro and in experimentally infected bovine udders. J. Am. Vet. Res. 45, 1398-1402.

50. Frost, A. J., Hill, A. W., Brooker, B. E., 1982. Pathogenesis of experimental bovine mastitis following a small inoculum of *Escherichia coli*. Res. Vet. Sci. 33, 105-112.

51. Gentilini, E., Denamiel, G., Llorente, P., Godaly, S.,Rebuelto, M., Degregorio, O., 2000. Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. J. Dairy Sci. 83, 1224-1227.

52. Gillespie, B. E., Oliver, S. P., 2005. Simultaneous detection of mastitis pathogens *Staphylococcus aureus*, *Streptococcus uberis*, *and Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. J. Dairy Sci. 88, 3510–3518.

53. Goff, J.P., Horts, R.L., 1997. Physiological changes at parturition and their relationship to metabolic disorders. J. Dairy Sci. 80, 1260-1268.

54. Graber, H. U., Casey, M. G., Naskova, J., Steiner, A., Schaeren, W., 2007. Development of a highly sensitive and specific assay to detect *Staphylococcus aureus* in bovine mastitic milk. J. Dairy Sci. 90, 4661–4669.

55. Grave, K., Greko, C., Nilsson, L., Odensvik, K., Mørk, T., Rønning, M., 1999. The usage of veterinary antibacterial drugs for mastitis in cattle in Norway and Sweden during 1990–1997. Prev. Vet. Med. 42, 45-55.

56. Green, M. J., Leach, K. A., Breen, J. E., Green, L. E.,Bradely, A. J., 2007. National intervention study of mastitis control on dairy herds in England and Wales. Vet. Rec. 160, 287-293.

57. Green, M, Bradley, A. 2010. Practical methods to evaluate treatment outcomes. In the proceedings of the 49th National Mastitis Council Annual Meeting, Albuquerqe, New Mexico, 131-140.

58. Gruet P., Maincent, P., Berthelot, X., Kaltsatos, V., 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. Adv. Drug Deliv. Rev. 50, 245-259.

59. Gröhn, Y. T., Wilson, D. J., González, R. N., Hertl, J. A., Schulte, H., Bennett, G., Schukken, Y. H., 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87, 3358-3374.

60. Grönlund, U., Hulten, C., Eckersall, P. D., Hogarth, C., Persson Waller, K., 2003. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis from dairy cows with chronic sub-clinical mastitis. J. Dairy Res. 70, 379-386.

61. Guerin-Faublee, V., Carret, G., Houffschmitt, P., 2003. *In vitro* activity of 10 antimicrobial agents against bacteria isolated from cows with clinical mastitis. Vet. Rec. 152, 466–471.

62. Güler, L., Ok, Ü., Gündüz, K., Gülcü, Y., Hadimli, H. H. 2005. Antimicrobial susceptibility and coagulase gene typing of *Staphylococcus aureus* isolated from bovine clinical mastitis cases in Turkey. J. Dairy Sci. 88, 3149-3154.

63. Haenni, M., Galofaro, L., Ythier, M., Gidde,y M., Majcherczyk, P., Moreillon, P., Madec, J.Y., 2010. Penicillin-binding protein gene alterations in *Streptococcus uberis* isolates presenting decreased susceptibility to penicillin. Antimicrob Agents Chemother. 54, 1140-1145.

64. Halasa, T., Huijps, K., Osterås, O., Hogeveen, H. 2007. Economic effect of bovine mastitis management: a review. Veterinary Quarterly. 29, 18-31.

65. Hallen-Sandgren, C., Persson Waller, K., Emanuelson, U., 2008. Therapeutic effects of systemic or intramammary antimicrobial treatment of bovine subclinical mastitis during lactation. Vet. J. 175, 108-117.

66. Haltia, L., Honkanen-Buzalski, T., Spiridonova, I., Olkonen, A., Myllys, V., 2006. A study of bovine mastitis, milking procedures and management practices on 25 Estonian dairy herds. Acta Vet. Scan. 48, 22.

67. Haveri, M., Roslöf, A., Rantala L., Pyörälä, S., 2005. Toxin genes of Staphylococcus aureus isolated from bovine intramammary infection of different clinical characteristics and outcome. In: Proceedings of the 4th IDF International Mastitis Conference. Mastitis in dairy production. Current knowledge and future solutions, June 12-15, Maastricht, Netherlands, 149-154.

68. Haveri, M., Hovinen, M., Roslöf, A., Pyörälä, S., 2008. Molecular types and genetic profiles of Staphylococcus aureus strains isolated from bovine intramammary infections and extramammary sites. J. Clin. Microbiol. 46, 3728-3735.

69. Hendriksen, R. S., Mevius, D. J., Schroeter, A., Teale, C., Meunier, D., Buitaye, P., Franco, A., Ultinane, A., Amado, A., Moreno, M., Greko,C., Stärk, K., Berghold, C., Myllyniemi, A.-L., Wasyl, D., Sunde, M., Aarestrup, F. M., 2008. Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet. Scand. 50, 28.

70. Hillerton, J. E., 1999. Redefining mastitis based on somatic cell count. In: International Dairy Federation Bulletin, 245, 4-6.

71. Hillerton, J. E., Kliem, K. E., 2002. Effective treatment of *Streptococcus uberis* clinical mastitis to minimize the use of antibiotics. J. Dairy Sci. 85, 1009-1014.

72. Hiss, S., Mielenz, M., Bruckmaier, M. R., Sauerwein, H., 2004. Haptoglobin concentration in blood and milk after endotoxin challenge and quantification of mammary Hp and mRNA expression. J. Dairy Sci. 87, 3778-3784.

73. Hogan, J. S., Smith, K. L., Hoblet, K. H.,Schonberger, P. S., Todhunter, D.A., Hueston, W.D., Pritchard, D. E., Bowan, G. L., Heider, L. E., Brockett, B.L., 1989. Filed survey of clinical mastitis in low somatic cell counts herds. J. Dairy Sci. 72, 1547-1556.

74. Hovinen, M., Simojoki, H., Pösö, R., Suolaniemi, J., Pyörälä, S., 2010. N-acetyl-β-D-glucosaminidase activity in normal cow milk. In: proceedings of the 8th European Colloquium on Acute Phase Proteins, August 25-27, Helsinki, Finland, 16.

75. Hultgren, J., 2002. Foot/leg and udder health in relation to housing changes in Swedish dairy herds. Prev. Vet. Med. 53, 167-189.

76. Hunter, P. A., Dawson, S., French, G. L., Goossens, H., Hawkey, P. M., Kuijper, E. J., Nathwani, D., Taylor, D. J., Teale, C. J., Warren, R. E., Wilcox, M. H., Woodford, N., Wulf, M. W., Piddock, L. J. V., 2010. Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies. J. Antimicrob. Chemother. 65 (Suppl. 1), 3–17.

77. Hyvönen, P., Suojala, L., Orro, T., Haaranen, J., Simola, O., Røntved, C., Pyörälä, S., 2006. Transgenic cows that produce recombinant human lactoferrin in milk are not protected from experimental *Escherichia coli* intramammary infection. Infect. Immun. 74, 6206-6212.

78. International Dairy Federation. 1999. Suggested interpretation of mastitis terminology. International Dairy Federation Bulletin 338, 3–26.

79. Jarp, J., Bugge, H. P., Larsen, S., 1989. Clinical trial of three therapeutic regimens for bovine mastitis. Vet. Rec. 124, 630-634

80. Jonsson, P., Olsson, S. O., Olofson, A. S., Fätlh, C., Holmberg, O., Funke, H., 1991. Bacteriological investigation of clinical mastitis in heifers in Sweden. J. Dairy Res. 58, 179-185.

81. Kitchen, B. J., Seng Kwee, W., Middleton, G., Andrews, R.J., 1984. Relationship between the level of N-acetyl-β-d-glucosaminidase (NAGase) in bovine milk and the presence of mastitis pathogens. J. Dairy Res. 51, 11–16.

82. Koj, A., 1996. Initiation of acute phase response and synthesis of cytokines. Biochim. Biophys. Acta 1317, 84-94.

83. Koskinen, M. T., Holopainen, J., Pyörälä, S., Bredbacka, P., Pitkälä, A., Barkema, H. W., Bexiga, R., Roberson, J., Sølverod, L., Piccinini, R., Kelton, D., Lehmusto, H., Niskala, S., Salmikivi, L. 2009. Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. J. Dairy Sci. 92, 952-959.

84. Koskinen, M. T., Wellenberg, G. J., Sampimon, O. C., Holopainen, J., Rothkamp, A., Salmikivi, L., van Haeringen, W. A., Lam, T. J. G. M., Pyörälä, S., 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J. Dairy Sci. 93, 5705-5715.

85. Koskiniemi, K., 1982. Observation on the incidence of teat injuries in different cowshed. Nordisk Veterinaermedicin. 34, 13-19.

86. Krömker, V., Grabowski, N. T., Redetzky, R., Hamann, J. 2001. Detection of mastitis using selected quarter milk parameters. In: proceedings of the 2nd International Symposium of Bovine Mastitis and Milk Quality, Vancouver, Canada, September 13-15, 486-487.

87. Krömker, V., Friedrich, J., 2009.Teat canal closure in non-lactating heifers and its association with udder health in the consecutive lactation.Vet. Microbiol. 134, 100-105.

88. Krömker, V., Paduch, J. H., Klocke, D., Friedrich, J., Zinke, C., 2010. Efficacy of extended intramammary therapy to treat moderate and severe clinical mastitis in lactating dairy cows. Berl. Munch. Tierarztl. Wochenschr. 123, 147-152.

89. Krömker, V., Pfannenschmidt, F., Helmke, K., Andersson, R., Grabowski, N. T., 2012. Risk factors for intramammary infections and subclinical mastitis in post-partum dairy heifers. J. Dairy Res. 79, 304-309.

90. Lai, I. H., Tsao, J. H., Lu, Y. P., Lee, J. W., Zhao, X., Chien, F. L., Mao, S. J. T., 2009. Neutrophils as one of major haptoglobin sources in mastitis affected milk. Vet. Res. 40, 17.

91. Landin, H., 2006. Treatment of mastitis in Swedish dairy production. Svensk Veterinärtidning. 58, 19-25.

92. Larsen, T., Rønntved, C. M., Ingvartsen, K. L., Vels, L., Bjerring, M., 2010. Enzyme activity and acute phase proteins in milk utilized as indicators of acute clinical *E.coli* LPS-induced mastitis. Animal 4, 1672-1679.

93. Lehtolainen, T., Schwimmer, A., Shpigel, N. Y., Honkanen-Buzalski, T., Pyörälä, S., 2002. In vitro antimicrobial susceptibility of *Escherichia coli* isolates from clinical bovine mastitis in Finland and Israel. J. Dairy Sci. 86, 3927-3932.

94. Louhi, M., Inkinen, K., Myllys, V., 1992. Relevance of sensitivity testings (MIC) of *S. aureus* to predict the antibacterial action in milk. J. Vet. Med. 39, 253-262.

95. Makimura, S., Suzuki, N. 1982. Quantitative determination of bovine serum haptoglobin and its elevation in some inflammatory diseases. Nippon Juigaku Zasshi 44, 15-21.

96. Makovec, J. A., Ruegg, P. L., 2003. Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994-2001). J. Am. Vet. Med. Assoc. 222, 1582-1589.

97. MARAN, 2008. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2008. www.cvi.wur.nl.

98. Matthews, K. R., Harmon, R. J., Langlois, B. E., 1992. Prevalence of *Staphylococcus* species during the periparturient period in primiparous and multiparous cows. J. Dairy Sci. 75, 1835-1837.

99. Matzke, P., Holzer, A., Deneke, J., 1992. The effect of environmental factors on the occurrence of udder diseases. Tierartztliche Praxis. 20, 21-23.

100. Mattila, T., Sandholm, M., 1986. Antitrypsin and N-acetyl-β-D-glucoseaminidase as markers of mastitis in herd of Ayrshire cows. Am. J. Vet. Res. 46, 2453-2456.

101. McDougall, S. M., 1998. Efficacy of two antibiotic treatments in curing clinical and subclinical mastitis in lactating dairy cows. New Zealand Vet. J. 46, 226-232.

102. McDougall S., 2003. Intramammary treatment of clinical mastitis of dairy cows with a combination of lincomycin and neomycin, or penicillin and dihydrostreptomycin. New Zealand Vet. J. 51, 111-116.

103. McDougall, S. M., Arthur, D. G., Bryan, M. A., Vermunt, J. J., Weir, A. M., 2007 a. Clinical and bacteriological response to treatment of clinical mastitis with one of three intramammary antibiotics. New Zealand Vet. J. 55, 161-170.

104. McDougall, S. M., Agnew, K. E., Cursons, R., Hou, X. X., Compton, C. R., 2007 b. Parenteral treatment of clinical mastitis with tylosin base or penethamate hydriodide in dairy cattle. J. Dairy Sci. 90, 779-789.

105. Middelton, J. R., Luby, C. D., 2008. Short communication *Escherichia coli* mastitis in cattle being treated for *Staphylococcus aureus* intramammary infection. Vet Rec. 162, 156-157.

106. Minst, K., Märtlbauer, E., Miller, T., Meyer, C., 2012. Short communication: *Streptococcus* species isolated from mastitis milk samples in Germany and their resistance to antimicrobial agents. J. Dairy Sci. 95, 6957-6962.

107. More, S. J., Clegg T. A., O´Grady, L. 2012. Insights into udder health and intramammary antibiotic usage on Irish dairy farms during 2003-2010. Irish Vet J. 65, 7.

108. Moretain, J. P., Boisseau, J., 1989. Excretion of penicillins and cephalexin in bovine milk following intramammary administration. Food Add. Contamin. 6, 79-90.

109. Murata, H., Shimada, N., Yoshioka, M., 2004. Current reseach on acute phase proteins in veterinary diagnosis: an overview. Vet. J. 168, 28-40.

110. Myllys, V., 1995. Staphylococci in heifer mastitis before and after parturition. J. Dairy Sci. 62, 51-60.

111. Myllys, V., Rautala. H., 1995. Characterization of clinical mastitis in primiparous heifers. J. Dairy Sci. 78, 538-545.

112. Myllys, V., Asplund, K., Brofeldt, E., Hirvelä-Koski, V., Honkanen-Buzalski, T., Junttila, J., Kulkas, L., Myllykangas, O., Niskanen, M., Saloniemi, H., Sandholm, M., Saranpää, T., 1998. Bovine mastitis in Finland in 1988 and 1995--changes in prevalence and antimicrobial resistance. Acta Vet. Scand. 39, 119-126.

113. National Mastitis Council, 2004. Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality. In: National Mastitis Council publications, 4th edition, Madison, WI, USA.

114. Nevala, M., Taponen, S., Pyörälä, S., 2004: Bacterial etiology of bovine clinical mastitis- data from Saari Ambulatory Clinic in 2002-2003. Suomen Eläinlääkarilehti. 110, 363-369.

115. Nickerson, S. C., Owens, E., Boddie, R. L., 1995. Symposium: mastitis in dairy heifers. Mastitis in dairy heifers: initial studies on prevalence and control. J Dairy Sci. 78, 1607-167.

116. Nielsen, B. H., Jacobsen, S., Andersen, P. H., Niewold, T. A., Heegaard, P. M., 2004. Acute phase protein concentrations in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions. Vet. Rec. 20, 361-365.

117. NORM/NORM-VET., 2003: Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø / Oslo 2004.

118. Nyman, A. K. Emanuellson, U., Gustafsson, A. H., Persson Waller, K., 2009. Management practises associated with udder health of first-parity dairy cows in early lactatation. Prev. Vet. Med. 88, 138-149.

119. OIE., 2012 Guidelines on the responsible and prudent use of antimicrobial agents in veterinary medicine. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.6.9.htm. Accessed in February 2013.

120. Oliver S, Mitchell, B. A., 1983. Intramammary infection in primigravid heifers near parturition. J. Dairy Sci. 66, 1180.

121. Oliver, S. P., Almeida, R. A., Gillespie, B. E., Ivey, S. J., Moorehead, H., Lunn, P., Dowlen, H. H., Johnson, D. L., Lamar, K. C., 2003. Efficacy of extended pirlimycin therapy for treatment of experimentally induced Streptococcus uberis intramammary infections in lactating dairy cattle. Vet Ther. 4, 299-308.

122. Oliver, S. P., Almeida, R. A., Gillespie, B. E., Headrick, S. J., Dowlen, H. H., Johnson, D. L., Lamar, K. C., Chester, S. T., Moseley, W. M., 2004. Extended ceftiofur therapy for treatment of experimentally-induced *Streptococcus uberis* mastitis in lactating dairy cattle. J. Dairy Sci. 87, 3322-3329.

123. Oltenacu, P. A., Bendixen, P. H., Vilson, B., Eksebo, I., 1990. Tramped teats-clinical mastitis disease complex in tied cows. Environmental risk factors and interrelationships with other diseases. Acta Vet.Scan. 31, 471-478.

124. Osterås, O., Sølverød, L., Reksen, O., 2006. Milk culture results in a large Norwegian survey- effects of season, parity, days in milk, resistance, and clustering. J. Dairy Sci. 89, 1010-102.

125. Owens, W. E., Watts, J. L., Boddie, R. L., Nickerson, S. C., 1998. Antibiotic treatment of mastitis: comparison of intramammary and intramammary plus intramuscular therapies. J. Dairy Sci. 71, 3143-3147.

126. Pankey, J. W., Drechsler, P. A., Wildeman, E. E., 1991. Mastitis prevalence in primigravid heifers at parturition. J. Dairy Sci. 74, 1550-1552.

127. Parker, K. L., Compton C. W., Annis F. M, Weir A. M., Mc Dougall S., 2007. Management of dairy heifers and its realtionships with the incidence of clinical mastitis. New Zealand Vet. J. 55, 208-216.

128. Pedersen, L. H., Aalbæk, B., Røntved, C. M., Ingvartsen, K. L., Sorensen, N. S., Heegaard,P. M. H., Jensen, H. E., 2003. Early pathogenesis and inflammatory response in experimental bovine mastitis due to *Streptococcus uberis*. J. Comp. Pathol. 128, 156-164.

129. Peeler, E. J., Green, M. J., Fitzpatrick, J. L., Morgan, K. L., Green, L. E., 2000. Risk factors associated with clinical mastitis in low somatic cell count British dairy herds. J. Dairy Sci. 83, 2464-2472.

130. Persson Waller, K., Colditz, I. G., Flapper, P., Seow, H. F., 1997. Leukocyte and cytokine accumulation in the ovine teat and udder during endotoxin-induced inflammation. Vet.Res. Comm. 20, 101-115.

131. Persson Waller, K., Bengtsson, B., Lindberg, A., Nyman, A., Ericsson Unnerstad, H., 2009 Incidence of mastitis and bacterial findings at clinical mastitis in Swedish primiparous cows – influence of breed and stage of lactation. Vet. Microbiol. 134, 89-94.

132. Persson Waller, K., Nyman, A., Persson, Y., Grönlund Andersson, U., 2011. CNS species and antimicrobial resistance in clinical and subclinical bovine mastitis. Vet. Microbiol. 152, 112-116.

133. Phuektes, P., Mansell, P. D., Browning, G. F., 2001. Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. J. Dairy Sci. 84, 1140–1148.

134. Piepers, S., De Meulemeester, L., de Kruif, A., Opsomer, G., Barkema, H. W., De Vliegher, S., 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J. Dairy Res. 74, 478-483.

135. Pitkälä, A., Haveri, M., Pyörälä, S., Myllys, V., Honkanen-Buzalski, T., 2004. Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. J. Dairy Sci. 87, 2433-2441.

136. Pol, M., Ruegg, P.L., 2007. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. J. Dairy Sci. 90, 262-273

137. Poutrel, B., Stegemann, M. R., Roy, O., 2008. Evaluation of the efficiacy of systemic danofloxacin in the treatment of induced acute *Echerichia coli* bovine mastitis. J. Dairy Res. 75, 310-318.

138. Prescott, J. 2007. Beta-lactam antibiotics: Penam penicillins. In: Antimicrobial Therapy in Veterinary Medicine, 4th edition. S. Giguère, J. F. Prescott, J. D. Baggot, R. D. Walker and P. M. Dowling, Blackwell Publishing Ltd., Oxford, UK, 121-137.

139. Pyörälä, S., Pyörälä, E., 1997. Accuracy of methods using somatic cell count and milk N-acetyl-ß-D-Glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis. J. Dairy Sci. 80, 2820-2825.

140. Pyörälä, S. Pyörälä, E., 1998. Efficacy of parenteral administration of three antimicrobial agents in treatment of clinical mastitis in lactating cows: 487 cases (1989-1995). J. Am. Vet. Med. Assoc., 1998, 212, 407-412.

141. Pyörälä, S., 2003. Indicators of inflammation in the diagnosis of mastitis. Vet. Res. 34, 565-578.

142. Pyörälä, S., Taponen, S., 2009. Coagulase-negative staphylococci – emerging mastitis pathogens. Vet. Mic.134, 3-8.

143. Pyörälä, S., 2011 Treatment of mastitis during lactation. Irish Vet. J. 64, 41-44.

144. Pyörälä, S., Hovinen, M., Simojoki, H., Fitzpatrick, J., Eckersall, P. D., Orro, T., 2011. Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens. Vet. Rec. 168, 535.

145. Pyörälä, S. 2012. Letter to the editor: Comments on Schwaiger et al. (2012). J. Dairy Sci. 95, 4185.

146. Rantala, M., Kaartinen, L., Välimäki, E., Stryrman, M., Hiekkaranta, M., Niemi, A., Saari, L., Pyörälä, S., 2002. Efficacy and pharmacokinetics of enrofloxacin and flunixin meglumine for treatment of cows with experimentally induced *Escherichia coli* mastitis. J. Vet. Pharmacol Ther. 25, 251-258.

147. Ruegg, P. 2010. The application of evidence based medicine to mastitis therapy. Updates on Ruminant Production and Medicine. In: Proceedings of the XXVI World Buiatrics Congress, Santiago, Chile, November 14-18, Santiago, Chile.

148. Rainard P., Riollet, C., 2006. Innate immunity of the bovine mammary gland. Vet. Res. 37, 369–400.

149. Reksen, O., Sølverod, L., Branscum, A. J., Østerås, O., 2006. Relationships between milk culture results and treatment for clinical mastitis or culling in Norwegian dairy cattle. J. Dairy Sci. 89, 2928–2937.

150. Riekerink, O., Barkema, H. W., Kelton, D. F., Scholl, D. T., 2008. Incidence rate of clinical mastitis on Canadian dairy farms J. Dairy Sci. 91, 1366–1377.

151. Roberson, J. R., 2008. Apparent efficacy of blanket clinical mastitis treatment. In: Proceedings of the 41th Annual Convention American Association of Bovine Practitioners, September 25-28, Charlotte, North Carolina, 288.

152. Roberson, J. R., 2012. Treatment of clinical mastitis. Vet. Clin North Am. Food Animal Pract. 28, 271-288.

153. Roesch, M., Doherr, M. G., Schären, W., Schällibaum, M., Blum, J. V., 2007. Subclinical mastitis in dairy cows in Swiss organic and conventional production systems. J. Dairy Res. 74, 86-92.

154. Saini, V., Mc Clure, J. T., Léger, D., Keefe, G. P., Scholl, D. T., Morck, D. W., Barkema, H. W., 2012 Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. J. Dairy Sci. 95, 4319-4332.

155. Sargeant, J. M., Morgan, S. H., Leslie, K. E., Ireland, M. J., Bashiri, A., 1998. Clinical mastitis in dairy cattle in Ontario: Frequency of occurrence and bacteriological isolates. Can. Vet. J. 39, 33-39.

156. Schröder, A., Hoedemaker, M., Klein, G., 2005. Resistance of mastitis pathogens in Northern Germany. Berl. Münch. Tierärztl. Wochenschr. 9/10, 393–398.

157. Schukken, Y. H., Grommers, F. J., Van de Geer, D., Erb, H. N., Brand, A., 1990. Risk factors for clinical mastitis with a low bulk milk somatic cell count: I. Data and risk factors in all cases. J. Dairy Sci. 73, 3463–3471.

158. Schukken, Y. H., Deluyker, H. A., 1995. Design of field trials for the evaluation of antibacterial products for therapy of bovine clinical mastitis. J. Vet. Pharmacol. Ther. 18, 274-283.

159. Schukken, Y. H., Kremer, W. D. J. 2001. Monitoring udder health: objectives, materials and methods. In: Herd health and production management in dairy practise. Wageningen Press, Wageningen, Netherlands, 351-352.

160. Schwarz, S., Silley, P., Shabbir, S., Woodword, N., van Duijkeren, E., Johnson, A. P., Gaastra, W. 2009.Editorial. Assessing the antimicrobial susceptibility of bacteria obtained from animals. Vet. Microbiol. 141, 1-4.

161. Sears, P. M., Mc Carthy, K. K., 2003. Management and treatment of staphylococcal mastitis. Vet. Clin. North Am. Food Anim. Pract. 19, 171-185.

162. Serieys, F., Raquet, Y., Goby, L., Schmidt, H., Friton, G., 2005. Comparative efficacy of local and systemic antibiotic treatment in lactating cows with clinical mastitis. J. Dairy Sci. 88, 93-99.

163. Shpigel, N.Y., Winkler, M., Ziv, G., Saran, A., 1998. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. Prev Vet Med., 35, 1-9.

164. Simojoki, H., Orro, T., Pyörälä, S. 2009. Bovine experimental infection induced by coagulase-negative staphylococci. Vet. Microbiol. 134, 95-99.

165. Smith G. W. 2010. The pharmacologic aspects of mastitis treatment. In: Proceedings of the 49th National Mastitis Council Annual Meeting, Albuquerqe, New Mexico, 98-108.

166. Sol. J., Sampimon, O. C., Barkema, H. W., Schukken, Y. H., 2000. Factors associated with cure after therapy of clinical mastitis caused by Staphylococcus aureus. J. Dairy Sci. 83, 278-284.

167. Sordillo, L., 2005. Factors affecting mammary gland immunity and mastitis susceptibility. Lifestock Prod. Sci. 89, 89-99.

168. Suojala, L., Orro, T., Järvinen, H., Saatsi, J., Pyörälä, S., 2008. Acute phase response in two consecutive experimentally induced *E. coli* intramammary infections in dairy cows. Acta Vet. Scand. 50, 18.

169. Suojala, L., Simojoki, H., Mustonen, K., Kaartinen, L., Pyörälä. S., 2010. Efficacy of enrofloxacin in the treatment of naturally occurring acute clinical *Escherichia coli* mastitis. J. Dairy Sci. 93, 1960-1969.

170. Schröder, A., Hoedemaker, M., Klein, G., 2005. Resistance of mastitis pathogens in Northern Germany. Berl. Münch. Tierärztl. Wochenschr. 9/10, 393–398.

171. SVARM, 2004. Swedish veterinary antimicrobial resistance monitoring. The National Veterinary Institute (SVA), Uppsala, Sweden. ISSN 1650-6332.

172. SVARM, 2007. Swedish veterinary antimicrobial resistance monitoring. The National Veterinary Institute (SVA), Uppsala, Sweden. ISSN 1650-6332.

173. SVARM, 2011. Swedish veterinary antimicrobial resistance monitoring. The National Veterinary Institute (SVA), Uppsala, Sweden. ISSN 1650-6332.

174. Svensson, C., Nyman, A. K., Persson Waller, K., Emanuellson, U., 2006. Effect of housing, management, and healt of dairy heifers on first-lactation udder health in southwest Sweden. J. Dairy Sci. 89, 1990-1999.

175. Swinkels, J. M., Cox, P., Schukken, Lam, T. J. G. H., 2013. Efficacy of extended cefquinome treatmentof clinical *Staphylococcus aureus* mastitis. J. Dairy Sci. 96, 1–10.

176. Ziv, G., 1980. Drug selection and use in mastitis: systemic vs. local therapy. J. Am. Vet. Med. Assoc. 176, 1109-1115.

177. Ziv, G., Storper, M., 1985. Intramuscular treatment of subclinical staphylococcal mastitis in lactating cows with penicillin G, methicillin and their esters. J. Vet. Pharmacol. Therap. 8, 276-283.

178. Taponen, S., Dredge, K., Henriksson, B., Pyyhtiä, A., Suojala, L., Junni, R., Heinonen, K., Pyörälä, S., 2003 a. Efficacy of intramammary treatment with procaine penicillin G vs. procaine penicillin plus neomycin in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria – a double blind field study. J. Vet. Pharm. Ther. 26, 193-198.

179. Taponen, S., Jantunen, A., Pyörälä, E., Pyörälä, S., 2003 b. Efficacy of targeted 5-day combined parenteral and intramammary treatment of clinical mastitis caused by penicillin-susceptible or penicillin-resistant *Staphylococcus aureus.* Acta Vet Scand. 44, 53-62.

180. Taponen, S., Simojoki, H., Haveri, M., Larsen, H. D., Pyörälä, S., 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. Vet Microbiol. 115, 199-207.

181. Taponen, S., Björkroth, J., Pyörälä, S., 2008. Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in single dairy herd. J. Dairy Res. 75, 422-429.

182. Taponen, S., Salmikivi, L., Simojoki, H., Koskinen, T., Pyörälä, S., 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J. Dairy Sci. 92, 2610–2617.

183. Taponen, S., Pyörälä, S., 2009. Coagulase-negative staphylococci as cause of bovine mastitis—Not so different from *Staphylococcus aureus*? Vet. Microbiol. 134, 29-36.

184. Tenhagen, B.- A., Köster, G., Wallmann, J., Heuwieser, W., 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci. 89, 2542-2551.

185. Tenhagen B.- A., Hansen, I., Reinecke, A., Heuwieser, W., 2009. Prevalence of pathogens in milk samples of dairy cows with clinical mastitis and in heifers at first parturition. J. Dairy Res.76, 179-187.

186. Thomson, K., Rantala, M., Hautala, M., Pyörälä, S., Kaartinen, L., 2008. Cross-sectional prospective survey to study indication-based usage of antimicrobials in animals: results of use in cattle. BMC Veterinary Research, 4, 15.

187. Thorberg, B. M., Danielsson-Tham, M. L., Emanuelson, U., Persson Waller, K., 2009. Bovine subclinical mastitis caused by different types of coagulase-negative staphylococci. J. Dairy Sci. 92, 4962-4970

188. Trinidad, P., Nickerson, S. C., Alley, T. K., 1990. Prevalence of intramammary infection and teat canal colonisation in unbred and primigravid dairy heifers. J. Dairy Sci. 73, 465-470.

189. Unnerstad, H. E., Lindberg, A., Persson Waller, K., Ekman, T., Artursson, K., Nilsson-Ost, M., Bengtsson, B. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. Vet. Microbiol. 137, 90-97.

190. Valde, J. P., Hird, D. W., Thurmond, M. C., Österås, O., 1997. Comparison of ketosis, clinical mastitis, somatic cell count, and reproductive performance between free stall and tie stall barns in Norwegian dairy herds with automatic feeding. Acta Vet. Scan. 38, 181-192.

191. van den Borne, B., van Schaik, G., Nielen, M., Lam, T. J. G. M., 2007. Prevalence and incidence of sub clinical mastitis in heifers in a random samples of dairy herds in the Netherlands. In: Heifer mastitis conference. Ghent, Belgium, June 24-26, 65-66.

192. van den Borne B., Nielen, M., van Schaik, M., Melchior, B., Lam, T. J. G. M., Zadoks, R. N., 2010. Host adaptation of bovine Staphylococcus aureus seems associated with bacteriological cure after lactational antimicrobial treatment. J. Dairy Sci. 93, 2550-2558.

193. Vasil, M., 1994. Therapy of clinically apparent forms of mastitis in lactating dairy cows using intra-mammary Sulphamycin (Polfa, PR). Vet Med (Praha). 39, 511-517.

194. Waage, S., Sviland, S., Ødegaard, S. A., 1998. Identification of risk factors for clinical mastitis in dairy heifers: J. Dairy Sci. 81, 1275-1284.

195. Waage, S., Mørk, T., Røros, A., Aasland, D., Hunshamar, A., Ødegaard, S. A., 1999. Bacteria associated with clinical mastitis in dairy heifers. J. Dairy Sci. 82, 712-719.

196. Waage, S., 2001. Case -control study of risk factor for clinical mastitis in postpartum dairy heifers. J. Dairy Sci. 84, 392-399.

197. Wang, Y., Wu, G.-M., Lu, L.-M., Na Ren, G.-W., Cao, X.-Y., Shen, J.-Z., 2008. Marcolide-lincosamide-resistant phenotype and genotype of *Staphylococcus aureus* isolated from bovine clinical mastitis. Vet. Microbiol. 130, 118-125.

198. Watts, J. L., Yancey, R.J., 1994. Identification of veterinary pathogens by use of commercial identification systems and new trends in antimicrobial susceptibility testing of veterinary pathogens. Clin. Microbiol. Rev. 34, 6-56.

199. Wenz, J. R., Garry, F. B., Lombard, J. E., Elia, R., Prentice, D., Dinsmore, R. P., 2005. Short communication: Efficacy of parenteral ceftiofur for treatment of systemically mild clinical mastitis in dairy cattle. J. Dairy Sci. 88, 3496-3499.

200. Wenz, J. R., Fox, L. K., Muller, F. J., Rinaldi, M., Zeng, R., Bannerman, D. D., 2010. Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis. J. Dairy Sci. 93, 2458–2470.

201. Wilson, D. J., Gonzales, R. N., Das, H. H., 1997. Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. J. Dairy Sci. 80, 2592–2598.

202. Witte, W., 2000. Selective pressure by antibiotic use in livestock. Int J Antimicrob Agents. Suppl 1, 19-24.

203. Wolfova, M., Stipkova, M., Wolf, J., 2006. Incidence and economics of clinical mastitis in five Holstein herds in the Czech Republic. Prev. Vet. Med. 77, 48-64.

204. Yassin, A. F., Hupfer, H., Siering, C., Schumann, P., 2011. Comparative chemotaxonomic and phylogenetic studies on the genus *Arcanobacterium* Collins et al. 1982 emend. Lehnen et al. 2006: proposal for Trueperella gen. nov. and emended description of the genus Arcanobacterium. Int. J. Syst. Evol. Microbiol. 61, 1265-1274.

9. SUMMARY IN ESTONIAN

Kliiniliste mastiitide diagnoosimine, ravi tulemuslikkus ja patogeenide antimikroobne resistentsus Eestis

Piimakarjakasvatus on üks tähtsamaid põllumajandusharusid Eestis. Lüpsilehmade tervise säilitamine ja kvaliteetse piima tootmine suurendab Eesti piimatoodete konkurentsivõimet nii Euroopa kui ka maailma turul. Mastiit ehk udarapõletik on piimalehmade levinuim haigus, põhjustades suurt majanduslikku kahju, eeskätt suurenenud ravikulude, saamatajäänud piima ja lehmade enneaegse karjast praakimise tõttu.

Peamiseks põletiku vallandajaks udaras on nisajuha kaudu udarasse tunginud mikroobid. Udara vastuvõtlikkust nakkusele soodustavad lehma vähenenud vastupanuvõime, puudulik lüpsihügieen ja ebasoodsad keskkonnatingimused. Organismi vastureaktsiooniks eri udaranakkustele on erineva raskusastmega põletiku tekkimine. Mastiidid jagatakse kliinilise avaldumise alusel kliinilisteks ja subkliinilisteks. Kliiniliseks mastiidiks nimetatakse põletikku, mille korral vähemalt üks järgnevalt loetletud tunnustest on kliinilisel uurimisel nähtav: üldhaigestumise tunnused (palavik, isutus), lokaalsed tunnused (udara kuumus, paistetus, valulikkus) või makroskoopilised muutused haigestunud udaraveerandi sekreedis (vesisus, kämbud, helbed). Subkliinilise mastiidi korral kliinilised haigustunnused puuduvad, kuid piimas on põletikuindikaatorite sisaldus suurenenud. Peamiseks määratavaks põletikuindikaatoriks on somaatiliste rakkude arv (SRA). Rahvusvahelise Piimandusföderatsiooni (IDF) soovituste kohaselt loetakse udaraveerand põletikuliseks, kui udaraveerandi SRA on piimas üle 100 000 raku milliliitri kohta ja piimast isoleeritakse haigusttekitav mikroob.

Tänapäeval on teada üle 150 mastiiti põhjustava mikroobiliigi, kuid nendest 18–20 mikroobiliiki põhjustavad ligikaudu 90% kõikidest kliinilistest ja subkliinilistest mastiidijuhtudest. Udarapatogeenide mikrobioloogilise diagnoosimismeetodi kõrval on väga kiiresti arenemas molekulaarsed diagnostikameetodid. 2010. aastast saadik on nii Eestis kui ka mitmel pool maailmas rutiinselt kasutusele võetud polümeraasi ahelreaktsiooni (PCR)-meetodil põhinev mastiididiagnostika. See väga tundlik ja spetsiifiline diagnostikameetod võimaldab piimaproovist määrata 11 erinevat mastiidipatogeeni ja beetalaktamaasi produktsiooni

kodeerivat geeni. Kuivõrd mitmed udaranakkusi põhjustavad mikroobid elavad ka terve lehma nisa nahal või lehmi ümbritsevas keskkonnas, siis nende mikroobide tuvastamine piimaproovis võib olla põhjustatud proovi saastumisest. Seetõttu ei ole harv olukord, kus ühest piimaproovist määratakse enam kui ühe mastiidipatogeeni DNA ja selliste analüüsivastuste tõlgendamine on väga keeruline.

Kliinilise mastiidi esinemissagedus varieerub, sõltudes farmist ja isegi regioonist, kõikudes 2–60 juhuni 100 lehma kohta aastas. Subkliinilise mastiidi esinemissagedus võib olla veelgi suurem. Kliinilisse mastiiti võivad haigestuda igas vanuses ja laktatsioonijärgus olevad lehmad, kusjuures suurimat majanduslikku kahju põhjustab esimest laktatsiooni lüpsvate lehmade laktatsiooni alguses tekkiv kliiniline mastiit. Üldiselt arvatakse, et mullika udar on nakkusvaba ja udaratervisega seotud probleemid tekivad hiljem. Siiski näitavad mitmed uuringud udaranakkuste olemasolu mullikate udaras juba enne poegimist.

Lüpsilehmade ja -karjade arv Eestis on viimaste aastate jooksul vähenenud. Eesti Jõudluskontrolli Keskuse statistikast selgub, et 2009. aastal oli Eestis kokku 1024 veisekarja, kus peeti ligemale 95 000 lüpsilehma. 2012. aastaks oli karjade arv 833, milles peeti kokku 90 274 lehma. 1990. aastate keskpaigani peeti lehmi peamiselt lõastatult, kuid alates 2000. aastast on järjest suurenenud vabapidamisega lüpsilautade osakaal. Vaatamata pidamisviisi muutmisele, uute lüpsiseadmete paigaldamisele ja hügieenivõtete täiustumisele farmides ei ole Eesti lüpsikarjade keskmine SRA oluliselt vähenenud. Samas ei ole Eestis senini täpsemalt uuritud kliiniliste mastiitide esinemissagedust, mastiiditekitajate jaotumist ega nende antibiootikumiresistentsust.

Kliiniline mastiit põhjustab lehmale valu, mistõttu õigeaegne ja adekvaatne ravi on lehma heaolu seisukohalt väga tähtis. Antibakteriaalse ravi eesmärgiks on kiire bakterite elimineerimine udarast ja seeläbi kudede kahjustuse vähendamine. Ligikaudu 70% kogu lehmadele manustatavast antibiootikumikogusest kasutatakse just mastiidiravis. Erinevaid antibakteriaalse ravi skeeme on mastiidiravis kasutatud enam kui 50 aastat, kuid üksmeelt kõige efektiivsema, ohutuma ja majanduslikult parima ravi osas ei ole saavutatud.

Antibiootikumide pideva kasutamise negatiivse kõrvalmõjuna on mikroobidel arenenud resistentsus antibiootikumide suhtes.

Rahvusvahelised organisatsioonid, nagu Rahvusvaheline Episootiate Büroo (OIE) ja Maailma Terviseorganisatsioon (WHO), pööravad suurt tähelepanu resistentsusalase olukorra analüüsimisele ning mitmed riigid on välja töötanud juhendmaterjalid antibiootikumide otstarbekaks kasutamiseks loomadel. Eestis on viimastel aastatel mastiidiravis kasutatud valdavalt laia toimespektriga antibiootikume. Näiteks 2009. aastal oli kliinilise mastiidi raviks registreeritud 18 erinevat nisasüstalt, mis sisaldasid 15 erinevat toimeainet või nende kombinatsiooni. Laia toimespektriga antibiootikumide sage ja põhjendamatu kasutamine lehmadel võib kaasa tuua resistentsete mikroobide leviku, kelle levik inimestele on võimalik toiduahela kaudu.

Käesoleva uurimistöö eesmärgid olid:

1) hinnata esmaspoegijatel kliinilisse mastiiti haigestumise esinemust erinevate pidamistehnoloogiate korral ja selgitada välja kliinilise mastiidi haigustekitajad poegimise päeval nii esmaspoegijatel kui ka korduvpoegijatel (artikkel I);
2) leida seoseid kvantitatiivse PCR meetodiga diagnoositud kliinilist mastiiti põhjustavate mikroobide ja udaras tekkiva paikse põletikureaktsiooni vahel (artikkel II);
3) välja selgitada Eesti karjades levivad mastiidipatogeenid ja nende antibiootikumiresistentsus (artikkel III);
4) hinnata nisajuha kaudu ja lihasesisese penitsilliinravi tulemuslikkust grampositiivsete haigustekitajate põhjustatud kliinilise mastiidi korral (artikkel IV).

Esmaspoegijate ja korduvpoegijate kliinilise mastiidi hindamiseks koguti andmeid üheteistkümnest Eesti karjast üheaastase katseperioodi (2004–2005) jooksul. Kokku poegis katseperioodil 1063 mullikat ja 2355 lehma. Läbiviidud juhtkontrolluuringus loeti juhtudeks (n = 68) kõik äsjapoeginud 1. laktatsiooni lehmad, kellel diagnoositi poegimise päeval kliiniline mastiit. Ülejäänud äsjapoeginud 1. laktatsiooni lehmi kasutati kontrollidena (n = 995). Kliinilise mastiidi diagnoosimise järel võeti kõikidelt esmas- ja korduvpoegijatelt steriilselt piimaproovid bakterioloogiliseks uuringuks. Logistilise regressioonanalüüsiga hinnati kliinilise mastiidi, laudatüübi ja mullikate poegimislauta viimise omavahelisi seoseid. X^2 testiga hinnati esmas- ja korduvpoegijatelt isoleeritud haigustekitajate erinevust.

Poegimise päeval diagnoositi kliiniline mastiit 6,4%-l esmaspoegijatest, mis on samaväärne teiste riikide uurimustulemustega. Risk haigestuda kliinilisse mastiiti oli üle kahe korra suurem (OR 2,44, $p < 0,001$) neil mullikatel, keda peeti lõaspidamisega lüpsilautades võrreldes vabapidamislautades poeginud mullikatega. Suurim risk haigestuda kliinilisse mastiiti poegimise päeval (OR = 3,74 $p < 0,0001$) oli nendel mullikatel, kes toodi lõaspidamisega lüpsilauta vähem kui kaks nädalat enne loodetavat poegimist. Vabapidamisega lüpsilauta toomise ja kliinilise mastiidi tekkimise vahel olulist seost ei leitud. Esmaspoegijate peamisteks udarapatogeenideks oli *Escherichia coli* (*E. coli*) (22,1%), *Streptococcus uberis (Str.uberis)* (19,1%) ja koagulaasnegatiivsed stafülokokid (KNS) (8,8%). Korduvpoegijatelt võetud piimaproovidest seevastu isoleeriti poegimise päeval kõige enam *Staphylococcus aureus´t (S. aureus)* (11,2%). *E. coli*, *Str. uberis* ja *S. aureus* olid haigustekitajad, mille esinemus poegimise päeval esmaspoegijatel ja korduvpoegijatel oluliselt erines. *E. coli* põhjustas enim kliinilisi mastiite vabapidamisega lüpsilautades, koagulaasnegatiivsed stafülokokid ja *Str. uberis* aga lõaspidamisega lüpsilautades. Statistiliselt olulist erinevust pidamistehnoloogiate ja mastiidipatogeenide esinemuse vahel siiski ei tuvastatud.

Väitekirja teine uurimus otsis seoseid kvantitatiivse PCR-meetodiga diagnoositud kliinilist mastiiti põhjustavate mikroobide ja udaras tekkiva paikse põletikureaktsiooni vahel. Piimaproovid (n = 281) koguti kliinilist mastiiti põdevatelt lehmadelt kolmest Eesti piimafarmist aastatel 2007–2009. Piimaproovide bakterioloogiline analüüs viidi läbi PCR metoodikaga (Pathoproof Mastitis PCR Assay, Thermo Fisher, Finland). Udara paikse põletikuvastuse hindamiseks määrati piimaproovidest ägeda faasi valgud: piima amüloidA (MAA) ja haptoglobiin ning kahjustatud udarakoest ja valgelibledest vabanev N-atsetüül-β-D-glükoosaminidaasi (NAGaas) aktiivsus. Logistilise regressioonanalüüsiga hinnati mastiidi kliiniliste tunnuste ja ägeda faasi valkude omavahelist seost. Lineaarse segamudeliga otsiti seoseid piima amüloidA ja haptoglobiini kontsentratsiooni, NAGaasi aktiivsuse ja PCR meetodil isoleeritud haigustekitajate vahel.

Kogutud 281st kliinilise mastiidi piimaproovist 28 proovi olid bakterioloogiliselt negatiivsed ja 27 proovi sisaldas enam kui nelja haigustekitajat (saastunud proovid). Allesjäänud 226 proovist tuvastati üks bakteriliik 68 proovis (30,1%), kaks bakteriliiki 99 (43,8%) ja kolm

bakteriliiki 59 (26,1%) proovis. Mida tugevamad oli kliinilised tunnused, seda suurem oli ka ägeda faasi valkude ja NAGaasi kontsentratsioon piimas. Uurimistulemused näitasid, et piimaproovis sisalduva haigustekitaja bakteriaalse DNA hulk ja udara paikne põletikuvastus on omavahel suurel määral seotud. Isoleeritud haigustekitajad põhjustasid erineva ulatusega põletikuvastuse. Nende udaraveerandite piimaproovides, kus leiti suures koguses *E. coli* või *Streptococcus dysgalactiae (Str. dysgalactiae)* DNAd, oli ägeda faasi valkude kontsentratsioon oluliselt suurem võrreldes nende udaraveerandite piimaproovidega, kus nimetatud baktereid ei esinenud. Udaraveerandites, kus oli suures koguses *Trueperella pyogenes´e* (*T. pyogenes'e)* ja *Str. uberis'e* bakteritest pärinevat DNAd, oli oluliselt suurem haptoglobiini ja NAGaasi aktiivsus, kuid MAA ei reageerinud peaaegu üldse. Piimaproovides, kus esines vähesel määral KNSi või *Corynebacterium bovis't* (*C. bovis*) oli ka oluliselt väiksem ägeda faasi valkude ja NAGaasi aktiivsus võrreldes proovidega, kus neid bakteriliike ei leitud. Uurimistulemustest saab järeldada, et mida suurem oli piimaproovides *E. coli*, *Str. dysgalactiae* ja *Str. uberis'e* hulk, seda suurema tõenäosusega need bakterid põletikureaktsiooni esile kutsuvad. Kui piimaproovis on väiksemas kontsentratsioonis veel mõni bakteriliik lisaks, siis selle mõju põletikureaktsiooni tekkimisele on ebaoluline. KNSi ja *C. bovis* olemasolu proovis aga hoopis vähendas põletikureaktsiooni tugevust.

Eesti karjades levivate mastiidipatogeenide ning nende antibiootikumiresistentsuse väljaselgitamiseks kasutati 2007–2009 aastatel Eesti veterinaar- ja toidulaboratooriumisse (VTL) saadetud kliiniliste ja subkliiniliste mastiitide korral võetud piimaproovide andmeid. Antibiootikumitundlikkust hinnati disk-difusiooni meetodil, kus grampositiivsetel mikroobidel määrati antibiootikumitundlikkus penitsilliini, ampitsilliini, tsefalotiini, klindamütsiini, erütromütsiini, gentamütsiini, trimetoprimi/sulfametoksasooli ja tetratsükliini suhtes. Gramnegatiivseid baktereid testiti ampitsilliini, gentamütsiini, trimetoprimi/sulfametoksasooli, tetratsükliini, enrofloksatsiini, streptomütsiini, neomütsiini ja tsefaperasooni suhtes. Logistilise regressioonanalüüsiga hinnati bakterioloogilise uuringu tulemuse seost farmi suuruse, geograafilise asukoha ja uuringuaastaga.

Kokku uuriti kolme aasta jooksul VTLis bakterioloogiliselt 3058t kliinilise mastiidi piimaproovi 190 karjast ja 5145t subkliinilise mastiidi korral võetud piimaproovi 274 karjast. Mastiiti põhjustavate mikroobide

puhaskultuurid isoleeriti 57%-l proovidest, bakterioloogiliselt negatiivsete proovide osakaal oli 22,3% ja segakasvuga proove kogunes 20,6%. Mikroobide segakasvu sisaldavaid piimaproove oli palju rohkem subkliinilise mastiidi korral võetud piimaproovide hulgas võrreldes kliinilise mastiidi korral võetud proovidega. Seevastu tõenäosus leida bakterioloogiliselt negatiivne proov oli suurem kliinilise mastiidi korral võetud piimaproovidest. Kliinilisi mastiite põhjustasid enim *Str. uberis* (18,4%), *E. coli* (15,9%) ja *Streptococcus agalactiae (Str.agalactiae)* (11,9%), subkliinilisi aga *S. aureus* (20%) ja koagulaasnegatiivsed stafülokokid (15,4%). *Str. agalactiae* isoleeriti oluliselt sagedamini ($p < 0,05$) piimaproovidest, mis pärinesid üle 600 lehmaga farmidest. *S. aureus'e* esinemine seostus ($p < 0,05$) väikeste, alla 30 lehmaga farmidega. Selle uuringu tulemused näitasid, et Eestis on kliinilisi mastiite põhjustavate bakterite resistentsus antibiootikumide suhtes väga suur. Isoleeritud *S. aureus'e* ($n = 174$) resistentsus penitsilliini suhtes oli 61,6% ja koagulaasnegatiivsete stafülokokkide resistentsus ($n = 173$) 38,5%. Streptokokkide hulgas esines resistentsust tetratsükliini ja gentamütsiini suhtes. Suur oli ka stafülokokkide ja streptokokkide resistentsus makroliidide suhtes. *E. coli* isolaatidest 24,3%, 15,6% ja 13,5% olid resistentsed vastavalt ampitsilliini, streptomütsiini ja tetratsükliinide suhtes.

Väitekirja neljandas uurimuses hinnati kliinilise udarapõletiku korral läbiviidava lihasesisese ja nisajuhakaudse penitsilliinravi efektiivsust grampositiivsetesse haigustekitajatesse. Kliinilise mastiidi ravikatse viidi läbi neljas eesti lüpsikarjas 2007–2009 aastatel. Kliinilise mastiidi diagnoosimise järel võeti lehmalt piimaproov ja olenevalt ravirühmast alustati ravi kas lihasesisese või nisajuhakaudse ainult bensüülpenitsilliini sisaldava preparaadiga. Lihasesisesel ravil kasutati annust 20 mg/kg kehamassi kohta ja nisajuhakaudsel ravil 600 mg üks kord päevas. Ravi kestis mõlemas rühmas viis päeva. Kui esialgne bakterioloogiline uuring kinnitas gramnegatiivsete või penitsilliiniresistentsete stafülokokkide olemasolu, muudeti ravi. Lõplik piimaproovide bakterioloogiline uurimine viidi läbi PCR-metoodikal põhineva kommertsiaalse testiga (Pathoproof Mastitis PCR Assay, Thermo Fisher, Finland). Lehma paranemist hinnati kolmandal või neljandal nädalal kliiniliste tunnuste, piimaproovi bakterioloogilise leiu ja põletikureaktsiooni muutuse alusel. Põletikureaktsiooni hindamiseks määrati piimaproovidest NAGasi aktiivsus. Uuringus olevatelt lehmadelt määrati üldpiima SRA kuue

ravijärgse kuu jooksul ja hinnati lehmade karjas püsimist. Logistilise regressioonanalüüsiga hinnati bensüülpenitsilliini manustamise koha (lihasesisene või nisajuhakaudne) mõju lehma kliinilisele ja bakterioloogilisele tervistumisele. Lineaarseid segamudeleid kasutati põletikureaktsiooni, bensüülpenitsilliini manustamise koha ja isoleeritud haigustekitajate omavaheliste seoste leidmiseks.

Kliinilise mastiidi ravikatses oli 140 lehma. Lihasesisesi raviti bensüülpenitsilliiniga 61 ja nisasisesi 79t penitsilliinitundlike udarapatogeenide põhjustatud mastiidijuhtu. Ülejäänud juhtudel (n = 32) kasutati raviks kloksatsilliini. Kokku tervistus kliiniliselt 108 (77,1%) ja bakterioloogiliselt 77 (55.0%) bensüülpenitsilliiniga ravitud udaraveerandit. Uuringust selgus, et lehma kliiniline ja bakterioloogiline tervistumine ei sõltunud sellest, kas bensüülpenitsilliin manustati lihasesse või nisajuhasse. Põletikureaktsioon oli pärast ravi oluliselt nõrgem nendes udaraveerandites, kus bakterioloogiline leid oli negatiivne võrrelduna endiselt bakterioloogiliselt positiivsete (mittetervistunud) udaraveeranditega.

10. ACKNOWLEDGEMENTS

This study was carried out at the Institute of Veterinary Medicine and Animal Sciences, the Estonian University of Life Sciences in cooperation with the Department of Production Animal Medicine, Faculty of Veterinary Medicine, the University of Helsinki.

The study was supported by the Estonian Ministry of Agriculture (Research contract 8-2/T8010), the Estonian Ministry of Education and Research (Research project 8-2/T9001) and the Estonian Science Foundation (ETF) grant no 5733 and also was funded by grants from Walter Ehrström Foundation, Finnish Dairy Association, Finnish Veterinary Foundation and the Research Foundation of Veterinary Medicine.

Research projects are a team work and I wish to express my sincere gratitude to all co-authors of my research papers.

My deepest gratitude goes to my supervisor Toomas Orro. You came back to our institute in time and started guiding me even though I had lost faith in myself. Without your support, fantastic inspiration and patience this PhD project would have hardly been finished.
I would also render warm thanks to my second supervisor Kalle Kask for advice and encouragement to move forward.

My gratitude goes to the people of Estonian Food and Veterinary Laboratory departments in Tartu, Saaremaa and Tallinn. You have always been friendly and helped me a lot with collecting data. Liidia Häkkinen, I warmly thank you for your patience, friendship and invaluable support in any questions I had ever happened to ask.

I wish to express my sincere gratitude to my Finnish co-workers, Heli Simojoki, Suvi Taponen, Katja Mustonen and Jani Holopainen. It was great honour to work with you and to be a part of your mastitis research group. Thank you, Prof. Satu Pyörälä, for sharing your endless knowledge of mastitis, critical reading and very constructive revisions of my manuscripts. I learned a lot from you!

I owe my warm thanks to the academic staff and veterinarians of the

Department of Therapy and Large Animal Clinic, especially to Madis Aidnik for introducing me "a magical world of mastitis pathogens" first.

Thank you, my undergraduate students Kadri Veski, Kadri Kaugerand and Maiju Hänninen for your assistance in collecting milk samples, analysing data and teaching me how to be a supervisor.
This work could have not been possible without enormous support of farmers and veterinarians participating in different studies. Your understanding and interest changed my work very easy and I would continuo like to share my knowledge with you.

I am very grateful to Liisa Hansson for valuable language revision of my manuscript.

My parents, you have always agreed with my choices and I can never express enough gratitude for your support.

Finally, my lovely husband Kalmer and fantastic sons Ott-Erik and Uku Villem, thank you for helping, supporting and taking care of me in so many ways. You are always in my heart.

I

ORIGINAL PUBLICATIONS

Kalmus, P., Viltrop, A., Aasmäe, B., Kask, K., 2006.
OCCURRENCE OF CLINICAL MASTITIS IN PRIMIPAROUS
ESTONIAN DAIRY COWS IN DIFFERENT HOUSING
CONDITIONS.
Acta Veterinaria Scandinavica, 48, 21.

Acta Veterinaria Scandinavica

BioMed Central

Research

Open Access

Occurrence of clinical mastitis in primiparous Estonian dairy cows in different housing conditions

Piret Kalmus*†1, Arvo Viltrop†2, Birgit Aasmäe†3 and Kalle Kask†1

Address: [1]Department of Therapy, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, 51014 Tartu, Estonia, [2]Department of Infectious Diseases, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, 51014 Tartu, Estonia and [3]Department of Animal Environment and Health, Tartu, Estonia

Email: Piret Kalmus* - piret.kalmus@emu.ee; Arvo Viltrop - arvo.viltrop@emu.ee; Birgit Aasmäe - birgit.aasmae@emu.ee; Kalle Kask - kalle.kask@emu.ee

* Corresponding author †Equal contributors

Published: 21 November 2006

Acta Veterinaria Scandinavica 2006, **48**:21 doi:10.1186/1751-0147-48-21

Received: 14 August 2006
Accepted: 21 November 2006

This article is available from: http://www.actavetscand.com/content/48/1/21

Abstract

Background: Objectives of the study were to document the impact of some management factors on the occurrence of clinical mastitis in primiparous dairy cows and to identify common udder pathogens of clinical mastitis in freshly calved heifers and multiparous cows on the day of calving.

Methods: A one-year study was conducted during 2004 and 2005 in 11 selected Estonian dairy herds. Data consisted of 68 heifers with clinical mastitis and 995 heifers without clinical mastitis on the day of calving. Multivariable logistic regression with a random herd effect was used to investigate any association between housing system or the time interval from movement of heifers to the calving facility and day of calving on occurrence of clinical mastitis. Milk samples for bacteriological analysis were collected from affected heifers and multiparous cows on the day of calving

Results: Clinical mastitis occurrence in the study population of freshly calved heifers equalled 6.1 %. Housing system was not a significant risk factor for clinical mastitis of freshly calved heifers.

Moving heifers to the cowbarn less than two weeks before calving in tiestall farms increased risk (OR = 5.9 p = 0.001) for clinical mastitis at parturition. The most frequently isolated udder pathogens among heifers were *Escherichia coli* (22.1%), *Streptococcus uberis* (19.1%) and coagulase-negative staphylococci (8.8%). In comparison, the main pathogen in multiparous cows with clinical mastitis at parturition was *Staphylococcus aureus* (11.2%).

Conclusion: Moving heifers to the calving facilities too late in tiestall farms increased risk for clinical mastitis at parturition. The isolated udder pathogens did not differ significantly in tiestall farms compared to freestall farms in heifers, but differences were found between heifers and multiparous cows at parturition.

Background

Mastitis is an economically important disease for dairy cattle production worldwide. Although replacement heifers are generally expected to have good udder health, many studies have identified a high risk of their developing subclinical mastitis during early lactation and

reported that the prevalence of intramammary infections (IMI) is high in the peripartum period [1-7], mainly depending on infectious species [8]. At the same time, published reports on clinical mastitis incidence in freshly calved heifers are scarce and controversial. A nested case-control study in Norway showed that 5 % (6,410 out of 128,027) cases of clinical mastitis was treated in first calving heifers [9]. In Finland, the frequency of treatments for heifer mastitis from one week before to one week after calving was 3.9% for Ayrshires and 5.6% for Frisians [10]. In a study conducted in Netherlands the rate of clinical mastitis around parturition was found to be higher in heifers (>30%) compared to older cows (13%) [11].

Coagulase negative staphylococci (CNS), *Streptococcus dysgalactiae* (*Str. dysgalactiae*) and coliforms have been the most commonly identified pathogens of clinical mastitis during the periparturient period in heifers [12,13]. However, in the studies conducted in Norway, *Staphylococcus aureus (S. aureus*) was the most frequently isolated microorganism, followed by *Str. dysgalacatiae* and CNS [14]. At the same time, differences have been found in occurrence of staphylococcal mastitis between primiparous and multiparous cows, where CNS was more prevalent among cows and *S. aureus* in freshly calved heifers [15]. Some studies have suggested that udder pathogens found in heifers close to parturition are similar to mammary pathogens found in lactating cows [1,12]. On the other hand, the risk of *S. aureus* IMI was influenced by the amount of time the heifers were housed with older cows and by the proportion of *S. aureus*- infected cows in the herd [16]. In Estonia, the most common pathogens of clinical mastitis are *S. aureus* (20.5% of isolated bacteria), CNS (11%), *Streptococcus agalactiae* (*Str. agalactiae*) (10.7%) and *Streptococcus uberis* (*Str. uberis*) (10.5%) [17]. No data are available on udder health in freshly calved heifers and multiparous cows in Estonia, although clinical mastitis has frequently been observed at parturition. Management factors at the herd level, including housing, feeding and milking systems, affect the incidence of clinical mastitis [18-21]; whereas at the individual cow level, milk leakage, teat and udder oedema and blood in the milk are associated with mastitis incidence [22]. Both types of associations are dependent upon species of udder pathogens that are present [23]. The transition phase, typically defined as the period from 3 weeks before to 3 weeks after parturition, is viewed as a critical time in the lactation cycle of a dairy cow. During this period, the cow experiences a series of nutritional, physiological and social changes which render her more vulnerable to infectious and metabolic diseases [24].

The aims of this study were:

1) to study whether mastitis occurrence in first calving heifers differs between farms with different housing systems and whether it is affected by the time interval between movement of heifers to their calving facility and their day of calving.

2) to identify common udder pathogens of clinical mastitis in first-calving heifers and multiparous cows on the day of calving in Estonia

Methods

Study population and experimental design

The one year study was carried out during 2004 and 2005. Eleven large-scale Estonian dairy herds was used in this study. These herds were selected from among the herds who received regular herd health visits by the university large animal clinic (in total 25 herds). The herds having more than 100 cows and 50 replacement heifers calving per year were included into the study. In Table 1, the main characteristics of the selected herds are presented. All heifers that calved during the observation period (n = 1,063) were eligible for inclusion. Heifers with clinical mastitis on the day of calving were included as cases (n = 68), and the remaining freshly calved heifers (n = 995) were controls. Heifers on each farm were moved from their rearing facility to the milking farm according to the availability of space. The number of days between the day of transfer of the heifer to the cowshed and the day of calving was recorded.

Data collection in cases of clinical mastitis

Local trained veterinarians collected milk samples during the first milking from all freshly calved heifers and multiparous cows on the day of calving. If milk from a quarter had abnormal viscosity (watery, thicker than normal), color(yellow, blood-tinged) or consistency(flakes or

Table 1: Characteristics of farms used in the study

	Tied housing	Loose housing
Number of herds	6	5
Average herd size (min; max)	259(200–350)	318(130–460)
Average milk yield per herd kg/305 d (min; max)	8056(5822–9130)	7194(6206–8061)
Total number of freshly calved heifers	423	640
Average number of calved heifers per herd (min; max)	71(50–82)	128(50–270)

clots), clinical mastitis was diagnosed, and samples from diseased udder quaters were collected for bacteriological examination [25]. Before collection, the teat end was cleaned with 70%-ethanol swabs and allowed to dry. After discarding a few streams of milk, samples (2 to 4 ml) were collected into sterile 10 ml plastic tubes, either frozen at -20°C or cooled to +4°C and transported to the Estonian Veterinary and Food Laboratory. All bacteriological examinations of milk samples were performed according to the standards of the National Mastitis Council [26].

Data analysis

Logistic regression with a random herd effect for controlling clustering was used to analyze the effect of housing system (freestall, tiestall with short stall-length or tiestall with long stall-length) and length of time before calving that the heifers had been moved to the calving facility on the occurrence of clinical mastitis. To simplify the modelling, the continuous variable, number of days from moving heifers to the calving facility and expected parturition, was transformed to a dichotomous variable (≤14 days vs. >14 days classes) in the model. Odds ratios (OR) with a 95% confidence interval (95% CI) were calculated. Statistical significance was assumed at $p \leq 0.005$. These analyses were conducted using Stata 9.2 [27]. A two-sample proportion test was used to estimate statistical significance of differences in occurrence of udder pathogens between first-calving heifers and multiparous cows. These analyses were conducted using statistical software Statistix for Windows 2.0.

Results

Approximately 40% (423) of the first-calving heifers were in tiestall farms and approximately 60% (640) were in freestall farms. The overall occurrence of clinical mastitis at calving of the heifers was 6.4% (n = 68), being 9.7% (n = 41) in tiestall farms compared with 4.1 % (n = 27) in freestall farms. The range of days from moving heifers to the calving facility and expected parturition were from 0 to76, where the median day was 26. The results of logistic regression analysis are shown in Table 2. Housing system only was not a significant risk factor for clinical mastitis of freshly calved heifers. In tiestall farms heifers moved to the calving facility less than two weeks before expected parturition had a higher risk (OR = 5.9 p = 0.001) to develop clinical mastitis at calving than heifers moved more than 14 days before calving.

In total, 303 clinical mastitis cases were identified on the day of parturition in 2,355 multiparous cows (12.8%). Udder pathogens were isolated from 49 (72%) out of 68 cases of clinical mastitis in freshly calved heifers and from 185 (61%) out of 303 cases in multiparous cows.

Bacteriological findings are shown in Table 3. The most frequently isolated bacteria from milk samples of freshly calved heifers were *E.coli* and *Str. uberis*. No clinical mastitis caused by *Str. agalactiae* or *Corynebacterium spp*. was discovered, and only one case of *S. aureus* mastitis was found in heifers. In contrast, *S. aureus* was the most common bacterium isolated from milk of affected multiparous cows, followed by *Str. uberis* and Escherichia coli(*E. coli*). Occurrence differences between heifers and cows were statistically significant for *Str. uberis* (p = 0.037), coliforms (p = 0.0002) and *S. aureus* (p = 0.019).

Figure 1 shows the distribution in tiestall vs. freestall housing systems of udder pathogens isolated from quarter milk samples with clinical mastitis in freshly calved heifers.

Table 2: Summary of logistic modelling of risk factors for clinical mastitis in heifers on the day of calving in eleven Estonian dairy herds.

Risk factor	Number of cases(n = 68)	Number of controls(n = 995)	OR[1]	95% CI OR[2]	P-value
Model 1					
Tiestall, short stall-length (≤ 175 cm), vs. tiestall, long stall-length (> 175 cm)	27/14	214/168	2.12	0.32–14.2	0.43
Freestall vs.tiestall, long stall-length	27/14	613/168	0.60	0.09–3.75	0.58
Model 2					
Freestall	27	613	0.39	0.85–1.83	0.237
Tiestall	41	382	1		
>14 day between movement to calving facility and day of calving	32	419	3.39	1.42–8.07	0.006
>14 days between movement to calving facility and day of calving	36	576	1		
Tiestall and >14 days	16	260	1		
Tiestall and ≤14 days	25	122	5.91	1.98–17.66	0.001
Freestall and >14 days	20	284	0.78	0.13–4.57	0.79
Freestall and ≤14 day	7	329	1.08	0.16–7.05	0.94

[1] Odds ratio
[2] 95% confidence interval odds ratio

Table 3: Bacterial species isolated from milk samples from heifers and multiparous cows having clinical mastitis at parturition

	Heifers		Cows	
Pathogens	%	n	%	n
E.coli*	22.1	15	6.6	20
Str. uberis*	19.1	13	9.9	30
CNS	8.8	6	7.3	22
Lactococcus lactis	4.4	3	5.0	15
Klebisella spp.	4.4	3	2.3	7
Str. spp	2.9	2	3	9
Enterococcus spp	2.9	2	2.3	7
Pseudomonas spp	2.9	2	0.7	2
S.aureus*	1.5	1	11.2	34
Arcanobacterium spp	1.5	1	2.6	8
Str.dysgalactiae	1.5	1	3.6	11
Corynebacterium spp	0	0	2.0	6
Str. agalactiae	0	0	3.3	10
Candida spp	0	0	1.3	4
No growth	25	17	29.4	89
Mixed culture	2.9	2	9.6	29
Total	100.0	68	100.0	303

* The difference between heifers and multiparous cows is statistically significant (

In tiestall herds, 36.6% (n = 15) of the samples were bacteriologically negative or mixed cultures, while the corresponding proportion in freestall herds was 14.8% (n = 4). The most common udder pathogens in both housing systems were *Str. uberis, E. coli* and CNS. Occurrence of colimastitis was higher in freestall farms than in tiestall farms, but *Str. uberis* was more frequent in tiestall farms than

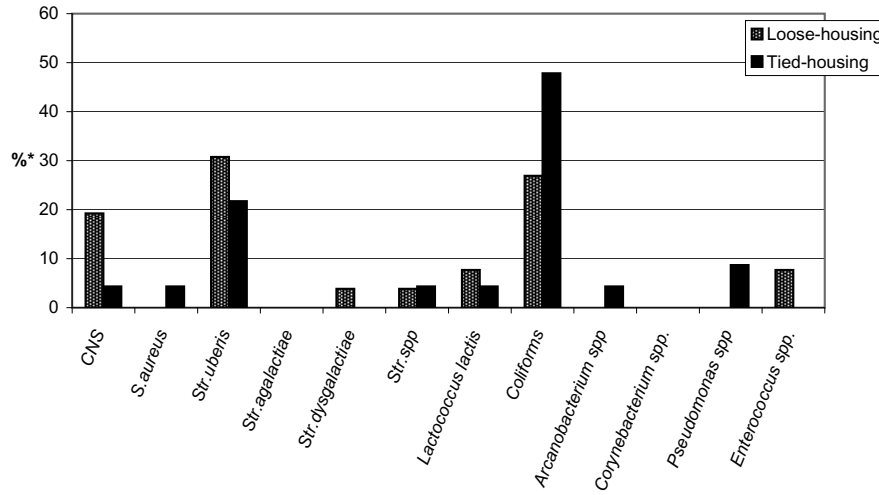

Figure 1
Distribution of udder pathogens in freshly calved heifers in two housing systems. Detailed legend: *calculated against the total number of isolates from heifers of each housing system.

freestall farms. The differences were not statistically significant.

Discussion

In 11 large herds using traditional Estonian dairy management, two housing systems did not differ significantly in clinical mastitis occurrence of first-calving heifers. Others, however, have reported higher incidence of clinical mastitis in tiestall than in freestall housing [21,28-30]. In tiestall farms, the main risk factors for clinical mastitis are teat injuries, short stalls and shortage of bedding material [31,32], especially during the periparturient period [33]. In one Swedish report, the incidence of clinical mastitis decreased across 18 months, after the management system was changed from the tiestall to the freestall system [34]. We did identify an association at the tie-stall farms between time of movement of close-to-term heifers to the milking farm and the occurrence of clinical mastitis. Stress and sudden changes in environmental and management conditions during the peripartum period could weaken natural defence mechanisms in animals, making them more susceptible to clinical mastitis. In tiestall systems, an increased frequency of lying down and rising may lead to increased risk of teat tramping, leading to increased clinical mastitis incidence [35]. Contrarily, in loose-housing systems, cows have sufficient space for lying down and standing up in a more natural way during parturition. The results of the present study reflect the situation in large commercial dairy herds in Estonia. However, the number of herds in the study was limited and because sample sizes were small in some herds, these results should be interpreted with caution. A larger study of longer duration and with more herds is needed for more reliable conclusions.

In the relatively few reports on clinical mastitis in heifers, occurrence of clinical mastitis has been variable. In Finnish studies by Myllys [10], the treatment of clinical mastitis in heifers from one week before through one week after calving increased from 1.8% to 4.4% between 1983 and 1991. In the USA, the incidence of clinical mastitis in heifers was 12.3%, and mostly coliforms and streptococci were isolated [36]. In 1,040 heifers, 1361 clinically affected quarters were found in a large-scale Norwegian study [14]. As to the present investigation, the occurrence of clinical mastitis in freshly calved heifers was a modest 6.1%.

Environmental bacteria dominated in our study. Mainly *E.coli* (22.1%), *Str.uberis* (19.1%) and CNS (9.2%) were isolated in cases of clinical mastitis of the freshly calved heifers. Similar results have been reported by others, in which common bacteria isolated after parturition were CNS, coliforms and streptococci [12,13,36].

In a Danish study, the most frequently isolated organism was *S. aureus* [14]. Our investigation did not show *S. aureus* clinical mastitis in freshly calved heifers, although *S. aureus* was the main pathogen among the multiparous cows. Despite that, the spread of this infection should not be underestimated. Comparing tiestall and freestall farms, the bacterial findings on the day of parturition were generally the same. Coliform infection was more common among loose-housed heifers, where the primary source of infection is bovine faeces and where the secondary multiplication of bacteria to high numbers in bedding and manure is often a risk factor [38]. Prevalence of *Str. uberis* infections depends on udder and calving hygiene, but immune response in the lower udder gland also plays an important role [37]. That might explain the higher prevalence of clinical mastitis in heifers. Altough more CNS infections were found in tiestall farms, we could not draw clear conclusions due to the small number of samples. Our findings confirmed that *S. aureus* could be the main pathogen causing mastitis in multiparous cows at the time of parturition in Estonia. The importance of environmental bacteria may increase if management systems evolve towards higher intensity of production.

Conclusion

Moving heifers to the calving fascilities too late in tiestall farms, increased risk for clinical mastitis at parturition. The isolated udder pathogens did not differ significantly in tiestall farms compared to freestall farms in heifers, but differences were found between heifers and multiparous cows at parturition.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

PK carried out the study, compiled the results and drafted the manuscript. AV participated in the designing the study and analysis of the data. BA coordinated data collection, and KK coordinated the study. All authors were significantly involved in designing the study, intepreting of data and composing the manuscript.

Acknowledgements

The authors are grateful to Estonian State Veterinary Laboratory. This study was financially supported by Estonian Ministry of Agriculture and Estonian Science Foundation (ETF) grant no 5733.

References

1. Oliver S, Mitchell BA: **Intramammary infection in primigravid heifers near parturition.** *J Dairy Sci* 1983, **66:**1180.
2. Trinidad P, Nickerson SC, Alley TK: **Prevalence of intramammary infection and teat canal colonisation in unbred and primigravid dairy heifers.** *J Dairy Sci* 1990, **73:**107.
3. Pankey JW, Drechsler PA, Wildeman EE: **Mastitis prevalence in primigravid heifers at parturition.** *J Dairy Sci* 1991, **74:**1550-1552.

4. Oliver SP, Lewis MJ, Gillespie BE, Dowlen HH: **Influence of prepartum antibiotic therapy on intramammary infections in primigravid heifers during early lactation.** *J Dairy Sci* 1992, **75:**406-408.
5. Roberson JR, Fox LG, Hancock DD, Gay CC, Besser TE: **Coagulase-positive Staphylococcus intramammary infections in primiparous dairy cows.** *J Dairy Sci* 1994, **77:**958-969.
6. Nickerson SC, Owens E, Boddie RL: **Symposium: mastitis in dairy heifers. Mastitis in dairy heifers: initial studies on prevalence and control.** *J Dairy Sci* 1995, **78:**1607-167.
7. De Vliegher S, Barkema HW, Stryhn H, Opsomer G, De Kruif A: **Impact of early lactation somatic cell count in heifers on milk yield over the first lactation.** *J Dairy Sci* 2005, **88:**938-947.
8. Aarestrup FM, Jensen NE: **Prevalence and duration of intramammary infection in Danish heifers during the peripartum period.** *J Dairy Sci* 1997, **80:**307-312.
9. Waage S, Sviland S, Ødegaard SA: **Identification of risk factors for clinical mastitis in dairy heifers.** *J Dairy Sci* 1998, **81:**1275-1284.
10. Myllys V, Rautala H: **Characterization of clinical mastitis in primiparous heifers.** *J Dairy Sci* 1995, **78:**538-545.
11. Barkema HW, Schukken YH, Lam TJ, Beiober GM, Wilmink ML, Benedictus G, Brand A: **Incidence of clinical mastitis in dairy herds grouped in three catergories by bulk milk somatic cell counts.** *J Dairy Sci* 1998, **81:**411-419.
12. Jonsson P, Olsson SO, Olofson AS, Fätlh C, Holmberg O, Funke H: **Bacteriological investigation of clinical mastitis in heifers in Sweden.** *J Dairy Research* 1991, **58:**179.
13. Myllys V: **Staphylocci in heifer mastitis before and after parturition.** *J Dairy Sci* 1995, **62:**51-60.
14. Waage S, Mørk T, Røros A, Aasland D, Hunshamar A, Ødegaard SA: **Bacteria associated with clinical mastitis in dairy heifers.** *J Dairy Sci* 1999, **82:**712-719.
15. Matthews KR, Harmon RJ, Langlois BE: **Prevalence of Staphylococcus species during the periparturient period in primiparous and multiparous cows.** *J Dairy Sci* 1992, **75:**1835-1837.
16. Bassel L, Kelton D, Godkin A, Leslie K, Lissemore K: **Risk factors for intramammary infection at first calving in Ontario dairy heifers.** *Proceedings of the 36th Ann Conv. AABP, Columbus, Ohio, United States of America* 2003:177-178.
17. **Estonian Veterinary and Food Laboratory: Annual report. Tartu.** 2004.
18. Aland A: **Lüpsikarja tervise seiremudel ning selle rakendamine loomade tervise hindamisel ja parandamisel.** *PhD thesis. Estonian Agricultural University, Tartu* 2003.
19. Schukken YH, Grommers FJ, Van de Geer D, Erb HN, Brand A: **Risk factors for clinical mastitis with a low bulk milk somatic cell count: I. Data and risk factors in all cases.** *J Dairy Sci* 1990, **73:**3463-3471.
20. Barkema HW, Van der Ploeg JD, Schukken YH, Lam TJ, Benedictus G, Brand A: **Management style and its association with bulk milk somatic cell count and incidence rate of clinical mastitis.** *J Dairy Sci* 1999, **82:**1655-1663.
21. Valde JP, Hird DW, Thurmond MC, Österås O: **Comparison of ketosis, clinical mastitis, somatic cell count, and reproductive performance between free stall and tie stall barns in Norwegian dairy herds with automatic feeding.** *Acta Vet Scan* 1997, **38:**181-192.
22. Waage S: **Case -control study of risk factor for clinical mastitis in postpartum dairy heifers.** *J Dairy Sci* 2001, **84:**392-399.
23. Barkema HW, Shaken YH, Lam TJ, Briber GM, Benedict's G, Brand A: **Management practices associated with the incidence rate of clinical mastitis.** *J Dairy Sci* 1999, **82:**1643-1654.
24. Goff JP, Horts RL: **Physiological changes at parturition and their relationship to metabolic disorders.** *J Dairy Sci* 1997, **80:**1260-1268.
25. **Bulletin of the International Dairy Federation. No.217.** *Brussels* 1987.
26. Hogan JS, Gonzales RN, Harmon RJ, Nickerson SC, Oliver SP, Smith KL: **Laboratory Handbook on Bovine Mastitis.** *National Mastitis Council Inc, Madison, WI* 1987.
27. **Stata 9.2. 2005 Stata® Statacorp LP, College Station, USA.** .
28. Ekesbo I: **Disease incidence in tied and loose housed dairy cattle.** *Acta Agric Scan* 1966. Thesis
29. Matzke P, Holzer A, Deneke J: **The effect of enviromental factors on the occurence of udder diseases.** *Tierartztliche Praxis* 1992, **20:**21-23.
30. Bakken G, Røn I, Østerås O: **Clinical disease in dairy cows in relation to housing systems.** In *the Proceedings of the 6th Int Congr Anim Hygiene Sweden, Skara* 1988:18.
31. Koskiniemi K: **Observation on the incidence of teat injuries in different cowshed.** *Nordisk Veterinaermedicin* 1982, **34:**13-19.
32. Bendixen PH, Wilson B, Eksebo I: **Disease frequencies in dairy cows in Sweden. V. Tramped teat.** *Preventive Veterinary Medicine* 1988, **6:**17-25.
33. Elbers ARW, Miltenburg JD, De Lange, De Crauwels APP, Barkema HW, Shukken YH: **Risk factors for clinical mastitis in a random sample of dairy herds from the southern part of the Netherlands.** *J Dairy Sci* 1997, **87:**3358-3374.
34. Hultgren J: **Foot/leg and udder health in relation to housing changes in Swedish dairy herds.** *Preventive Veterinary Medicine* 2002, **53:**167-189.
35. Oltenacu PA, Bendixen PH, Vilson B, Eksebo I: **Tramped teats-clinical mastitis disease complex in tied cows. Enviromental risk factors and interrelationships with other diseases.** *Acta Vet Scan* 1990, **31:**471-478.
36. Gröhn YT, Wilson DJ, González RN, Hertl JA, Schulte H, Bennett G, Schukken YH: **Effect of pathogen-specific clinical mastitis on milk yield in dairy cows.** *J Dairy Sci* 2004, **87:**3358-3374.
37. Andrews AH, Blowey RW, Boyd H, Eddy RG: **Bovine Medicine. Diseases and Husbandry of Cattle.** Second edition. Blackwell Science Ltd; 2004.

II

Kalmus, P., Simojoki, H., Pyörälä, S.,
Taponen, S., Holopainen, J., Orro, T., 2013.
MILK HAPTOGLOBIN, MILK AMYLOID A AND NAGASE
ACTIVITY IN BOVINE NATURALLY OCCURING CLINICAL
MASTITIS DIAGNOSED WITH A QUANTITATIVE PCR TEST.
Journal of Dairy Science, 96, 3662 – 3670
Reprinted with permission from The Journal of Dairy Science
(License Number: 3212481301190).

J. Dairy Sci. 96:3662–3670
http://dx.doi.org/10.3168/jds.2012-6177

Milk haptoglobin, milk amyloid A, and *N*-acetyl-β-D-glucosaminidase activity in bovines with naturally occurring clinical mastitis diagnosed with a quantitative PCR test

P. Kalmus,*[1] H. Simojoki,† S. Pyörälä,† S. Taponen,† J. Holopainen,‡ and T. Orro*
*Department of Therapy, Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Tartu, 51014, Estonia
†Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Leissantie 43, FI-04920 Saarentaus, Finland
‡Finnzymes Oy, Keilaranta 16A, FI-02150 Espoo, Finland

ABSTRACT

The associations between quantitative bacteriological results from a real-time PCR test and concentrations of acute-phase proteins (APP) and *N*-acetyl-β-D-glucosaminidase (NAGase) activity in milk in naturally occurring clinical mastitis were investigated. Milk APP concentrations and NAGase activity in clinical mastitis caused by different udder pathogens were studied. The associations between the severity of the clinical signs and concentrations of APP and NAGase activity were estimated. Milk samples from 281 cases of clinical mastitis were collected from 3 Estonian dairy farms and analyzed by PCR to identify pathogens. Twenty-seven samples out of 281 (9.6%) were PCR negative. Milk samples containing 4 or more bacterial species (n = 28) were considered possibly contaminated and excluded from all further analyses. In total, 443 bacterial identifications were made from the remaining 226 milk samples. A single bacterial species was detected in 68 samples (30.1%), 2 species were detected in 99 samples (43.8%), and 3 species were detected in 59 (26.1%) samples. To determine the inflammatory response in the udder, the concentrations of milk amyloid A (MAA) and haptoglobin (Hp) and NAGase activity in the milk were analyzed. A significant positive association was found between the severity of the clinical signs and inflammatory markers in the milk. Milk amyloid A and Hp concentrations and NAGase activity were significantly higher in samples with large quantities of bacterial DNA from *Escherichia coli* or *Streptococcus dysgalactiae* compared with milk samples not containing those species. Large quantities of bacterial DNA from *Trueperella pyogenes* or *Streptococcus uberis* in the milk were associated with elevated concentrations of Hp and high NAGase activity, but not with increased MAA concentrations. Milk samples containing *Corynebacterium bovis* and coagulase-negative staphylococci had significantly lower concentrations of MAA and Hp and lower NAGase activity compared with samples where these species were not detected. It can be concluded that concentrations of APP and NAGase activity in the milk were associated with the quantity of bacterial DNA in the milk samples.

Key words: clinical mastitis, acute-phase protein, *N*-acetyl-β-D-glucosaminidase (NAGase) activity, real-time PCR

INTRODUCTION

Knowledge of the causative pathogens is required for appropriate control and treatment of mastitis. Bacterial culture has been the gold standard for mastitis diagnostics (NMC, 2004), but a commercial PCR-based method has been introduced as a routine method for detection of mastitis-causing bacteria (PathoProof Mastitis PCR Assay; Thermo Fisher Scientific, Espoo, Finland). Due to the greater sensitivity of the PCR test compared with the conventional methods, often resulting in detection of more species per sample, the interpretation of the PCR results is challenging (Koskinen et al., 2010). More research concerning the use and interpretation of PCR mastitis tests in routine use is warranted.

Mastitis-causing bacteria entering the udder quarter via the teat canal, establish IMI with varying degrees of tissue injury. Tissue injury and inflammation initiate an acute-phase response (**APR**), which most commonly begins by releasing inflammatory mediators from tissue macrophages or blood monocytes that gather at the site of damage (Baumann and Gauldie, 1994; Koj, 1996). An APR results in an increase in systemic and local concentrations of acute-phase proteins (**APP**). Two of those proteins, haptoglobin (**Hp**) and serum amyloid A, play a significant role in the early response of the mammary gland to pathogenic bacteria (Eckersall et al., 2001; Nielsen et al., 2004). Haptoglobin is diffused from blood into the milk, but also originates from milk

Received September 18, 2012.
Accepted February 12, 2013.
[1]Corresponding author: piret.kalmus@emu.ee

leukocytes and epithelial cells in the mammary gland (Hiss et al., 2004). Serum amyloid A is secreted by hepatocytes and, in addition, the mammary gland epithelium appears to secrete a mammary gland-specific isoform mammary-associated serum amyloid A 3 (M-SAA3) milk amyloid A (**MAA**; Eckersall et al., 2001).

Local APR in the udder have mostly been studied using experimental models in which pathogenic bacteria such as *Escherichia coli* (Hyvönen et al., 2006; Suojala et al., 2008; Larsen et al., 2010) or staphylococci (Grönlund et al., 2003; Simojoki et al., 2009) have been infused into the udder quarter. These studies showed that *E. coli* increases concentrations of APP in the milk to a greater extent than CNS or *Staphylococcus aureus*. A field study by Pyörälä et al. (2011) concluded that the concentrations of Hp and MAA in milk vary depending on which pathogens are isolated. Concentrations of APP were the highest in cases where mastitis was caused by *E. coli* and significantly lower when mastitis was caused by streptococci or *Staph. aureus*. Milk amyloid A and Hp inflammatory responses were very mild in mastitis caused by CNS. *N*-Acetyl-β-D-glucosaminidase (**NAGase**) is an intracellular, lysosomal enzyme that is released into milk from neutrophils during phagocytosis and cell lysis, but also from damaged epithelial cells, indicating udder tissue destruction (Kitchen et al., 1984). Milk NAGase activity correlates very closely with SCC and can be analyzed also from frozen milk samples (Kitchen et al., 1984).

The first objective of the study was to investigate associations between different quantities of bacterial DNA detected using a PCR-based method and concentrations of APP and NAGase activity in milk from bovines with naturally occurring clinical mastitis. The second aim was to compare milk APP concentrations and NAGase activity in clinical mastitis caused by different udder pathogens and to study the effect of the severity of clinical signs.

MATERIALS AND METHODS

Milk Samples and Clinical Examination

Milk samples from cows diagnosed with clinical mastitis were collected for a treatment study, which was conducted during the period 2007 to 2009 in Estonia. Milk samples originated from 3 different loose-housing dairy farms. Herd sizes ranged from 300 to 1,000 cows. Among those herds, annual mean milk production was 8,500 to 9,800 kg, and the average bulk milk SCC was between 180,000 and 450,000 cells/mL. All herds were milked 3 times per day. Clinical mastitis was diagnosed by trained farm personnel at milking time. Clinical mastitis was defined according to the International Dairy Federation (IDF, 1999), which specifies that at least some visible signs should be present in the udder or in the milk. Systemic and local signs were recorded and categorized on a 3-point scale as follows: (1) mild clinical mastitis: milk from a quarter had abnormal viscosity (watery or thicker than normal), color (yellow or blood-tinged), or consistency (flakes or clots), but no udder swelling or systemic signs; (2) moderate clinical mastitis: similar to mild clinical mastitis, but with the addition of visible or palpable changes in the udder (swelling or pain) without systemic signs; and (3) severe clinical mastitis: both local and systemic signs (fever above 39.2°C). All cow data (affected quarter, score of clinical mastitis, age, and DIM) were recorded.

Once clinical mastitis was diagnosed, a milk sample from the diseased udder quarter was collected for bacteriological examination. Before collection, the teat end was cleaned with 70% ethanol swabs and allowed to dry. After discarding a few streams of milk, samples (2 to 4 mL) were collected into sterile 10-mL plastic tubes and frozen at −20°C until further investigation. Only 1 diagnosed case of clinical mastitis per cow was included in the study.

During the study period, milk samples were collected from 35 cows at farm 1, 123 cows at farm 2, and 105 cows at farm 3; a total of 281 quarter milk samples from 263 cows were included. Eighteen cows had 2 affected quarters.

Real-Time PCR Assay

A commercial real-time PCR test kit (PathoProof Mastitis PCR Assay; Thermo Fisher Scientific) was used for direct analysis of all milk samples. The kit protocol involved 4 separate multiplex real-time PCR reactions, which targeted 11 bacterial species and groups (covering more than 25 mastitis-causing species in total): *Staphylococcus* spp. (including *Staph. aureus* and all relevant CNS species), *Enterococcus* spp. (including *Enterococcus faecalis* and *Enterococcus faecium*), *Corynebacterium bovis*, *E. coli*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis*, *Trueperella (*formerly *Arcanobacterium) pyogenes*/*Peptoniphilus indolicus* (Yassin et al., 2011), *Klebsiella* spp. (including *Klebsiella oxytoca* and *Klebsiella pneumoniae*), and *Serratia marcescens*. The testing was done according to the user's manual and described by Koskinen et al. (2010). Based on the cycle threshold (Ct) values, the bacterial DNA quantity in each targeted bacterial species was grouped into 3 classes: small quantity (+), intermediate quantity (++), or large quantity (+++), according to the manufacturer's manual.

Analytical Methods for Determination of Inflammatory Response in Milk

The concentration of MAA in milk was determined using a commercial ELISA kit (Phase MAA Assay Kit; Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland). Milk samples were initially diluted to 1:500. If the concentration was above the range of a standard curve, they were further diluted as necessary. For very high MAA values, milk samples were diluted up to 1:10,000 (maximum concentration 1,500 mg/L). A 1:100 dilution was used (minimum concentration 0.94 mg/L) for very low values. Intra- and interassay coefficients of variation were <13 and <12%, respectively.

Milk Hp concentrations (mg/L) were determined by a method based on the ability of Hp to bind to hemoglobin (Makimura and Suzuki, 1982) and using tetramethylbenzidine as a substrate (Alsemgeest et al., 1994). The assay is meant to determine concentrations of Hp in the serum, but was adapted here to be used for milk, as described by Hyvönen et al. (2006). Optical densities of the formed complex were measured at 450 nm using a spectrophotometer. Lyophilized bovine acute-phase serum was used as a standard, and calibration was carried out according to the European Union concerted action on the standardization of animal APP (number QLK5-CT-1999-0153). The working range of the assay was 60 to 1,900 mg/L. The interassay and intraassay coefficient of variation values for Hp analysis were <8 and <13%, respectively.

Milk NAGase Activity Determination

Milk NAGase activity was measured by a fluoro-optical method using an in-house microplate modification developed by Mattila and Sandholm (1985) and further modified by Hovinen et al. (2010). The calibrated milk sample was replaced with a control milk sample with a known 4-methyl-umbelliferon (**4-MU**) concentration, and NAGase activity was expressed as picomoles of 4-MU/min per microliter of milk at 25°C. The upper detection limit for NAGase activity was 24.5 pmol of 4-MU/min per microliter. Interassay and intraassay coefficients of variation for NAGase activity were 5 and 4%, respectively.

Statistical Analysis

Stata 10.0 (StataCorp, TX) software was used for statistical analyses. Only milk samples with PCR-negative results or ≤3 pathogen species were analyzed (n = 253). Generalized linear mixed models (GLMM) were used to investigate associations between milk APP and PCR results. The outcome variable MAA was logarithmically transformed, and the inverse square root transformation of Hp was used. As Hp is evaluated using a model in inverse scale, negative model estimates represent higher Hp concentrations. The full models included lactation number as a 4-level categorical variable (1, 2, 3, and ≥4 lactations), DIM was categorized as a 4-level variable according to quartiles (1–19, 20–59, 60–118, and 119–412 DIM), farm as a 3-level variable, and affected quarter as a 2-level variable (fore and hind quarters). These variables were kept in all models to control possible confounding effects. As these variables were not significant in any of the models (except affected quarters in the MAA model), they are not shown in our results. The PCR results by pathogen were included as categorical variables (negative, +, ++, or +++). If PCR results were under 6 cases per level, they were consolidated with the previous factor level as follows: *C. bovis* ++ with *C. bovis* +; *Strep. agalactiae* +++ with *Strep. agalactiae* ++; *Strep. dysgalactiae* +++ with *Strep. dysgalactiae* ++; *T. pyogenes* +++ with *T. pyogenes* ++; and CNS +++ with CNS ++. To account for clustering of data (13 cows had 2 samples from different quarters), cow was included as random factor. The Wald test was used to evaluate the overall significance of the categorical variables with more than 2 levels. Nonsignificant PCR result variables were removed using the stepwise backward elimination procedure. Both final models were tested for interactions between minor pathogens (*C. bovis* and CNS) and major pathogens (*E. coli*, *T. pyogenes*, *Strep. uberis*, and *Strep. dysgalactiae*).

The random effects Tobit regression model for censored data was used to investigate associations between milk NAGase activity and PCR results. The Tobit regression model was chosen because >40% of the NAGase results were over the maximum working limit of the assay (24.5 pmol of 4-MU/min per microliter), which would violate the regression model's assumptions. In the Tobit regression, all cases falling above (or below) a specified threshold value are censored, although these cases remain in the analysis (Long, 1997). A more detailed explanation using Tobit regression for analyzing milk NAGase activity data are given in Pyörälä et al. (2011). Square root transformation of milk NAGase activities was used to achieve a normal distribution of uncensored data; 104 samples were censored at the level of 24.5 pmol of 4-MU/min per microliter. All other model building strategies and variables in each model were as described for the APP models above.

A linear regression model for APP and a Tobit regression model for NAGase were used to investigate the associations of milk APP concentrations and milk NAGase activities with the severity of clinical mastitis (mild, moderate, and severe signs). Assumptions of all

Table 1. Bacterial DNA quantities of udder pathogens detected in 226 milk samples from cases of cows with naturally occurring clinical mastitis[1]

Identified mastitis pathogen (n = 443)	% of all DNA-positive milk samples (n = 226)	No. (%); mean Ct value[2]		
		+	++	+++
Staphylococcus aureus (n = 49)	21.6	12 (24.4); 33.9	19 (38.7); 27.1	18 (36.7); 21.9
CNS (n = 91)	40.2	78 (85.7); 34.6	12 (13.1); 28.3	1 (1.1); 19.3
Streptococcus agalactiae (n = 12)	5.3	2 (16.6); 26.3	6 (50.0); 29.4	4 (33.3); 17.1
Streptococcus dysgalactiae (n = 33)	14.6	14 (42.4); 34.1	17 (51.5); 25.7	2 (6.1); 16.3
Streptococcus uberis (n = 98)	43.3	33 (33.6); 35.0	29 (29.6); 27.3	36 (36.7); 18.1
Trueperella pyogenes (n = 34)	15.0	24 (70.5); 35.1	5 (14.7); 25.1	5 (14.7); 19.4
Corynebacterium bovis (n = 53)	23.4	50 (94.3); 33.7	3 (5.7); 24.6	0
Enterococcus spp. (n = 4)	1.7	4 (100); 35.6	0	0
Escherichia coli (n = 67)	29.6	19 (28.3); 35.6	30 (44.8); 30.2	18 (26.8); 18.5
Klebsiella spp. (n = 2)	0.9	2 (100); 32.5	0	0

[1]+ = small quantities of bacterial DNA [range of cycle threshold (Ct) values: 28.3–37]; ++ = intermediate quantities of bacterial DNA (range of Ct values: 22.1–33.7); +++ = large quantities of bacterial DNA (range of Ct values: 13.4–27.4).
[2]Cycle threshold values of each pathogen were based on the quantity of bacterial DNA.

models were controlled using normality and scatter plots of model residuals.

RESULTS

Bacteriological Findings

Ten different species of udder pathogens were detected in the 281 quarter milk samples using the real-time PCR kit. A total of 27 milk samples (9.6%) were PCR negative, and 254 (90.4%) samples contained DNA of at least 1 target species. Milk samples containing DNA of 4 or more bacterial species (n = 28) were considered possibly contaminated and excluded from further analysis. In total, 443 bacterial identifications were made from the remaining 226 milk samples. A single bacterial species was found in 68 (30.1%), 2 species were found in 99 (40.8%), and 3 species were found in 59 (26.1%) of the DNA-positive milk samples.

The most prevalent bacterial species among the milk samples containing a single pathogen were *Strep. uberis* (n = 20; 29.4%), *Staph. aureus* (n = 14; 20.5%), and *E. coli* (n = 13; 19.1%). *Streptococcus uberis* was detected in 45 (45.4%) and CNS was detected in 40 (40.4%) of the milk samples with 2 bacterial species.

Different quantities and proportions of mastitis-causing bacteria were detected in the milk samples (Table 1). None of the milk samples contained 2 or 3 bacterial species in large quantities simultaneously. Of the 98 milk samples containing DNA from CNS, the quantity was small (+) in 78 of them (85.7%). Bacterial DNA from *Enterococcus* spp. and *Klebsiella* spp. was detected rarely and only in small quantities.

Clinical Signs, APP, and NAGase Activity in Milk

In a total of 253 analyzed mastitis cases, 63.6% (n = 161) exhibited mild clinical signs and 31.2% (n = 79) moderate clinical signs. Severe clinical signs were recorded in 5.1% (n = 13) of the cases. All samples collected from cows with severe mastitis were positive for bacteria, and *E. coli* was detected in 10 of the 13 samples. *Streptococcus uberis* and CNS were the main bacterial species in moderate and mild clinical mastitis. Clinical signs in the cows yielding PCR-negative samples were moderate or mild.

The concentration of MAA in milk ranged between 0.94 and 1,500 mg/L [median: 43.3 mg/L; interquartile range (**IQR**): 16.9–183.3 mg/L], and Hp varied from 59 to 1,890 mg/L (median: 214.1 mg/L; IQR: 105.7–398.6). Of the 253 milk samples, 2 samples contained an MAA concentration below the working limits of the assay, and 13 samples contained an MAA concentration above the working limits of the assay (0.94 and 1,500 mg/L, respectively). Twenty-one samples had Hp concentrations under the working limit of the assay (59 mg/L). The activity of NAGase in milk ranged between 0.53 and 24.5 pmol of 4-MU/min per microliter (median: 19.5 pmol of 4-MU/min per microliter; IQR: 7.9–24.5 pmol of 4-MU/min per microliter), and in 104 milk samples, the NAGase activity was above the working range of the assay (24.5 pmol of 4-MU/min per microliter).

An association between the severity of clinical signs and the concentrations of APP and NAGase activity in the milk was found. In cases of severe clinical mastitis, MAA and Hp concentrations and NAGase activity values were significantly higher compared with cases of clinical mastitis with mild or moderate signs ($P < 0.001$, $P = 0.006$, and $P = 0.021$, respectively). Concentrations of MAA and Hp and NAGase activity in milk from cows with moderate clinical mastitis were significantly higher than values measured in milk from cows with mild clinical signs ($P < 0.001$, $P = 0.007$, and $P < 0.001$, respectively).

Descriptive statistics of APP concentrations and NAGase activities in milk samples from clinical mas-

Table 2. Concentrations of milk amyloid A (MAA), haptoglobin (Hp), and *N*-acetyl-β-D-glucosaminidase (NAGase) activity in milk samples from cows with clinical mastitis (n = 147)[1]

Pathogen	Median (minimum; maximum) value		
	MAA (mg/L)	Hp (mg/L)	NAGase activity (pmol of 4-MU/min per microliter)[2]
Staphylococcus aureus (n = 18)	35.8 (6.7; 1,500)	201.1 (59.0; 756.2)	22.5 (2.7; 24.5)
CNS[3] (n = 11)	29.2 (8.5; 1,500)	123 (59.0; 775.5)	11.86 (6.6; 24.5)
Streptococcus agalactiae (n = 4)	53.5 (28.9; 735.7)	295.5 (172.1; 506.1)	20.6 (6.6; 24.5)
Streptococcus dysgalactiae (n = 19)	147.6 (7.2; 905.2)	248.9 (74.8; 1,118.2)	20.8 (3.28; 24.5)
Streptococcus uberis (n = 36)	53.1 (4.5; 1,500)	385.6 (59.0; 970.3)	24.5 (3.36; 24.5)
Trueperella pyogenes (n = 10)	25.6 (0.95; 348.9)	618.5 (5.0; 1,155.8)	24.5 (1.73; 24.5)
Corynebacterium bovis[4] (n = 4)	12.4 (0.09; 107.3)	95.8 (59.0; 108.6)	9.9 (0.5; 14.7)
Escherichia coli (n = 18)	394.1 (1.75; 1,500)	575.4 (59.0; 1,288.1)	24.5 (2.5; 24.5)
PCR negative (n = 27)	23.2 (7.4; 152.2)	164.1 (59.0; 548.3)	9.2 (1.3; 24.5)

[1]Only milk samples with 1 bacterial species only or 1 bacterial species in large quantity (+++) are included in the table.

[2]4-MU = 4-methyl-umbelliferon.

[3]Milk samples where only bacterial DNA of small/intermediate quantity (+/++) of CNS was detected.

[4]Milk samples where only bacterial DNA of *C. bovis* +/++ was detected.

titis is presented in Table 2. Associations between the concentrations of APP and NAGase activities in the milk and the PCR results are presented in Tables 3, 4, and 5. Concentrations of Hp and MAA and NAGase activity in the milk were not affected by farm, lactation number, or DIM ($P > 0.05$; data not shown). The affected quarter (fore or hind) did not affect the Hp concentrations or the NAGase activities in milk ($P > 0.05$; data not shown). Significantly higher concentrations of MAA were found in the hind quarters compared with the fore quarters (Table 3).

Haptoglobin and MAA concentrations and NAGase activity in the milk were significantly higher when *E. coli* ++ and +++ or *Strep. dysgalactiae* ++/+++ were detected, compared with all other milk samples where these species were not detected. In addition, a small quantity (+) of *E. coli* in milk samples was associated with an elevated NAGase activity and MAA concentration. The presence of *T. pyogenes* at high levels in milk samples caused a significant elevation in Hp concentration, as well as an increased NAGase activity in the milk ($P < 0.001$ and $P < 0.001$, respectively) compared with milk samples without *T. pyogenes*. However, no association between the MAA concentration and the presence of *T. pyogenes* was found ($P > 0.05$; data not shown). Milk samples containing DNA from *C. bovis* or

Table 3. Final generalized linear mixed model (GLMM) of associations between the concentration of milk amyloid A (MAA) in the milk and the pathogens detected with PCR (n = 253) from cows with naturally occurring clinical mastitis

Variable[1]	Estimate[2]	95% CI	*P*-value	Wald test (*P*-value)
Quarter				
Fore quarters (n = 102)	0			
Hind quarters (n = 179)	0.539	0.176; 1.011	0.005	
Escherichia coli				<0.001
E. coli negative[3] (n = 186)	0			
+ (n = 19)	0.857	0.104; 1.610	0.026	
++ (n = 30)	0.636	−0.025; 1.299	0.059	
+++ (n = 18)	1.68	0.843; 2.525	0.000	
Streptococcus dysgalactiae				0.001
Strep. dysgalactiae negative (n = 220)	0			
+ (n = 14)	0.124	−0.737; 0.985	0.788	
++/+++ (n = 19)	1.386	0.635; 2.136	0.000	
Corynebacterium bovis				
C. bovis negative (n = 200)	0			
+/++ (n = 53)	−0.664	−1.151; −0.140	0.012	
Intercept	3.116	2.192; 4.040	0.000	

[1]+ = small quantities of bacterial DNA; ++ = intermediate quantities of bacterial DNA; +++ = large quantities of bacterial DNA.

[2]Estimates are on a logarithmic scale.

[3]Number of milk samples not containing DNA from detected bacteria.

Table 4. Final generalized linear mixed model (GLMM) of associations between concentration of haptoglobin (Hp) in the milk and the pathogens detected with PCR (n = 253) from cows with naturally occurring clinical mastitis

Variable[1]	Estimate[2]	95% CI	*P*-value	Wald test (*P*-value)
Trueperella pyogenes				<0.001
T. pyogenes negative[3] (n = 219)	0			
+ (n = 24)	−0.005	−0.017; 0.007	0.415	
++/+++ (n = 10)	−0.037	−0.055; −0.019	0.000	
Escherichia coli				<0.001
E. coli negative (n = 186)	0			
+ (n = 19)	−0.004	−0.018; 0.009	0.532	
++ (n = 30)	−0.021	−0.033; −0.008	0.001	
+++ (n = 18)	−0.029	−0.047; −0.014	0.000	
Streptococcus uberis				<0.001
Strep. uberis negative (n = 164)	0			
+ (n = 33)	0.002	−0.009; 0.012	0.784	
++ (n = 29)	−0.009	−0.021; 0.003	0.150	
+++ (n = 36)	−0.023	−0.034; −0.012	0.000	
Streptococcus dysgalactiae				0.008
Strep. dysgalactiae negative (n = 220)	0			
+ (n = 14)	0.006	−0.010; 0.021	0.465	
++/+++ (n = 19)	−0.020	−0.034; −0.007	0.003	
CNS				0.10
CNS negative (n = 162)	0			
+ (n = 78)	0.008	0.0003; 0.016	0.040	
++/+++ (n = 13)	0.008	−0.009; 0.024	0.358	
Corynebacterium bovis				
C. bovis negative (n = 200)	0			
+/++ (n = 53)	0.013	0.005; 0.023	0.002	
Intercept	0.089	0.072; 0.107	0.000	

[1]+ = small quantities of bacterial DNA; ++ = intermediate quantities of bacterial DNA; +++ = large quantities of bacterial DNA.

[2]Estimates are on an inverse square root scale (negative estimate means higher concentration of Hp).

[3]Number of milk samples not containing DNA from detected bacteria.

a small quantity of DNA from CNS had a significantly lower concentration of APP and lower NAGase activity compared with all other milk samples where other species were detected.

No interaction was detected in the models between minor (*C. bovis* and CNS) and major (*E. coli, Strep. dysgalactiae, T. pyogenes*, and *Strep. uberis*) pathogens. This means that any association between major pathogen DNA and the concentrations of the inflammatory markers was not influenced by the presence of minor pathogen DNA in the milk samples.

DISCUSSION

This study describes the associations between concentrations of APP in the milk and PCR-based bacteriological findings in cases of clinical mastitis. The amount of bacterial DNA detected in the samples from mastitis caused by certain species was associated with MAA and Hp concentrations and NAGase activity in the milk. The highest concentrations of MAA and the highest NAGase activities in milk were found in cows with large quantities of *E. coli* in their milk. This is in accordance with experimental studies showing a strong inflammatory response to *E. coli* (Hyvönen et al., 2006; Suojala et al., 2008). Wenz et al. (2010) found that the concentration of Hp was the highest in *E. coli*-induced mastitis compared with mastitis caused by environmental streptococci or CNS. However, even small quantities of *E. coli* resulted in elevated concentrations of MAA and increased NAGase activity in the milk. Experimental studies have shown that even a small quantity of *E. coli* can induce an acute inflammatory reaction in the udder (Frost et al., 1982). *Escherichia coli* bacteria are generally eliminated rapidly from the udder, but trigger a strong inflammatory reaction, which is mainly due to endotoxin (Burvenich et al., 2003). In practice, the time of sampling after the onset of clinical mastitis could also influence the quantity of DNA of *E. coli* in the milk. Sampling late during the course of infection could explain the small quantity of *E. coli* detected in bacteriological examination, despite a strong inflammatory response.

Our findings support the results reported by Pyörälä et al. (2011), who found that higher concentrations of Hp and NAGase corresponded to the detection of *T. pyogenes* in mastitic milk samples but could establish no association between *T. pyogenes* and MAA. This could indicate that IMI due to *T. pyogenes* does not induce significant local production of MAA. Release of

Table 5. Random effects Tobit regression model of associations between *N*-acetyl-β-D-glucosaminidase (NAGase) activity in the milk and the pathogens detected with PCR (n = 253) from cows with naturally occurring clinical mastitis

Variable[1]	Estimate[2]	95% CI	*P*-value	Wald test (*P*-value)
Trueperella pyogenes				0.006
T. pyogenes negative[3] (n = 219)	0			
+ (n = 24)	0.041	−0.721; 0.804	0.91	
++/+++ (n = 10)	2.173	0.847; 3.499	0.001	
Escherichia coli				0.002
E. coli negative (n = 186)	0			
+ (n = 19)	0.806	−0.025; 1.638	0.057	
++ (n = 30)	1.247	0.486; 2.007	0.001	
+++ (n = 18)	1.464	0.500; 2.428	0.003	
Streptococcus uberis				<0.001
Strep. uberis negative (n = 164)	0			
+ (n = 18)	−0.276	−0.934; 0.382	0.41	
++ (n = 17)	0.159	−0.544; 0.861	0.66	
+++ (n = 30)	2.416	1.555; 3.276	0.000	
Streptococcus dysgalactiae				0.003
Strep. dysgalactiae negative (n = 220)	0			
+ (n = 14)	−0.966	−1.882; −0.049	0.039	
++/+++ (n = 19)	1.100	0.274; 1.926	0.009	
CNS				<0.001
CNS negative (n = 162)	0			
+ (n = 78)	−0.908	−1.390; −0.425	0.000	
++/+++ (n = 13)	−1.239	−2.162; −0.316	0.009	
Corynebacterium bovis				
C. bovis negative (n = 200)	0			
+/++ (n = 53)	−1.323	−1.855; −0.791	0.000	
PCR positive (n = 226)	0			
PCR negative (n = 27)	−0.918	−1.687; −0.149	0.019	
Intercept	3.116	2.018; 4.214	0.000	

[1]+ = small quantities of bacterial DNA; ++ = intermediate quantities of bacterial DNA; +++ = large quantities of bacterial DNA.

[2]Estimates are on a square root scale.

[3]Number of milk samples not containing DNA from detected bacteria.

different APP may depend on the pathogens present. The major producers of Hp and NAGase in milk are neutrophils and epithelial cells, whereas only mammary gland epithelial cells appear to secrete MAA in cows with mastitis (Kitchen et al., 1984; Eckersall et al., 2006; Lai et al., 2009). Epithelial damage may manifest differently in different infections, which in turn could affect MAA concentrations in the milk.

The presence of *Staph. aureus* in the udder increased the concentrations of APP and the NAGase activity in the milk less than other major pathogens, indicating a mild inflammatory response in this infection. In experimentally induced *Staph. aureus* mastitis, the concentrations of Hp and MAA ranged between 52 and 323 mg/dL and between 34 and 286 mg/dL, respectively (Grönlund et al., 2003), and were lower than those found in experimentally induced *Strep. uberis* or *E. coli* mastitis (Pedersen et al., 2003; Suojala et al., 2008). Concentrations of Hp and MAA and NAGase activity in naturally acquired *Staph. aureus* mastitis were also lower than in streptococcal or *E. coli* mastitis (Pyörälä et al., 2011). In the present study, mastitis caused by *Staph. aureus* may have been very mild, which could explain the weak inflammatory response detected in the udder quarters. A small quantity of *Staph. aureus* DNA in the milk samples could also indicate that the bacteria were just skin contaminants and not the actual cause of the mastitis (Haveri et al., 2008).

In the present study, CNS and *C. bovis* were common bacterial species detected, mainly in small quantities, in milk samples positive for several species. *C. bovis* and CNS were the main pathogens detected using PCR from milk samples without growth (Taponen et al., 2009) and in the study by Koskinen et al. (2010) comparing conventional bacterial culturing and PCR in mastitis milk diagnostics. The frequent detection of these bacteria may be due to their extramammary origin. *Corynebacterium bovis* and CNS are generally considered to be opportunistic bacteria inhabiting teat skin and canals (Taponen et al., 2008). Nevertheless, the presence of CNS and *C. bovis* in the milk samples alone could increase concentrations of APP and NAGase activity in the milk, indicating that these bacteria are able to invade the udder and induce an inflammatory reaction. The PCR method allows the quantitative detection of udder pathogens and is especially useful

when bacteria are present in small quantities and may be undetectable using conventional methods. On the other hand, the high sensitivity of the PCR analysis and the methods used to collect milk samples can cause false-positive results, especially in large herds when many staff members are involved in the sampling. The presence of microbes in a milk sample does not necessarily prove that those microbes caused the IMI. Interpretation of PCR results can be challenging and needs more guidance, even though PCR-based diagnostics are already in routine use in some countries. In the interpretation of PCR results, detection of a single species, preferably in moderate or large quantities, or detection of one dominating species with some other species provides a likely bacteriological diagnosis. The final diagnosis of mastitis always requires a full complement of supporting information, such as knowledge of the clinical signs and inflammation in the quarter (Pyörälä, 2012).

In the present study, Hp performed better than MAA in describing bovine inflammatory response. A constant increase in concentrations of Hp in the milk along with increasing quantities of DNA (except CNS and *C. bovis*) was observed. Haptoglobin could, thus, be a better marker than MAA for indicating the local inflammatory response in clinical mastitis caused by different pathogens.

CONCLUSIONS

The quantity of bacterial DNA in milk samples was associated with concentrations of APP and NAGase activity in the milk. These indicators reflect the inflammatory reaction in the mammary gland, and their concentrations increased with increasing severity of mastitis. Concentrations of APP and NAGase activity in milk significantly differed between different mastitis-causing bacterial species. Indicators of inflammation in milk, such as APP concentration and NAGase activity, may be useful to complete and support the bacteriological diagnosis of mastitis.

ACKNOWLEDGMENTS

This study was supported by the Research Foundation of Veterinary Medicine (Helsinki, Finland) and by the Walter Ehrström Foundation (Helsinki, Finland). Additional financial support was provided by the Estonian Ministry of Agriculture (Tallinn, Estonia; research contract 8-2/T8010) and the Estonian Ministry of Education (Tartu, Estonia; research project 8-2/T9001). Invaluable help of personnel from participating farms is deeply appreciated.

REFERENCES

Alsemgeest, S. P. M., H. C. Kalsbeek, T. Wensing, J. P. Koeman, A. M. van Ederen, and E. Gruys. 1994. Concentration of serum amyloid A (SAA) and haptoglobin (Hp) as parameters of inflammatory diseases in cattle. Vet. Q. 16:21–23.

Baumann, H., and J. Gauldie. 1994. The acute phase response. Immunol. Today 15:74–80.

Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile, and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. Vet. Res. 34:521–564.

Eckersall, P. D., F. J. Young, C. McComb, C. J. Hogarth, S. Safi, A. Weber, T. McDonald, A. M. Nolan, and J. L. Fitzpatrick. 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. Vet. Rec. 148:35–41.

Eckersall, P., F. J. Young, A. M. Nolan, C. H. Knight, C. McComb, M. M. Waterston, C. J. Hogarth, E. M. Scott, and J. L. Fitzpatrick. 2006. Acute phase proteins in bovine milk in an experimental model of *Staphylococcus aureus* subclinical mastitis. J. Dairy Sci. 89:1488–1501.

Frost, A. J., A. W. Hill, and B. E. Brooker. 1982. Pathogenesis of experimental bovine mastitis following a small inoculum of *Escherichia coli*. Res. Vet. Sci. 33:105–112.

Grönlund, U., C. Hultén, P. D. Eckersall, C. Hogarth, and K. Persson Waller. 2003. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis from dairy cows with chronic sub-clinical mastitis. J. Dairy Res. 70:379–386.

Haveri, M., M. Hovinen, A. Roslöf, and S. Pyörälä. 2008. Molecular types and genetic profiles of *Staphylococcus aureus* strains isolated from bovine intramammary infections and extramammary sites. J. Clin. Microbiol. 46:3728–3735.

Hiss, S., M. Mielenz, M. R. Bruckmaier, and H. Sauerwein. 2004. Haptoglobin concentration in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. J. Dairy Sci. 87:3778–3784.

Hovinen, M., H. Simojoki, R. Pösö, J. Suolaniemi, and S. Pyörälä. 2010. N-Acetyl-β-D-glucosaminidase activity in normal cow milk. In the 8th European Colloquium on acute phase proteins, Helsinki, Finland. University of Helsinki, Finland.

Hyvönen, P., L. Suojala, T. Orro, J. Haaranen, O. Simola, C. Røntved, and S. Pyörälä. 2006. Transgenic cows that produce recombinant human lactoferrin in milk are not protected from experimental *Escherichia coli* intramammary infection. Infect. Immun. 74:6206–6212.

IDF (International Dairy Federation). 1999. Suggested interpretation of mastitis terminology. Bull. Int. Dairy Fed. 338:3–26.

Kitchen, B. J., W. Seng Kwee, G. Middleton, and R. J. Andrews. 1984. Relationship between the level of N-acetyl-β-D-glucosaminidase (NAGase) in bovine milk and the presence of mastitis pathogens. J. Dairy Res. 51:11–16.

Koj, A. 1996. Initiation of acute phase response and synthesis of cytokines. Biochim. Biophys. Acta 1317:84–94.

Koskinen, M. T., G. J. Wellenberg, O. C. Sampimon, J. Holopainen, A. Rothkamp, L. Salmikivi, W. A. van Haeringen, T. J. G. M. Lam, and S. Pyörälä. 2010. Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J. Dairy Sci. 93:5707–5715.

Lai, I.-H., J. H. Tsao, Y. P. Lu, J. W. Lee, X. Zhao, F. L. Chien, and S. J. T. Mao. 2009. Neutrophils as one of major haptoglobin sources in mastitis affected milk. Vet. Res. 40:17.

Larsen, T., C. M. Røntved, K. L. Ingvartsen, L. Vels, and M. Bjerring. 2010. Enzyme activity and acute phase proteins in milk utilized as indicators of acute clinical *E. coli* LPS-induced mastitis. Animal 4:1672–1679.

Long, J. S. 1997. Regression Models for Categorical and Limited Dependent Variables. Sage Publications Inc., Thousand Oaks, CA.

Makimura, S., and N. Suzuki. 1982. Quantitative determination of bovine serum haptoglobin and it elevation in some inflammatory diseases. Nippon Juigaku Zasshi 44:15–21.

Mattila, T., and M. Sandholm. 1985. Antitrypsin and N-acetyl-β-D-glucosaminidase as markers of mastitis in herd of Ayrshire cows. Am. J. Vet. Res. 46:2453–2456.

Nielsen, B. H., S. Jacobsen, P. H. Andersen, T. A. Niewold, and P. M. H. Heegaard. 2004. Acute phase protein concentrations in serum and milk from healthy cows, cows with clinical mastitis and cows with extramammary inflammatory conditions. Vet. Rec. 154:361–365.

NMC (National Mastitis Council). 2004. Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality. 4th ed. NMC, Madison, WI.

Pedersen, L. H., B. Aalbæk, C. M. Røntved, K. L. Ingvartsen, N. S. Sorensen, P. M. H. Heegaard, and H. E. Jensen. 2003. Early pathogenesis and inflammatory response in experimental bovine mastitis due to *Streptococcus uberis*. J. Comp. Pathol. 128:156–164.

Pyörälä, S. 2012. Letter to the editor: Comments on Schwaiger et al. (2012). J. Dairy Sci. 95:4185.

Pyörälä, S., M. Hovinen, H. Simojoki, J. Fitzpatrick, P. D. Eckersall, and T. Orro. 2011. Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens. Vet. Rec. 168:535.

Simojoki, H., T. Orro, and S. Pyörälä. 2009. Bovine experimental infection induced by coagulase-negative staphylococci. Vet. Microbiol. 134:95–99.

Suojala, L., T. Orro, H. Järvinen, J. Saatsi, and S. Pyörälä. 2008. Acute phase response in two consecutive experimentally induced *E. coli* intramammary infections in dairy cows. Acta Vet. Scand. 50:18.

Taponen, S., J. Björkroth, and S. Pyörälä. 2008. Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herds. J. Dairy Res. 75:422–429.

Taponen, S., L. Salmikivi, H. Simojoki, T. Koskinen, and S. Pyörälä. 2009. Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J. Dairy Sci. 92:2610–2617.

Wenz, J. R., L. K. Fox, F. J. Muller, M. Rinaldi, R. Zeng, and D. D. Bannerman. 2010. Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis. J. Dairy Sci. 93:2458–2470.

Yassin, A. F., H. Hupfer, C. Siering, and P. Schumann. 2011. Comparative chemotaxonomic and phylogenetic studies on the genus *Arcanobacterium* Collins et al. 1982 emend. Lehnen et al. 2006: Proposal for *Trueperella* gen. nov. and emended description of the genus *Arcanobacterium*. Int. J. Syst. Evol. Microbiol. 61:1265–1274.

III

Kalmus, P., Aasmäe, B., Kärssin, A., Orro, T., Kask, K., 2011. UDDER PATHOGENS AND THEIR RESISTANCE TO ANTIMICROBIAL AGENTS IN DAIRY COWS IN ESTONIA. Acta Veterinaria Scandinavica, 53, 4.

Kalmus et al. Acta Veterinaria Scandinavica 2011, 53:4
http://www.actavetscand.com/content/53/1/4

AVS Acta Veterinaria Scandinavica ACTA VETERINARIA SCANDINAVICA

RESEARCH Open Access

Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia

Piret Kalmus[1*†], Birgit Aasmäe[2†], Age Kärssin[3†], Toomas Orro[2†], Kalle Kask[1†]

Abstract

Background: The goal of this study was to estimate the distribution of udder pathogens and their antibiotic resistance in Estonia during the years 2007-2009.

Methods: The bacteriological findings reported in this study originate from quarter milk samples collected from cows on Estonian dairy farms that had clinical or subclinical mastitis. The samples were submitted by local veterinarians to the Estonian Veterinary and Food Laboratory during 2007-2009. Milk samples were examined by conventional bacteriology. *In vitro* antimicrobial susceptibility testing was performed with the disc diffusion test. Logistic regression with a random herd effect to control for clustering was used for statistical analysis.

Results: During the study period, 3058 clinical mastitis samples from 190 farms and 5146 subclinical mastitis samples from 274 farms were investigated. Positive results were found in 57% of the samples (4680 out of 8204), and the proportion did not differ according to year ($p > 0.05$). The proportion of bacteriologically negative samples was 22.3% and that of mixed growth was 20.6%. *Streptococcus uberis* (*Str. uberis*) was the bacterium isolated most frequently (18.4%) from cases of clinical mastitis, followed by *Escherichia coli* (*E. coli*) (15.9%) and *Streptococcus agalactiae* (*Str. agalactiae*) (11.9%). The bacteria that caused subclinical mastitis were mainly *Staphylococcus aureus* (*S. aureus*) (20%) and coagulase-negative staphylococci (CNS) (15.4%). The probability of isolating *S. aureus* from milk samples was significantly higher on farms that had fewer than 30 cows, when compared with farms that had more than 100 cows ($p < 0.005$). A significantly higher risk of *Str. agalactiae* infection was found on farms with more than 600 cows ($p = 0.034$) compared with smaller farms. The proportion of *S. aureus* and CNS isolates that were resistant to penicillin was 61.4% and 38.5%, respectively. Among the *E. coli* isolates, ampicillin, streptomycin and tetracycline resistance were observed in 24.3%, 15.6% and 13.5%, respectively.

Conclusions: This study showed that the main pathogens associated with clinical mastitis were *Str. uberis* and *E. coli*. Subclinical mastitis was caused mainly by *S. aureus* and CNS. The number of *S. aureus* and *Str. agalactiae* isolates depended on herd size. Antimicrobial resistance was highly prevalent, especially penicillin resistance in *S. aureus* and CNS.

Background

Bovine mastitis is the most common disease in dairy cows worldwide, and antimicrobial therapy is the primary tool for the treatment of mastitis. The prevalence of mastitis pathogens and their antimicrobial resistance have been investigated in numerous studies around the world. The main pathogens that cause subclinical mastitis are coagulase-negative staphylococci (CNS), *Corynebacterium bovis* (*C. bovis*) and *Staphylococcus aureus* (*S. aureus*) [1-5]. Coliforms, *Streptococcus uberis* (*Str. uberis*) and *S. aureus* are the pathogens isolated most frequently from clinical mastitis samples [6-8]. *Streptococcus agalactiae* (*Str. agalactiae*) has been largely eradicated from herds in Europe [3], but in studies from the United States, 7.7% and 13.1% of samples contained *Str. agalactiae* [9,10].

Several methods, such as disc diffusion, agar dilution, broth dilution and broth microdilution are suitable for *in vitro* antimicrobial susceptibility testing. Depending on the study design and the methodology used, the antimicrobial susceptibility of udder pathogens varies greatly between studies. For example, studies from France and

* Correspondence: piret.kalmus@emu.ee
† Contributed equally
[1]Department of Therapy, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, Tartu, 51014, Estonia
Full list of author information is available at the end of the article

the UK have reported a high prevalence of penicillin-resistant *S. aureus* (36.2%, 56%) [11,12], whereas a low percentage of resistant isolates (4-9%) were found in the Netherlands and Norway [13,14]. The streptococci that cause mastitis are susceptible to β-lactam antibiotics; however, resistance to macrolides and lincosamides is notable [13,15]. *In vitro* resistance of *E. coli* to different antimicrobials has been reported to be low [13,14,16,17].

National studies of mastitis prevalence provide important information through the monitoring of national udder health status, and they enable national guidelines to be developed for the prudent use of antibiotics in each country [18]. During recent decades, only broad-spectrum antibiotics have been used for the treatment of clinical mastitis in Estonia. For example, in the years 2006-2009, 15 different combinations of antibiotics were available for use in 18 intramammary preparations that were authorised by the Estonian State Medical Agency [19]. Given that a large overview of udder pathogens and their antibiotic resistance has not been performed in Estonia, the goal of this study was to estimate the distribution of udder pathogens and their antibiotic resistance during the years 2007-2009 in Estonia.

Methods

Sample collection

Milk samples were submitted to the Estonian Veterinary and Food Laboratory during the period 2007-2009. Quarter milk samples were collected from cows on Estonian dairy farms by local veterinarians or farmers. Clinical mastitis was diagnosed when visible abnormalities of udder (swelling) were detected or milk from a quarter had abnormal viscosity (watery, thicker than normal), colour (yellow, blood-tinged) or consistency (flakes or clots) [20]. Normal milk appearance, together with a positive California Mastitis Test result (score greater than 1), was used to make a diagnosis of subclinical mastitis.

The samples were sent to the laboratory either for isolation of the clinical mastitis pathogen and determination of its antimicrobial susceptibility or to determine the reason for an increased somatic cell count.

Laboratory analysis

Bacterial species were identified using accredited methodology based on the National Mastitis Council [21] standards. From each sample, 0.01 ml of milk was cultured on blood-esculin agar and incubated for 48 h at 37°C. The plates were examined after 24 and 48 h of incubation. A minimum of five colonies of the same type of bacterium was recorded as bacteriologically positive, and growth of more than two types of bacterial colonies was categorised as mixed growth. No bacterial growth was recorded when fewer than five colony-forming units were detected during 48 h of incubation.

Once they had been isolated and identified, pure cultures of udder pathogens were tested for antibacterial susceptibility with the disc diffusion assay on Mueller-Hinton agar. Testing was performed according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) document M31-A2 in the years 2007-2008 and M31-A3 in 2009 [22,23]. Quality control strains, *S. aureus* ATCC® 25923, *E. coli* ATCC® 25922, *Pseudomonas aeruginosa* ATCC® 27853 and *Streptococcus pneumoniae* ATCC® 49619, were included with each batch of isolates tested. The antimicrobial susceptibility of Gram-positive bacteria was tested with penicillin, ampicillin, cephalothin, clindamycin, erythromycin, gentamycin, trimethoprim/sulfa and tetracycline. The antimicrobial susceptibility of Gram-negative bacteria was tested with ampicillin, gentamycin, trimethoprim/sulfa, tetracycline, enrofloxacin, streptomycin, neomycin and cefaperazone. The list of antibiotics in susceptibility testing may vary, different veteriarians preferred different set of antibiotics in order to find accurate treatment after getting the laboratory test results.

The criteria for the interpretation of zone diameter used in this study are described in Table 1.

Data analysis

The farm, herd size and year were recorded and categorised before statistical analysis. A logistic regression model with a random herd effect for the control of clustering was used for all of the analyses in this study. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated. Statistical significance was set at $p \leq 0.005$.

The influence of milk samples with mixed growth or no bacterial growth on the occurrence of clinical or subclinical mastitis was assessed. Potential interactions (no growth or mixed growth × year) were assessed in the logistic regression model. The effects of herd size and year on the pathogens that caused clinical and subclinical mastitis were analysed. These analyses were conducted using Stata 10.2 [24].

Results

Isolation of mastitis pathogens

During the study period, 3058 clinical mastitis samples from 190 farms and 5146 subclinical mastitis samples from 274 farms were investigated (Table 2).

Positive results were found in 57% of the samples (4680 out of 8204), and this proportion did not differ according to year ($p > 0.05$). The proportion of bacteriologically negative samples was 22.3% and that of mixed growth 20.6%. There was a significantly higher chance (OR = 1.15, 95% CI = 1.01, 1.33, p = 0.042) of finding bacteriologically negative samples in presence of subclinical mastitis (n = 1317, 25.6%) in comparison with

Table 1 Zone diameter intepretive criteria

Disc content in µg	*Staphylococcus* spp.			*Streptococcus* spp.			*Enterococcus* spp.			*Enterobacteriaceae* spp.		
	S	I	R	S	I	R	S	I	R	S	I	R
Ampicillin 10 µg	≥ 29	-	≤28	≥ 26	19-25	≤18	≥ 17	-	≤16	≥ 17	15-16	≤14
Penicillin 10 µg	≥ 29	-	≥ 29	≥24	-	-	≥15	-	≤14	-	-	-
Cephalothin 30 µg				≥		≤	-	-	-			
Cefaperazone 75 µg	-	-	-	-	-	-	-	-	-	≥21	16-20	≤15
Clindamycin 2 µg	≥ 21	15-20	≥ 14	≥19	16-18	≤15	-	-	-	-	-	-
Erythromycin 15 µg	≥ 23	14-22	≥ 14	≥21	16-20	≤15	-	-	-	-	-	-
Gentamycin 10 µg	≥ 12	13-14	≥ 15	≥12	13-14	15≤	≥10	7-9	≤6	≥ 12	13-14	≥ 15
Tetracycline 30 µg	≥ 19	15-18	≥ 14	≥23	19-22	≤18	≥19	15-18	≤14	≥ 19	15-18	≥ 14
Enrofloxacin 5 µg										≥ 20	15-19	≤14
Trimethoprim/sulfa 1,25/23,75 µg	≥ 16	11-15	≥ 10	≥16	11-15	≤10	≥16	11-15	≤10	≥ 16	11-15	≥ 10

clinical mastitis (n = 554, 16.8%). The probability of obtaining mixed growth from milk samples was also significantly higher (OR = 2.2, 95% CI = 1.9, 2.6, p < 0.001) if subclinical mastitis was found. The distribution of bacterial species isolated from samples from cows with clinical and subclinical mastitis is shown in Table 3. Among the bacteriologically positive (n = 2016) clinical mastitis samples, *Str. uberis* was the bacterium isolated most frequently (n = 371; 18.4% of the positive samples), followed by *E. coli* (n = 321; 15.9%) and *Str. agalactiae* (n = 293; 11.9%). *S. aureus* (n = 532; 20%) and CNS (n = 411; 15.4%) were the bacteria isolated most commonly from milk in cases of subclinical mastitis, followed by *Corynebacterium* spp. (n = 395; 14.8%).

The probability of isolating *S. aureus* from milk samples was significantly higher on farms that had fewer than 30 cows, when compared with farms with more than 100 cows (OR = 0.2, 95% CI = 0.11, 0.53, p < 0.005). Also, there was a significantly higher risk of diagnosing *Str. agalactiae* on farms with more than 600 cows (OR = 17.6, 95% CI = 1.2, 259.1, p = 0.034) compared with smaller farms.

Table 2 Distribution of milk samples according to herd size

	Clinical mastitis				Subclinical mastitis			
Farm size category	Farms	%	Samples	%	Farms	%	Samples	%
1 (1-30 cows)	54	28.4	98	3.2	41	15	86	1.7
2 (31-99 cows)	35	18.4	149	4.9	51	18.6	268	5.2
3 (100-299 cows)	40	21.1	378	12.4	53	19.3	541	10.5
4 (300-599 cows)	44	23.2	1472	48.1	80	29.2	2426	47.1
5 (> 600 cows)	17	8.9	961	31.4	49	17.9	1825	35.5
Total	190	100	3058	100	274	100	5146	100

Antimicrobial susceptibility testing

The percentage of *S. aureus* isolates resistant to penicillin and ampicillin was 61.4% and 59.5%, respectively. In addition, CNS showed resistance to penicillin and ampicillin (38.5% and 34.4%), but resistance to erythromycin and lincomycin was also common (14.9% and 17.6%). Six isolates (3.8%) of *S. aureus* and three isolates (3.6%) of CNS were resistant to cephalothin (Table 4).

All streptococci (Table 5) were susceptible to penicillin, ampicillin and cephalothin, except for one isolate of *Str. uberis*. Of the 90 isolates of *Str. dysgalactiae*, 19.8% were classified with intermediate susceptibility and 32.2% with resistance to tetracycline. Of a total of 151 isolates of *Str. uberis*, 7.3% with intermediate susceptibility and 14.3% with resistance to tetracycline were recorded. Among the *E. coli* isolates (Table 6), the highest percentage of isolates showing intermediate susceptibility and resistance were observed with ampicillin, neomycin, streptomycin and tetracycline. *E. coli*. was 98.4% susceptible to enrofloxacin and 100% to cefaperazone.

Discussion

The results of the present study were based on an analysis of milk samples submitted to an Estonian National Veterinary Laboratory over a three-year period. The laboratory protocols did not change during the study period. Of the samples investigated, 22.3% were bacteriologically negative. Several other studies have also demonstrated bacteriologically negative findings in 17.7-26.5% cases of clinical mastitis [12,25] and as many as 28.7-38.6% of subclinical mastitis [12,26], which is in line with our results. The possible reasons for bacteriologically negative findings in milk samples could be the presence of antibacterial substances in the milk that lead to a decrease in the viability of bacteria in the culture [27], or failures in conventional culture compared with identification of bacteria using the real-time polymerase chain reaction [28].

Table 3 Distribution of bacterial species isolated from clinical and subclinical mastitis samples in 2007-2009

	Clinical mastitis			Subclinical mastitis		
Bacteria	**2007 (n = 598)**	**2008 (n = 692)**	**2009 (n = 726)**	**2007 (n = 939)**	**2008 (n = 1063)**	**2009 (n = 661)**
S. aureus	11.7	11.7	11.7	19.2	22.8	16.6
CNS	4.8	7.1	8.5	16.1	13.6	17.4
CPS*	3.8	3.3	1.6	4.6	2.8	5.1
Str. agalactiae	9.0	11.3	14.7	13.6	9.0	10.7
Str. dysgalactiae	8.0	7.8	7.2	3.6	4.0	5.6
Str. uberis	16.1	21.8	17.1	10.2	12.3	12.9
Str. spp	3.2	3.3	1.9	1.2	2.0	2.7
Lactococcus lactis	10.9	3.9	5.7	8.9	8.2	3.9
E. coli	14.4	16.6	16.5	1.6	2.0	3.8
Klebsiella spp.	7.0	1.3	2.3	0.7	0.6	0.9
Enterococcus spp.	1.3	2.3	1.1	1.5	2.8	4.2
Corynebacterium spp.	2.2	2.6	5.0	16.5	17.3	8.5
A. pyogenes	2.2	3.8	3.6	0.1	0.6	0.6
Pseudomonas spp.	1	0.3	0.3	0	0	0.6
Proteus spp.	0.2	0	0.2	0.4	0.1	0.6
Yeast	2.3	2	1.6	1.5	1.6	5.6
Other	1.8	0.9	1	0.3	0.3	0.3
Total	100%	100%	100%	100%	100%	100%

* CPS: coagulase-positive staphylococci (other than *S. aureus*).

In the present study, *E. coli* and *Str. uberis* were the pathogens isolated most frequently from clinical mastitis, while *S. aureus*, CNS and *Corynebacterium* spp. caused mainly subclinical mastitis. The same results were shown in an Estonian study ten years ago, where *C. bovis* (47.5%), *S. aureus* (21%) and CNS (15.8%) were the pathogens isolated most commonly from cases of subclinical mastitis [29]. The isolation rate of *Str. agalactiae* was surprisingly high in our study.

We found a strong association between the isolation of *Str. agalactiae* and very large-scale farms. In total, there are 98000 dairy cows in Estonia and the mean herd size is 88 cows [30]. Rapid changes in management style (from tie-stalls to free-stalls) have occurred during the last eight years, which may explain the coexistence of environmental pathogens together with *Str. agalactiae*. Although teat disinfection and dry cow therapy is a common routine on Estonian dairy farms, proper eradication programmes for *Str. agalactiae* have not been employed. In contrast, an increased probability of finding *S. aureus* was correlated with farms with fewer than 30 cows. The average age of cows on small farms was 5.3 years, compared with 4.3 years on farms on which more than 300 cows were kept [30]. The culling policy may be different, and the owners of smaller farms may keep (possibly chronically infected) cows in the herd for a longer period of time.

Table 4 Antimicrobial susceptibility of staphylococci isolated from bovine clinical mastitis

	S. aureus				CNS			
Disc content in µg	**n**	**S* (%)**	**I * (%)**	**R* (%)**	**n**	**S* %**	**I * (%)**	**R* (%)**
Ampicillin10 µg	173	40.5	-	59.5	91	61.5	-	38.5
Penicillin10 µg	174	38.6	-	61.4	93	65.5	-	34.4
Cephalothin 30 µg	160	96.2	-	3.8	84	96.4	-	3.6
Clindamycin 2 µg	169	81.9	0	18.1	91	82.4	0	17.6
Erythromycin15 µg	83	95.2	0	4.8	47	85.1	0	14.9
Tetracycline 30 µg	147	95.9	0	4.1	86	88.4	0	11.6
Trimethoprim/sulfa 1.25/ 23.75 µg	162	96.6	0	3.4	76	97.4	0	2.6
Gentamycin 10 µg	146	93.2	0	6.8	69	98.6	0	1.4

* Propotion of susceptible (S), intermediate susceptibility (I) and resistant (R) isolates.

The disc diffusion method for *in vitro* antimicrobial susceptibility testing was used in this study. This technique is the most widely used method for determination of the susceptibility of animal pathogens, especially in clinical work when it is necessary to determine the correct treatment. The primary disadvantage of using this method when monitoring development of resistance is that outcomes are reported on a qualitative basis (sensitive, intermediate, or resistant), and subtle changes in susceptibility may not be apparent. Therefore any comparison with studies that use other methods of susceptibility testing is not acceptable [31].

Generally in our study, the *in vitro* antimicrobial resistance of the isolates examined from samples of clinical

Table 5 Antimicrobial susceptibility of streptococci isolated from bovine clinical mastitis

	Str. agalactiae				*Str. dysgalactiae*				*Str. uberis*			
Disc content in µg	**n**	**S* (%)**	**I* (%)**	**R* (%)**	**n**	**S* (%)**	**I* (%)**	**R* (%)**	**n**	**S* (%)**	**I* (%)**	**R* (%)**
Ampicillin 10 µg	162	100	-	0	111	100	0	0	265	99.6	0	0.4
Penicillin 10 µg	168	100	-	0	111	100	0	0	267	99.6	0	0.4
Cephalothin 30 µg	143	100	-	0	101	100	0	0	254	99.6	0	0.4
Clindamycin 2 µg	161	91.9	1.9	6.2	115	92.2	0	7.8	273	92	1.4	6.6
Erythromycin 15 µg	77	96.1	2.6	1.3	60	88.3	5	6.7	134	89.6	2.2	8.2
Tetracycline 30 µg	151	78.1	7.3	14.6	90	48.9	18.9	32.2	234	79.9	3.4	19.7
Trimethoprim/sulfa 1.25/23.75 µg	140	93.6	0	6.4	103	99	0	1	223	95.9	0.9	3.2
Gentamycin 10 µg	143	63.6	11.9	24.5	88	88.6	0	11.4	210	71.9	9.5	18.6

* Propotion of susceptible (S), intermediate susceptibility (I) and resistant (R) isolates.

mastitis were high. Isolates of *S. aureus* had an alarming level of resistance to penicillin (61.4%) and ampicillin (59.5%), whereas CNS exhibited a lower degree of resistance to penicillin and ampicillin (38.5%; 34.4%). The reported percentages for penicillin resistant *S. aureus* in cases of clinical mastitis, detected by the disc diffusion method, are 50.4% and 35.4% in the USA [10,32], 63.3% in Turkey [33] and 12% in Northern Germany [34]. In addition, cephalothin resistance among staphylococci was found in our study. Although reports of methicillin-resistant staphylococci causing bovine mastitis are rare, those samples found in our study need further investigation in order to prove or exclude the presence of the *mecA* gene. In the present study, both staphylococci and streptococci showed resistance to erythromycin and lincomycin, but the figures for resistance in annual reports from some other countries show a low prevalence of lincomycin and erythromycin resistance in *S. aureus* and CNS [13,14,35]. Given that *S. aureus* and CNS were the pathogens isolated most frequently from cases of subclinical mastitis, one possible explanation for resistance to several antibiotics may be the collection and submission to the laboratory of milk samples from chronic clinical mastitis (which demonstrate poor treatment efficacy). Therefore, random sampling strategies should be used to provide a good evaluation of antimicrobial susceptibility.

Table 6 Antimicrobial susceptibility of *E. coli* and *Klebsiella* spp. isolated from bovine clinical mastitis

	E. coli				*Klebsiella* spp.			
Disc content in µg	**n**	**S* (%)**	**I * (%)**	**R* (%)**	**n**	**S* (%)**	**I* (%)**	**R* (%)**
Ampicillin 10 µg	201	68.7	7.0	24.3	39	15.4	7.7	76.9
Cefaperazone75 µg	137	100	0	0	32	100	0	0
Tetracycline 30 µg	184	77.8	8.7	13.5	39	79.6	10.2	10.2
Trimethoprim/sulfa 1.25/ 23.75 µg	191	84.3	3.7	12.0	40	97.5	0	2.5
Gentamycin 10 µg	161	94.3	2.5	2.2	40	95.0	0	5.0
Streptomycin 300 µg	154	78.6	5.8	15.6	37	73.0	8.1	18.9
Neomycin 30 µg	155	72.9	20.6	6.5	37	83.8	13.5	2.7
Enrofloxacin 5 µg	185	98.4	0	1.6	37	100	0	0

* Proportion of susceptible (S), intermediate susceptibility (I) and resistant (R) isolates.

The level of resistance of *E. coli* and *Klebsiella* spp. was high against all tested antimicrobials, except cefaperazone and enrofloxacin. Coliforms are often resistant to more than one antimicrobial [36,37], and the number of multi-resistant strains may influence the resistance figures. Coliform bacteria isolated from cases of mastitis may reflect the general situation of resistance in the herd and can be considered more as an indicator of the bacteria present than an indicator of specific pathogens from the udder [36]. All of the bacterial species investigated in the present study showed resistance to tetracycline. A possible explanation for this phenomenon could be that tetracycline has been the class of antimicrobial most widely used for treatment of several infections for many years. In addition, tetracycline has been found in multiresistant patterns with penicillin and streptomycin [33,37].

Statistical data from the Estonian State Medical Agency confirmed [19] that alltogether 209880 single intramammary syringes for lactating cows and 205648 for dry cow therapy were sold in the year 2009. Ampicillin and cloxacillin combinations, cephalosporins with aminoglycosides, and lincomycin with neomycin were the most common choices for the treatment of mastitis in lactating cows. For example, 255 grams of intramammary lincomycin (pure antimicrobial) and 44.2 grams of intramammary cephalosporins per thousand dairy cows were sold for the treatment of clinical mastitis in 2009 [19]. However, only 73.4 grams of penicillin G was used per thousand dairy cows for intramammary treatment of clinical mastitis. The use of broad-spectrum antibiotics and antibiotic combinations may influence the resistance of mastitis pathogens. In addition, bacteriological examination of milk samples before treatment of clinical mastitis is not a common practice in Estonia. According to

the available data in Sweden, intramammary and intramuscular penicillin G [38] are used in over 80% of cases for treatment of clinical mastitis, but the prevalence of resistance of *S. aureus* to penicillins is only 7.1% [36]. In Finland, penicillin G and some broad-spectrum β-lactam antibiotics are used in the treatment of clinical mastitis, but the prevalence of resistance in *S. aureus* is only 13% [39]. Bacteriological examination before treatment is common in both countries.

Considering these results, we can assume that the main reason for the occurrence of a high number of resistant strains in Estonian herds is the wide use of broad-spectrum antimicrobials and the long-term presence of infected cows in herds.

Conclusion

This study showed that the main pathogens that caused clinical mastitis were *Str. uberis* and *E. coli*. Subclinical mastitis was caused mainly by *S. aureus* and CNS. A relatively high number of isolates of *Str. agalactiae* were cultured from both types of case. The number of *S. aureus* and *Str. agalactiae* isolates depended on herd size. Among the bacteria investigated, the prevalence of antimicrobial resistance was extremely high, especially penicillin resistance in *S. aureus* and CNS.

Acknowledgements

The Estonian Ministry of Agricultural is acknowledged for financial support (research project No 10043VLVL)

Author details

[1]Department of Therapy, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, Tartu, 51014, Estonia. [2]Department of Environment and Animal Health, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, Tartu, 51014, Estonia. [3]Estonian Veterinary and Food Laboratory Tartu 51014, Estonia.

Authors' contributions

PK carried out the study, compiled the results and drafted the manuscript, BA participated in data collection and coordinated the laboratory analysis, TO participated in designing the study and statistical analysis of the data, AK performed bacteriological analysis, and KK coordinated the study. All authors were significantly involved in designing the study, interpreting data and composing the manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 5 October 2010 Accepted: 8 February 2011
Published: 8 February 2011

References

1. Pitkälä A, Haveri M, Pyörälä S, Myllys V, Honkanen-Buzalski T: **Bovine mastitis in Finland 2001–prevalence, distribution of bacteria, and antimicrobial resistance.** *J Dairy Sci* 2004, **87**:2433-2441.
2. Osterås O, Sølverød L, Reksen O: **Milk culture results in a large Norwegian survey-effects of season, parity, days in milk, resistance, and clustering.** *J Dairy Sci* 2006, **89**:1010-102.
3. Piepers S, De Meulemeester L, de Kruif A, Opsomer G, Barkema HW, De Vliegher S: **Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium.** *J Dairy Res* 2007, **74**:478-483.
4. Tenhagen BA, Köster G, Wallmann J, Heuwieser W: **Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany.** *J Dairy Sci* 2006, **89**:2542-2551.
5. Botrel MA, Haenni M, Morignat E, Sulpice P, Madec JY, Calavas D: **Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France.** *Foodborne Pathog Dis* 2009, 17.
6. Sylverod L, Branscum AJ, Østerås O: **Relationships between milk culture results and treatment for clinical mastitis or culling in Norwegian dairy cattle.** *J Dairy Sci* 2006, **89**:2928-2937.
7. Aarestrup FM, Jensen NE: **Development of penicillin resistance among *Staphylococcus aureus* isolated from bovine mastitis in Denmark and other countries.** *Microb Drug Resist* 1998, **4**:247-256.
8. Riekerink O, Barkema HW, Kelton DF, Scholl DT: **Incidence rate of clinical mastitis on Canadian dairy farms.** *J Dairy Sci* 2008, **91**:1366-1377.
9. Wilson DJ, Gonzales RN, Das HH: **Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production.** *J Dairy Sci* 1997, **80**:2592-2598.
10. Makovec JA, Ruegg PL: **Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994-2001).** *J Am Vet Med Assoc* 2003, **222**:1582-1589.
11. Guerin-Fauble V, Carret G, Houffschmitt P: ***In vitro* activity of 10 antimicrobial agents against bacteria isolated from cows with clinical mastitis.** *Vet Rec* 2003, **152**:466-471.
12. Bradley AJ, Leach KA, Breen JE, Green LE, Green MJ: **Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales.** *Vet Rec* 2007, **160**:253-257.
13. MARAN: *Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2008* 2008 [http://www.cvi.wur.nl].
14. NORM/NORM-VET 2003: **Usage of Antimicrobial Agents and Occurrence of AntimicrobialResistance in Norway.** *Tromsø/Oslo* 2004.
15. Guerin-Faublee V, Tardy F, Bouveron C, Carret C: **Antimicrobial susceptibility of *Streptococcus* species isolated from clinical mastitis in dairy cows.** *Int J Antimicrob Agents* 2002, **19**:219-226.
16. FINRES-Vet 2005-2006: **Finnish veterinary antimicrobial resistance monitoring and consumption of antimicrobial agents.** *Evira publications* 2007 [http://http:/evira.fi/uploads/WebshopFiles/11981412119 41.pdf].
17. SVARM, 2004: **Swedish veterinary antimicrobial resistance monitoring.** *The National Veterinary Institute(SVA), Uppsala, Sweden* , ISSN 1650-6332.
18. Sampimon O, Barkema HW, Berends I, Sol J, Lam T: **Prevalence of intramammary infection in Dutch dairy herds.** *J Dairy Res* 2009, **76**:129-136.
19. Estonia State Medical Agency: ***Official annual report. Usage of antimicrobial agents in animals.*** *Estonia* 2009.
20. IDF: **Suggested interpretation of mastitis terminology.** *Int Dairy Fed Bull* 1999, , **338:** 3-26.
21. Hogan JS, Gonzales RN, Harmon RJ, Nickerson SC, Oliver SP, Smith KL: **Laboratory Handbook on Bovine Mastitis.** National Mastitis Council Inc, Madison, WI;, Revised 1999.
22. Clinical and Laboratory Standard Institute (CLSI): **Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved standard.** *NCCLS document M31-A2.* Second edition. Clinical and Laboratory Standard Institute, Wayne, PA, USA; 2002.
23. Clinical and Laboratory Standard Institute (CLSI): **Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals: Approved standard.** *CLSI document M31-A3.* Third edition. Clinical and Laboratory Standard Institute, Wayne, PA, USA; 2008.
24. *Stata 10.2. 2008 Stata® Statacorp LP, College Station, USA* .
25. Sargeant JM, Morgan SH, Leslie KE, Ireland MJ, Anna Bashiri A: **Clinical mastitis in dairy cattle in Ontario: Frequency of occurrence and bacteriological isolates.** *Can Vet J* 1998, **39**:33-39.
26. Roesch M, Doherr MG, Schären W, Schällibaum M, Blum JV: **Subclinical mastitis in dairy cows in Swiss organic and conventional production systems.** *J Dairy Res* 2007, **74**:86-92.
27. Rainard P, Riollet C: **Innate immunity of bovine mammary gland.** *Vet Res* 2006, **37**:369-400.
28. Taponen S, Salmikivi L, Simojoki H, Koskinen MT, Pyörälä S: **Real-time polymerase chain reaction-based identification of bacteria in milk**

samples from bovine clinical mastitis with no growth in conventional culturing in milk. *J Dairy Sci* 2009, **92**:2610-2617.
29. Haltia L, Honkanen-Buzalski T, Spiridonova I, Olkonen A, Myllys V: **A study of bovine mastitis, milking procedures and management practises on 25 Estonian dairy herds.** *Acta Vet Scan* 2006, **48**:22.
30. Animal Recording Centre: *Annual Report Estonia* 2009.
31. Schwarz S, Silley P, Shabbir S, Woodword N, van Duijkeren E, Johnson AP, Gaastra W: **Editorial. Assessing the antimicrobial susceptibilty of bacteria obtained from animals.** *Vet Microbiol* 2009, **141**:1-4.
32. Erskine RJ, Walker RD, Bolin CA, Bartlett PC, White DG: **Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period.** *J Dairy Sci* **85**:1111-1118.
33. Güler L, Ok Ü, Gündüz K, Gülcü Y, Hadimli HH: **Antimicrobial susceptibility and coagulase gene typing of Staphylococcus aureus isolated from bovine clinical mastitis cases in Turkey.** *Dairy Sci* 2005, **88**:3149-3154.
34. Schröder A, Hoedemaker M, Klein G: **Resistance of mastitis pathogens in Northern Germany.** *Berl Münch Tierärztl Wochenschr* 2005, **9/10**:393-398.
35. SVARM: **Swedish veterinary antimicrobial resistance monitoring.** The National Veterinary Institute(SVA), Uppsala, Sweden; 2002, ISSN 1650-6332.
36. Bengsston B, Unnerstad HE, Ekman T, Artursson K, Nilsson-Öst M, Persson Waller K: **Antimicrobial suspectibility of udder pathogens from cases of acute clinical mastitis in dairy cows.** *Vet Microbiol* 2009, **36**:142-149.
37. Lehtolainen T, Schwimmer A, Shpigel NY, Honkanen-Buzalski T, Pyörälä S: ***In vitro* antimicrobial susceptibility of *Escherichia coli* isolates from clinical bovine mastitis in Finland and Israel.** *J Dairy Sci* 2002, **86**:3927-3932.
38. Landin H: **Treatment of mastitis in Swedish dairy production.** *Svensk Veterinärtidning* 2006, **58**:19-25.
39. Nevala M, Taponen S, Pyörälä S: **Bacterial etiology of bovine clinical mastitis- data from Saari Ambulatory Clinic in 2002-2003.** *Suomen Eläinlääkarilehti* **110**:363-369.

doi:10.1186/1751-0147-53-4
Cite this article as: Kalmus *et al.*: Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. *Acta Veterinaria Scandinavica* 2011 **53**:4.

IV

Kalmus, P., Simojoki, H., Orro, T., Taponen, S., Mustonen, K., Holopainen, J., Pyörälä, S., 2013. EFFICACY OF 5-DAY PARENTERAL VS. INTRAMAMMARY BENZYLPENICILLIN TREATMENT OF CLINICAL MASTITIS CAUSED BY GRAM-POSITIVE BACTERIA SUSCEPTIBLE TO PENICILLIN.
Journal of Dairy Science, accepted 18 December 2013.

Efficacy of 5-day parenteral vs. intramammary benzylpenicillin for treatment of clinical mastitis caused by Gram-positive bacteria susceptible to penicillin in vitro

Journal:	*Journal of Dairy Science*
Manuscript ID:	JDS-13-7338.R3
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Kalmus, Piret; Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Science Simojoki, Heli; University of Helsinki, Department of Production Animal Medicine Orro, Toomas; Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Science Taponen, Suvi; University of Helsinki, Department of Production Animal Medicine Mustonen, Katja; University of Helsinki, Department of Production Animal Medicine Holopainen, Jani; Thermo Fisher Scientific, Pyorala, Satu; University of Helsinki, Department of Production Animal Medicine;
Key Words:	dairy cow, clinical mastitis, treatment route, benzylpenicillin

Interpretive summary

Efficacy of 5-day parenteral vs. intramammary benzylpenicillin treatment of clinical mastitis caused by Gram-positive bacteria susceptible to penicillin *in vitro*. Kalmus

The objective of this study was to compare the efficacy of parenteral (intramuscular) and intramammary (IMM) treatment with benzylpenicillin in clinical mastitis caused by Gram-positive bacteria susceptible to penicillin *in vitro*. Cows were randomly placed into two groups and treated with parenteral or IMM benzylpenicillin for five days. Cure from mastitis was assessed using clinical, bacteriological and inflammatory (milk N-acetyl-β-D-glucosaminidase (NAGase) activity) parameters. Bacteriological diagnosis was based on a real-time polymerase chain reaction assay. No association between the route of benzylpenicillin treatment and clinical and bacteriological cure was observed. Milk NAGase activities in the post-treatment samples did not differ between the two treatments; however, the milk NAGase activity was significantly lower in either the clinically or bacteriologically cured animals compared to animals that were not cured.

TREATMENT OF CLINICAL MASTITIS

Efficacy of 5-day parenteral vs. intramammary benzylpenicillin for treatment of clinical mastitis caused by Gram-positive bacteria susceptible to penicillin *in vitro*.

P. Kalmus,[1]* H. Simojoki,† T. Orro,* S. Taponen,† K. Mustonen,† J. Holopainen,‡ and S. Pyörälä†

* Department of Therapy, Institute of Veterinary Medicine and Animal Science, Estonian University of Life Sciences, Tartu, 51014, Estonia

† Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Paroninkuja 20, FI-04920 Saarentaus, Finland

‡ Thermo Fisher Scientific, FI-016210 Vantaa, Finland

[1]Corresponding author:
Piret Kalmus
Estonian University of Life Sciences
Kreutzwaldi 62, 51014 Tartu
Estonia
Phone: +3727313219
Fax: +3727313706
piret.kalmus@emu.ee

ABSTRACT

The efficacy of parenteral (intramuscular) or intramammary (IMM) benzylpenicillin treatment for clinical mastitis caused by Gram-positive bacteria susceptible to penicillin *in vitro* was investigated. Cows with clinical mastitis in one udder quarter were randomly placed into two treatment groups. The preliminary bacteriological diagnosis of intramammary infection (IMI) was based on on-farm culturing, and the bacteriological diagnoses were later confirmed by a quantitative polymerase chain reaction (PCR) assay.

Clinical mastitis caused by Gram-positive bacteria susceptible to benzylpenicillin was treated with penicillin via either the parenteral route (20 mg/ kg) or IMM route (600 mg) once a day for 5 days. The outcome of the treatment was evaluated 3-4 weeks after the onset of the treatment. The affected quarter was examined to assess the clinical cure, and milk samples were collected from the affected quarter to determine the bacteriological cure and milk N-acetyl-β-D-glucosaminidase (NAGase) activity. The survival and the composite milk somatic cell counts (CMSCC) of the treated cows were followed-up for 6 months and 3 months after treatment, respectively.

A total of 140 cows with clinical mastitis were included in the study, 61 being treated with benzylpenicillin parenterally and 79 via the IMM route. From all quarters treated, 108 of 140 (77.1%) were cured clinically and 77 of 140 (55.0%) were cured bacteriologically. The route of treatment did not significantly affect the outcome of the treatment; 80.3% of the quarters with parenteral treatment and 74.7% of the quarters with IMM treatment showed a clinical cure, and 54.1% and 55.7% a bacteriological cure, respectively. The milk NAGase activity was significantly lower in the quarters with a clinical or a bacteriological cure than in the quarters with no cure. The 6-month survival and the proportion of cows with CMSCC < 200,000/mL among the treated cows during the 3-month follow-up period did not significantly differ between the treatment groups.

In conclusion, the outcome of either parenteral or IMM benzylpenicillin treatment of clinical mastitis caused by penicillin-susceptible bacteria was similar.

Keywords: dairy cow, clinical mastitis, treatment route, benzylpenicillin

INTRODUCTION

Bovine mastitis is the most common reason for the use of antimicrobials in dairy cows (Thomson et al., 2008). The most common route of administration for the treatment of mastitis is the intramammary (**IMM**) route (Gruet et al., 2001; Ruegg, 2010). Parenteral treatment of mastitis has been suggested to be more efficient than IMM treatment because of the improved distribution of the drug throughout the mammary gland (Ziv, 1980; Erskine et al., 2003). This would particularly apply to invasive infections, such as mastitis caused by *Staphylococcus aureus* (Erskine et al., 2003; Smith, 2010). Advantages of the IMM route over the parenteral route include high concentrations of the substance in the milk (Moretain et al., 1989; Smith, 2010) and lower consumption of the antimicrobial, because the dose of the drug directly infused into the quarter is small compared with parenteral treatment. A disadvantage of IMM treatment could be an uneven distribution of the antimicrobial to the upper parts of the affected quarter (Ehringer and Kietzmann, 2000) and risk for contamination when infusing the drug via the teat canal.

For the antimicrobial treatment of animal infections, such as mastitis, targeting the treatment toward the causative agents is recommended (OIE, 2013). If the causative agent of infection is susceptible to the so-called first-line antimicrobials, such as agents with a relatively narrow spectrum, including benzylpenicillin, they should be used for treatment (Anon., 2003; Constable et al., 2008). However, in the majority of countries, the treatment of mastitis remains reliant on the routine use of combinations of several active substance or broad-spectrum antimicrobials (Ruegg et al., 2010). Selection pressure for the development of antimicrobial resistance among bacteria is greater when broad-spectrum agents are used (Hunter et al., 2010). Parenteral treatment with benzylpenicillin has been the treatment of choice for clinical mastitis in Nordic countries (Grave et al., 1999; Thomson et al., 2008;

Pyörälä, 2013). Thus far, studies comparing the outcome of parenteral and IMM treatment of clinical mastitis with benzylpenicillin have not been published. One Swedish study compared IMM and parenteral penicillin treatment with no treatment of subclinical mastitis and the bacteriological cure did not differ between the two antimicrobial treatment groups (Hallén Sandgren et al., 2008).

The gold standard to assess cure after treatment is bacteriological culturing, possibly completed with the determination of some indicator of inflammation in the milk, primarily the milk somatic cell count (**SCC**) (Green and Bradley 2010; Ruegg, 2010). Other indicators of inflammation such as milk N-acetyl-*β*-D-glucosaminidase (**NAGase**) activity may also be used (Mattila and Sandholm, 1986). DNA-based bacteriological tests have become available for diagnostic use in bovine mastitis providing an alternative to conventional culturing and are also useful for assessing bacteriological cure after antimicrobial treatment of mastitis (Koskinen et al., 2010).

The objective of the study was to compare the outcome of parenteral and IMM treatment with benzylpenicillin in clinical mastitis caused by Gram-positive bacteria susceptible to penicillin *in vitro*.

MATERIALS AND METHODS

Characteristics of study herds

The study was performed in four dairy herds in the practice area of the Large Animal Clinic of the Estonian University of Life Sciences during 2007-2009 in Estonia. The study period was one year in each farm. The study was carried out in year 2007 in two herds, in 2008 in one herd and 2009 in one herd. The herd size ranged from 300 to 1,000 dairy cows. All farms were loose-housing system farms with a side by side milking parlor where the cows were milked three times per day. An average annual milk yield was 8,387 kg (min. 6,900 kg;

max. 9,850 kg). The mean herd bulk milk somatic cell count per month ranged between 198,000-408,000 cells/mL during the study period.

Collection and analysis of milk samples

Any lactating dairy cow with clinical mastitis was considered for enrollment in the study. Initial exclusion criteria were cows with more than one quarter affected and cows with known chronic mastitis, defined as cows with known high somatic SCC or cows having had more than three mastitis episodes before the beginning of the study. Additionally, cows with a visible teat injury, and cows treated with antimicrobials within one week before the onset of clinical mastitis were excluded. Clinical mastitis was defined as any mastitis in which the cow shows visible, even mild, clinical signs (IDF 1999). Before treatment, the cow was examined clinically and the results recorded on a study form. The clinical signs of the cow were assigned to the following three categories: mild clinical mastitis (1) = changes in the milk appearance, in which the milk from the quarter had abnormal viscosity (watery or thicker than normal), abnormal color (yellow or blood-tinged), or abnormal consistency (flakes or clots), but no udder swelling or systemic signs; moderate clinical mastitis (2) = the same changes in the milk appearance and local signs in the quarter, such as swelling or pain but no systemic signs, such as a lack of appetite, depressed rumen function, or body temperature greater than 39.2°C; and severe clinical mastitis (3) = both local and systemic signs.

Handling of milk samples

Clinical examination of the cows, sampling and on-farm culturing were made by the local farm veterinarians. Before the study, all veterinarians were trained to collect aseptic milk samples and use on-farm culturing techniques. An aseptic milk sample was collected from the affected quarter before treatment. After discarding a few streams of milk, the samples (5-7 mL) were collected into sterile 10 mL plastic tubes. Approximately 2 mL milk was separated

for preliminary on-farm bacteriology and the remaining sample was stored at -20°C. All stored milk samples were submitted to the Thermo Fisher laboratory for DNA-based diagnosis (Pathoproof Mastitis PCR Assay, Thermo Fisher Scientific, Vantaa, Finland) and to the laboratory of the Department of Production Animal Medicine, University of Helsinki, for determination of milk NAGase activity.

Bacteriological culturing at the farm

Preliminary bacteriological examination was performed on the farm every evening using triplate selective mastitis agars, including blood-aesculin agar, mannitol-salt agar, and McConkey agar (Estonian Veterinary and Food Laboratory, Tartu, Estonia). The preliminary on-farm bacteriology was used to differentiate between Gram-positive and Gram-negative bacteria. Ten µL of milk was streaked onto each section and plates were cultured for 12-24h. Bacterial growth was evaluated at first on the blood-esculin agar and then on the McConkey agar (the media for detection of Gram-negative bacteria) or on the salt-mannitol agar (the media for detection of staphylococci). After detection of staphylococci, penicillin resistance indicated by *β*-lactamase production was determined using a chromogenic nitrocefin test (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) (NCCLS 2002).

Bacteriological analysis using real-time PCR analysis

Bacteriological diagnosis of mastitis was based on the PCR results. A commercial real-time PCR test kit (Patho Proof Mastitis PCR Assay, Thermo Fisher Scientific, Vantaa, Finland) was used for direct analysis of all milk samples. The kit protocol involved 4 separate multiplex real-time PCR reactions, which targeted the following bacterial species and groups in total: *Staphylococcus* spp., including *Staph. aureus* and all relevant coagulase-negative staphylococci (**CNS**) species, *Enterococcus* spp., including *E. faecalis* and *E. faecium*, *Corynebacterium bovis*, *Escherichia coli*, *Strep. dysgalactiae*, *Strep. agalactiae*, *Strep. uberis*, *Trueperella (*formerly *Arcanobacterium) pyogenes*/*Peptoniphilus indolicus*, *Klebsiella* spp.,

179 including *K. oxytoca* and *K. pneumoniae*, and *Serratia marcescens*. The PCR assay also
180 detects the staphylococcal beta-lactamase gene *blaZ* coding for penicillin resistance. The
181 assay was performed according to the manufacturer's instructions and as described by
182 Koskinen et al. (2010). Based on the cycle threshold values, the bacterial DNA quantity in
183 each targeted bacterial species was grouped into three classes, +, ++ or +++.

184 ***Milk NAGase activity determination***

185 Milk NAGase activity was determined with a fluoro-optical method using an in-house
186 microplate modification (Hovinen et al., 2010) of the method developed by Mattila and
187 Sandholm (1986). The calibrated milk sample was replaced with a control milk sample with a
188 known concentration of 4-methyl-umbelliferon (**4-MU**), and the NAGase activity was
189 expressed as picomoles 4-MU/min/µL milk at 25°C. The upper detection limit for NAGase
190 activity was 24.49 pmol 4-MU/min/µL. Inter-assay and intra-assay coefficients of variation
191 (CV) for the NAGase activity were 5% and 4%, respectively.

192 ***Treatments***

193 All cows with clinical mastitis were allocated into treatment groups A and B using
194 cow ID (A: even numbers and B: odd numbers). The following two treatments were used:
195 parenteral treatment with benzylpenicillin procaine (Penovet® vet 300 mg/mL, Boehringer
196 Ingelheim Vetmedica, Denmark) in group A or IMM treatment with benzylpenicillin procaine
197 (Carepen® 600 mg, Vetcare Oy, Finland) in group B. One IMM tube was infused into the
198 affected quarter once a day. The dose of benzylpenicillin used for parenteral treatment was 20
199 mg (20, 000 IU) per kg intramuscularly once a day. The duration of treatment was 5 days in
200 both groups. The use of supportive treatment with non-steroidal anti-inflammatory agents
201 (**NSAID**) was possible, but treatment with corticosteroids was not allowed. Treatment with
202 penicillin according to the defined treatment groups began on the day of diagnosis. Treatment
203 was stopped on the next day if Gram-negative bacteria were detected on the selective media

or if the isolated staphylococci were resistant to penicillin (a positive nitrocefin test). Cases of clinical mastitis caused by Gram-negative bacteria were treated with NSAID, fluid therapy and if the case was severe, with fluoroquinolones. Udder quarters infected with penicillin-resistant staphylococci were treated with IMM cloxacillin (Wedeclox mastitis® 1,000 mg cloxacillin, WDT, Garbsen, Germany) once a day for 5 days.

Assessment of treatment outcome

The outcome of the treatment was assessed 3-4 weeks after the onset of treatment by using clinical, inflammatory and bacteriological criteria. The milk samples were collected as described above and frozen at -20°C. The cow was defined as clinically cured if the affected quarter was free from clinical signs. A quarter was defined as bacteriologically cured if the DNA of the same bacterial species detected in the pre-treatment milk sample was not present in the follow-up milk sample. A quarter with the DNA from the same bacterial species detected before the treatment was defined as not cured.

Inflammatory reaction in the affected quarters was studied using milk NAGase activity, which should return back to normal after recovery from inflammation (Mattila and Sandholm, 1986). Milk NAGase activity was determined in the pre-treatment and post-treatment milk samples, and used as an additional parameter to compare outcomes in the treatment groups (Hovinen et al., 2010).

Composite milk somatic cell counts (**CMSCC**) from the cows included in the study were collected from the study herds once each month during a 3 month period after the treatment; the mean number of recordings per cow was 2.6. The culling data were analyzed during a six month period after the treatment. These data originated from the routine herd health recording system.

The final enrollment criteria

Only cows with one affected udder quarter (n=140) with penicillin- susceptible Gram-

229 positive bacteria were included into the study based on the following criteria regarding the
230 species detected by the PCR assay: 1) DNA of one bacterial species only; or 2) DNA of one
231 bacterial species in proportion over 99% from DNA of all target bacterial species detected 3)
232 >90% DNA of a major pathogen combined with a low quantity (+) of DNA of a minor
233 pathogen (CNS or *C. bovis*).

234 PCR-negative samples (n = 25), contaminated samples (more than three different
235 species detected) (n = 27), and samples containing DNA from Gram-negative bacteria (n =
236 11) were removed from the study. Cows (n=44) treated with IMM cloxacillin, and cows (n =
237 26) with *blaZ* gene positive staphylococcal species, but treated unintendedly with
238 benzylpenicillin were excluded from the main material, but analyzed separately.

239 ***Statistical analysis***

240 Prior to the beginning of the study, the sample size necessary for statistical evaluation
241 was calculated as 106 in both treatment groups. The calculations were based on the hypothesis
242 that differences in the cure rates of the parenteral vs. IMM treatment are less than 20%
243 (bacteriological cure rates of 65% and 45%, respectively; two-sided *P*-level at 0.05 and a
244 study power of 80%). This hypothesis was based on the assumption that a large proportion of
245 cases would be caused by *Staph. aureus*. However, after collection of the data a large
246 proportion of the cows were lost, due to missing data or reasons for post-inclusion exclusions
247 and the power of the study to detect at least a 20% difference in the bacteriological cure was
248 59% (sample size of 61 in the parenteral group and 79 in the IMM group).

249 Logistic regression models were used to evaluate the associations between clinical and
250 bacteriological cures, with treatment route. Bacteriological and clinical cures were the
251 outcome variables. The treatment route (IMM, parenteral), bacteriological diagnosis as a 7-
252 level categorical variable (*Staph. aureus*, CNS, *Strep. agalactiae, Strep. dysgalactiae*, *Strep.*
253 *uberis*, *C. bovis*, *T. pyogenes),* and a continuous variable milk NAGase activity in the pre-

254 treatment milk samples (as a marker of the severity of the inflammation) were included as
255 independent variables. Additionally, the lactation number was used as a 4-level categorical
256 variable (1, 2, 3 and ≥ 4 lactations), the days in milk was used as a 4-level categorized
257 variable (1-30, 31-69, 70-140 and > 140 days in milk), and the farm and affected quarter were
258 used as a 4-level variable. Non-significant variables were removed using a stepwise backward
259 elimination procedure. The Wald test was used to evaluate the overall significance of the
260 categorical variables with more than two levels. No significant interactions were detected and
261 as no included variables were associated with any outcome variables both final models
262 included only treatment route as independent variable.

263 Differences in the number of culled cows between the treatment groups during the 6
264 months after treatment were analyzed with logistic model in which the treatment, farm, days
265 in milk, lactation number and bacteriological diagnosis were included. Variables were
266 categorized similarly to the previous models.

267 A linear regression model was used to investigate the associations between milk
268 NAGase activity in the post-treatment milk samples and the route of treatment. Before
269 analysis, the outcome variable milk NAGase activity was logarithmically transformed. The
270 full models included bacteriological recovery (yes/no), clinical recovery (yes/no), treatment
271 route, diagnosed pathogens and milk NAGase activity in clinical mastitis in the pre-treatment
272 milk samples, lactation number, days in milk, farm and affected quarter as fixed variables.
273 The variables categorized similarly to the previous logistic regression models. The Wald test
274 was used to evaluate the overall significance of the categorical variables with more than two
275 levels. Non-significant variables were removed using a stepwise backward elimination
276 procedure. Possible interaction effects of the treatment with diagnosed clinical mastitis
277 pathogens, bacteriological cure, clinical cure and farm were verified. No significant
278 interactions were detected. Assumptions of the model were controlled using normality and

scatter plots of the model residuals. Stata 11.0 (Stata Corp, Texas, USA) software was used for logistic regression models and linear regression model.

For analyzing associations between the treatment and low CMSCC (<200,000 cells/mL) occurrence during the 21-110 days after the mastitis cases, generalized linear mixed model was used. For this model, the GLIMMIX procedure in the SAS/STAT 9.1 software (SAS Institute Inc., Cary, NC, USA) was used. An auto-regressive correlation structure was used for modeling serial correlations of repeated measurements within cows. Treatment, time group after mastitis (21-50, 51-80 and 81-110 days) and their interaction, sample time in relation to mastitis, days in milk at the time of mastitis, and farm were included as fixed factors.

RESULTS

Outcome of benzylpenicillin treatment

In total, 140 quarters with clinical mastitis were included in the study. Clinical signs were defined as mild in 83 cows (59.2%) and moderate in 55 cows (39.2%). Mastitis was defined as severe in two cows (1.4%). Of 140 quarter cases with clinical mastitis, 61 (43.6%) were treated with benzylpenicillin via the parenteral route and 79 (56.4%) with benzylpenicillin via the IMM route. Distribution of the bacteria detected in the milk samples did not significantly differ between the treatment groups (Table 1). *Strep. uberis* was the most common bacteriological finding, followed by other streptococcal species.

No significant associations between the clinical cure (OR = 1.38; 95% CI 0.62, 3.12; P = 0.431) or bacteriological cure (OR = 0.94; 95% CI 0.48, 1.83; P = 0.851) and the route of treatment were observed. The cure rates for the 140 quarters with clinical mastitis infected by Gram-positive bacteria susceptible to benzylpenicillin *in vitro* are shown in table 1.

303 Milk NAGase activities in the post-treatment samples did not differ between the two
304 treatment groups (P = 0.688; table 2). Milk NAGase activity was significantly lower (P =
305 0.003) in the quarters with a clinical cure than the quarters with no clinical cure and in the
306 bacteriologically cured quarters compared with those without bacteriological cure (P = 0.002;
307 table 2). The median NAGase activities in the milk before treatment and in the post-treatment
308 samples are presented in table 3.

309 In total, the number of culled cows was 18 (13.1%) by the end of the 6 month follow-
310 up period after treatment. No data were available for 3 cows. No significant differences
311 between the treatment groups (OR = 0.91, 95% 0.33, 2.46, P = 0.507) were found.

312 ***Composite milk somatic cell count after treatment***

313 Individual cow CMSCC data from three routine test milkings (every 30 days) during
314 the 3-month follow-up period after treatment were available for 126 cows. The summary of
315 data and the proportion of cows with CMSCCs less than 200,000 cells/mL in the two
316 treatment groups at different time points after treatment is shown in table 4. No association (P
317 = 0.787) between the route of penicillin treatment and the proportion of cows with CMSCC
318 <200,000/mL after treatment was seen.

319

320 **DISCUSSION**

321 In this study, the outcome of benzylpenicillin treatment of clinical mastitis caused by
322 Gram-positive bacteria susceptible to penicillin *in vitro* was not affected by the route of
323 administration of the drug. Clinical studies comparing the efficacy of parenteral and IMM
324 treatment for clinical mastitis are in general rare. To the authors' knowledge, field trials
325 comparing the efficacy of parenteral and IMM benzylpenicillin treatment of bovine clinical
326 mastitis have not been published. Parenteral penethamate hydroiodide treatment was
327 compared to IMM penicillin-dihydrostreptomycin treatment in a study performed in New

328 Zealand, and no significant differences were observed (McDougall, 1998). The majority of
329 mastitis cases in that study were caused by *Strep. uberis*, a species susceptible to
330 benzylpenicillin. Sérieys et al. (2005) compared treatment with parenteral penethamate to
331 IMM ampicillin-cloxacillin, with no significant differences between the two treatment
332 regimens. Specific information regarding the *in vitro* susceptibility of the causative agents
333 was not available, and no real comparison can be made. In clinical mastitis experimentally
334 induced by *Strep. uberis* and treated with penicillin, bacteriological cure did not differ
335 between IMM, parenteral or combined treatment groups; however, the groups were so small
336 that no conclusions could be made (Hillerton and Kliem, 2002). The dose of benzylpenicillin
337 procaine used in that study was half of that used in our study, which could affect the
338 parenteral cure rates. No differences between parenteral benzylpenicillin and IMM
339 penethamate were found for the treatment of subclinical mastitis caused by penicillin-
340 sensitive *Staph. aureus* or streptococci (Hallen-Sandgren et al., 2008). In an old U.S. study,
341 the efficacy of IMM amoxicillin alone or combined with intramuscular benzylpenicillin was
342 compared for the treatment of subclinical *Staph. aureus* mastitis (Owens et al., 1988).
343 Bacteriological cure rates were approximately 50% and did not differ between the treatments;
344 however, because no information regarding penicillin susceptibility was available, drawing
345 any conclusions is difficult.

346 Overall, the bacteriological cure rates of clinical mastitis caused by staphylococci and
347 streptococci treated with different antimicrobials and routes of administration have ranged
348 from 56% to 84% (Jarp et al., 1989; Taponen et al., 2003a; Serieys et al., 2005; McDougall et
349 al., 2007; Apparao et al., 2009; Bradley and Green, 2009, Ruegg, 2010). Taponen et al.
350 (2003a) used a 4-day treatment with IMM benzylpenicillin for mastitis caused by penicillin-
351 susceptible Gram-positive bacteria, and reported a clinical cure rate of 75% and a
352 bacteriological cure rate of 73%. The clinical cure rate was similar to our study, but the

bacteriological cure rate was approximately 20% higher than in the present study, which may be due to the different clinical severity of mastitis or different methods used for the bacteriological follow-up. Jarp et al. (1989) reported a total bacteriological cure rate of 68% for clinical mastitis due to Gram-positive, penicillin susceptible bacteria, treated for 5 days with benzylpenicillin, using the same dose as here. The cure rate of that study was also higher than reported in our study, possibly due to the same reasons as mentioned earlier. Bacteriological cure rates of mastitis depend on the causative agent. McDougall et al. (2007) compared treatment of clinical mastitis with three different IMM products, one of them containing procaine penicillin alone. More than half of the cases were caused by *Strep. uberis*, and treatment with penicillin IMM for 1.5 d resulted in a cure rate as high as 91%, which is much higher than found here. Different conditions in the New Zealand such as much lower average milk production and less severe clinical signs may at least partly explain the difference.

Mastitis causing streptococcal species have remained susceptible to benzylpenicillin (Pitkälä et al., 2004; Hendriksen et al., 2008; Bengtsson et al., 2009; Kalmus et al., 2011). *Staph. aureus* and CNS isolated from bovine mastitis have developed resistance to penicillin (Hendriksen et al., 2008; Bagcigil et al., 2012), which may significantly influence the efficacy of treatment (Pyörälä and Pyörälä 1998; Sol et al.; 2000; Taponen et al., 2003b). In our study, 6 of 8 cases of mastitis caused by penicillin-susceptible *Staph. aureus* were cured using either intramuscular or IMM penicillin treatment. The bacteriological cure of 20 quarters with mastitis caused by penicillin-resistant *Staph. aureus* treated for 5 days with cloxacillin was in the present study zero (data not shown). It is known that mastitis caused by penicillin-resistant *Staph. aureus* is difficult to cure (Taponen et al., 2003b; Barkema et al., 2006). The poor treatment response of these cases is mainly not derived from antibiotic resistance. The ability of penicillin-resistant *Staph. aureus* isolates to cause persistent infections may be due to

several virulence factors, possibly linked to the β-lactamase gene of the resistant isolates (Haveri et al., 2005; Van den Borne et al., 2010). In the treatment of mastitis, tested or assumed *in vitro* susceptibility of the causing bacteria is considered a prerequisite for the use of a particular antibiotic, but pre-treatment susceptibility is not always predictive of treatment response *in vivo* (Barlow, 2011).

Benzylpenicillin is a weak acid, which after parenteral administration penetrates poorly into the mammary gland. However, because the MIC values for susceptible organisms are generally very low ($\leq$0.12 µg/mL for staphylococci and $\leq$0.06 µg/mL for streptococci (Prescott et al., 2007; Bengtsson et al., 2009), it is possible to achieve and maintain therapeutic concentrations in the milk using parenteral administration of 20 mg/kg benzylpenicillin procaine once a day as used in this study (Franklin et al., 1984; Ziv and Storper, 1985).

IMM infusion results in concentrations as high as 100-1000-fold of those obtained with parenteral administration, which is advantageous for infections of the milk compartment, such as streptococcal mastitis (Moretain et al., 1989; Erskine et al., 2003). The total dose of antimicrobials administered via the IMM route is considerably lower than that in parenteral treatment. Furthermore, painful injections can be avoided. When infusing IMMs containing narrow-spectrum antimicrobials antibiotics such as benzylpenicillin, strict hygienic measures should be used to avoid inducing mastitis (Middleton and Luby, 2012). IMM administration is the route of choice for mastitis caused by streptococcal species, which reside in the milk compartment (Erskine et al., 2003; Guardabassi et al., 2008). Parenteral or combined treatment has been suggested for mastitis caused by *Staph. aureus* (Erskine et al., 2003; Constable et al., 2008). Taponen et al. (2003b) reported a bacteriological cure of 72% for mastitis caused by penicillin-susceptible *Staph. aureus* treated with 5-day combined parenteral and IMM treatment with penicillin. In our study, no difference was observed

403 between the two routes of treatment, but the *Staph. aureus* group was too small to draw any
404 conclusions. Our group infected with CNS was also small, but based on the literature, IMM is
405 the route of choice in the treatment of CNS mastitis (Erskine et al., 2003; Pyörälä and
406 Taponen, 2009).

407 In this study, bacteriological diagnosis was based on a PCR assay. For the evaluation
408 of the bacteriological cure, strict criteria were used. If DNA of the same species detected
409 before treatment was found alone or together with the DNA of other species in the post-
410 treatment sample, the case was classified as not cured. It is known that the PCR-based assay is
411 more sensitive than a conventional culture (Koskinen et al., 2010). This may be reflected as
412 lower percentages of cure than in previous studies in which conventional culturing was used
413 for assessment. Excluding all samples with more than one species from the analysis would
414 result in the discarding of a considerable number of cases, because the PCR test often detects
415 more than one species (Koskinen et al., 2010).

416 Higher cure rates may have also been expected here because our 5-day treatment is
417 longer than standard treatments used for mastitis in many countries. Longer treatments have
418 been reported to result in higher cure rates, at least for mastitis caused by *Staph. aureus* and
419 *Strep. uberis* (Jarp et al., 1998; Oliver et al., 2004; Deluyker et al., 2005; Krömker et al.,
420 2010). Recently, 5-day treatment with cefquinome did not increase cure rates in clinical
421 *Staph. aureus* mastitis compared with 1.5-day treatment (Swinkels et al., 2013). This
422 discrepant result may be due to the drug used or differences in the virulence of the bacterial
423 strains causing IMIs.

424 In assessing cure rates, the possibility of contamination of the sample with the same
425 species as detected in the pre-treatment sample should also to be taken into account. This
426 could lead to a false positive sample and false classification of the case as not cured.
427 However, this affects both conventional and PCR-based tests. If PCR assays are used to

assess the outcome in treatment trials of mastitis, some adjustments to the tests may be necessary for the interpretation of results.

Combining bacteriology with some indicator of inflammation in the milk would be useful for confirming the assessment (Green and Bradley, 2010). The most common indicator used to monitor the inflammatory status of the udder is milk SCC. Milk NAGase activity is another good choice for this purpose (Pyörälä, 2003). NAGase originates from somatic cells but also from damaged epithelial cells (Mattila and Sandholm, 1986; Pyörälä, 2003). It correlates well with milk SCC and has the advantage that freezing the milk samples does not interfere with the analysis (Pyörälä, 2003). The threshold values of these parameters should perhaps be adjusted for the assessment of the response to mastitis treatment, because the inflammatory reaction of the quarter may last longer than elimination of the infection. The threshold levels of the markers used for screening of mastitis may be too high for monitoring the recovery of the quarter (Pyörälä and Pyörälä, 1997).

Generally, two post-treatment samples are recommended for the bacteriological evaluation of cure (Schukken and Deluyker, 1995). Here, only one sample was collected for practical reasons, but we used a sensitive PCR assay for bacteriology, which could somewhat compensate the lack of the second sampling. Including the cow survival data and cow composite milk SCCs follow-up provides information regarding the long-term effects of the treatments and can be recommended for field trials of mastitis. In the present study, the CMSCCs remained higher and the proportion of low CMSCC cows was numerically smaller in the IMM-treated group, even though no significant differences between the groups were found. A possible explanation for this result is that the cows had other quarters with subclinical IMI, which were also treated when the treatment was administered parenterally and this may have affected cow CMSCCs.

CONCLUSIONS

The outcome of parenteral or intramammary penicillin treatment of mastitis caused by penicillin-susceptible bacteria was found to be similar. We conclude that IMM could routinely be used for the treatment of clinical mastitis caused by streptococcal species. Streptococci reside in the milk compartment, and there are no pharmacokinetic grounds for the use of parenteral administration of the antimicrobial. Parenteral treatment is more invasive and significantly increases the dose of the antimicrobial. The number of quarters infected with *Staph. aureus* were too low to reach any conclusions regarding treatment of *Staph. aureus* mastitis. With a more sensitive PCR method, bacteriological cure rates may be lower, which should be considered by researchers, the pharmaceutical industry and authorities in the future.

ACKNOWLEDGMENTS

This work was funded by grants from the Walter Ehrström Foundation, Finnish Dairy Association, Finnish Veterinary Foundation and the Research Foundation of Veterinary Medicine. We thank Vetcare Oy Company for kindly providing the medicinal preparations. Additional financial support was provided by the Estonian Ministry of Agriculture (Research contract 8-2/T8010) and the Estonian Ministry of Education (Research project 8-2/T9001). Invaluable help of personnel from participated farms is deeply appreciated.

REFERENCES

478 Anonymous. 2003. Use of antimicrobial agents in animals. Report of the working group on
479 antimicrobial agents. Ministry of Agriculture and Forestry in Finland. MAFF Publications 9,
480 2003.
481 Apparao, M. D., P. L. Ruegg, A. Lago, S. Godden, R. Bey and K. Leslie. 2009. Relationship
482 between in vitro susceptibility test results and treatment outcomes for gram-positive mastitis
483 pathogens following treatment with cephapirin sodium. J. Dairy Sci. 92: 2589–2597.
484 Bagcigil, A. F., S. Taponen, J. Koort, B. Bengtsson, A. L. Myllyniemi, A. L. and S. Pyörälä.
485 2012. Genetic basis of penicillin resistance of *S. aureus* isolated in bovine mastitis. Acta Vet.
486 Scand. 54: 69.
487 Barkema, H., Y. H. Schukken and R. N. Zadoks. 2006. Invited review: the role of cow,
488 pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus*
489 mastitis. J. Dairy Sci. 89: 1877-1895.
490 Barlow, J. 2011. Mastitis therapy and antimicrobial susceptibility: a multispecies review with
491 a focus on antibiotic treatment of mastitis in dairy cattle. J. Mammary Gland Biol. Neoplasia,
492 16: 383-407.
493 Bengtsson B., H. E. Unnerstad; T. Ekman, K. Artursson; M. Nilsson-Öst and K. Persson
494 Waller .2009. Antimicrobial susceptibility of udder pathogens from cases of acute clinical
495 mastitis in dairy cows. Vet. Microbiol. 36: 142-149.
496 Bradley A. J. and M. J. Green. 2009. Factors affecting cure when treating bovine clinical
497 mastitis with cephalosporin-based intramammary preparation. J. Dairy Sci. 92: 1941-1953.
498 Constable, P., S. Pyörälä, and G. Smith. 2008. Guidelines for antimicrobial use in cattle.
499 Pages 143-160 in Guide to Antimicrobial Use in Animals. L. Guardabassi, L. B. Jensen and
500 H. Kruse, ed. Blackwell Publishing Ltd., Oxford, UK.

501 Deluyker, H. A., S. N. van Oye and J. F. Boucher. 2005. Factors affecting cure and somatic
502 cell count after pirlimycin treatment of subclinical mastitis in lactating cows. J. Dairy Sci. 88:
503 604–614.
504 Ehinger, A. M., M. Kietzmann, M. 2000. Tissue distribution of benzylpenicillin after
505 intramammary administration in the isolated perfused bovine udder. J. Vet. Pharm. Ther. 23:
506 303-310.
507 Erskine, R. J., S. Wagner and F. J. DeGraves. 2003. Mastitis therapy and pharmacology. Vet.
508 Clin. North Am. Food Anim. Pract. 19:109.
509 Franklin, A., O. Holmberg, M. Horn af Rantzien and G. Åström. 1984. Effect of procaine
510 benzylpenicillin alone or in combination with dihydrostreptomycin on udder pathogens in
511 vitro and in experimentally infected bovine udders. J. Am. Vet. Res. 45: 1398-1402.
512 Grave, K., C. Greko, L. Nilsson, K. Odensvik, T. Mørk and M. Rønning. 1999. The usage of
513 veterinary antibacterial drugs for mastitis in cattle in Norway and Sweden during 1990–1997.
514 Prev. Vet. Med. 42: 45-55.
515 Green, M. and A. Bradley. 2010. Practical methods to evaluate treatment outcomes. Pages
516 131-140 in Proc. National Mastitis Council 49th Annual Meeting, New Mexico, Albuquerque,
517 USA.
518 Gruet P., P. Maincent, X. Berthelot and V. Kaltsatos. 2001. Bovine mastitis and
519 intramammary drug delivery: review and perspectives. Adv. Drug Deliv. Rev. 50: 245-259.
520 Hallén Sandgren, C., K. Persson Waller and U. Emanuelson. 2008. Therapeutic effects of
521 systemic or intramammary antimicrobial treatment of bovine subclinical mastitis during
522 lactation. Vet. J. 175: 108-117.
523 Haveri, M., A. Roslöf, L. Rantala L and S. Pyörälä. 2005. Toxin genes of *Staphylococcus*
524 *aureus* isolated from bovine intramammary infection of different clinical characteristics and

525 outcome. Pages 149-154 in Proc. 4th IDF International Mastitis Conference. Mastitis in dairy
526 production. Current knowledge and future solutions. Maastricht, The Netherlands.
527 Hendriksen, R. S., D. J. Mevius, A. Schroeter, C. Teale, D. Meunier, P. Buitaye, A. Franco,
528 A. Ultinane, A. Amado, M. Moreno, C. Greko , K. Stärk, C. Berghold, A.-L. Myllyniemi, D.
529 Wasyl, M. Sunde and F. M. Aarestrup. 2008. Prevalence of antimicrobial resistance among
530 bacterial pathogens isolated from cattle in different European countries: 2002–2004. Acta Vet.
531 Scand. 50: 28
532 Hillerton, J. E. and K. E Kliem. 2002. Effective treatment of *Streptococcus uberis* clinical
533 mastitis to minimize the use of antibiotics. J. Dairy Sci. 85:1009-1014.
534 Hovinen M., H. Simojoki, R. Pösö, J. Suolaniemi and S. Pyörälä. 2010. N-acetyl-β-D-
535 glucosaminidase activity in normal cow milk. Page 16 in Proc. 8th European Colloquium on
536 Acute Phase Proteins, Helsinki, Finland.
537 Hunter, P. A., S. Dawson, G. L. French, H. Goossens, P. M. Hawkey, E. J. Kuijper, D.
538 Nathwani, D. J. Taylor, C. J. Teale, R. E. Warren, M. H. Wilcox, N. Woodford, M. W. Wulf
539 and L. J. V. Piddock. 2010. Antimicrobial-resistant pathogens in animals and man:
540 prescribing, practices and policies. J. Antimicrob. Chemother. 65 (Suppl. 1): 3–17.
541 IDF (International Dairy Federation). 1999. Suggested interpretation of mastitis terminology.
542 Bull. Int. Dairy Fed. 338: 3–26.
543 Jarp, J., H. P. Bugge, S. Larsen.1989. Clinical trial of three therapeutic regimens for bovine
544 mastitis. Vet. Record. 17.
545 Kalmus, P., B. Aasmäe, A. Kärssin, T. Orro, and K. Kask. 2011. Udder pathogens and their
546 resistance to antimicrobial agents in dairy cows in Estonia Acta Vet. Scand., 53:4.
547 Koskinen, M. T., G. J. Wellenberg, O. C. Sampimon, J. Holopainen, A. Rothkamp, L.
548 Salmikivi, W. A. van Haeringen, T. J. G. M. Lam and S. Pyörälä. 2010. Field comparison of

549 real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis
550 bacteria. J. Dairy Sci. 93: 5705-5715.
551 Krömker, V., J. H. Paduch, D. Klocke, J. Friedrich, C. Zinke. 2010. Efficacy of extended
552 intramammary therapy to treat moderate and severe clinical mastitis in lactating dairy cows.
553 Berl. Munch. Tierarztl. Wochenschr. 123:147-152
554 Mattila, T. and M. Sandholm. 1986. Antitrypsin and N-acetyl-β-D-glucoseaminidase as
555 markers of mastitis in herd of Ayshire cows. Am. J. Vet. Res. 46: 2453-2456.
556 Middelton, J. R. and C. D. Luby. 2012. Short communication *Escherichia coli* mastitis in
557 cattle being treated for *Staphylococcus aureus* intramammary infection Vet. Rec. 162: 156-
558 157.
559 McDougall, S. M. 1998. Efficacy of two antibiotic treatments in curing clinical and
560 subclinical mastitis in lactating dairy cows. N. Z. Vet. J. 46: 226-232.
561 McDougall, S. M., D. G. Arthur, M. A. Bryan, J. J. Vermunt and A. M. Weir. 2007. Clinical
562 and bacteriological response to treatment of clinical mastitis with one of three intramammary
563 antibiotics. N. Z. Vet J. 55: 161-170.
564 Moretain, J. P. and J. Boisseau. 1989. Excretion of penicillins and cephalexin in bovine milk
565 following intramammary administration. Food Add. Contamin. 6: 79-90.
566 OIE. Guidelines on the responsible and prudent use of antimicrobial agents in veterinary
567 medicine. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_1.6.9.htm. Accessed
568 in October 2013
569 Oliver, S. P., B. E. Gillespie, S. J. Headrick, H. Moorehead, P. Lunn, H. H. Dowlen, D. L.
570 Johnson, K. C. Lamar, S. T. Chester and W. M. Moseley. 2004. Efficacy of extended ceftiofur
571 intramammary therapy for treatment of subclinical mastitis in lactating dairy cows. J. Dairy
572 Sci. 87: 2393–2400.

573 Owens, W. E., J. L. Watts, R. L. Boddie, S.C. Nickerson.1998. Antibiotic treatment of
574 mastitis: comparison of intramammary and intramammary plus intramuscular therapies. J.
575 Dairy Sci. 71: 3143-3147.
576 Pitkälä A., M. Haveri, S. Pyörälä, V. Myllys and T. Honkanen-Buzalski. 2004. Bovine
577 mastitis in Finland 2001 — prevalence, distribution of bacteria and antimicrobial resistance. J.
578 Dairy Sci. 87: 2433-2441.
579 Prescott, J. 2007. Beta-lactam antibiotics: Penam penicillins. Pages 121-137 in Antimicrobial
580 Therapy in Veterinary Medicine, 4th edition. S. Giguère, J. F. Prescott, J. D. Baggot, R. D.
581 Walker and P. M. Dowling , ed. Blackwell Publishing Ltd., Oxford, UK.
582 Pyörälä, S. and E. Pyörälä. 1997. Accuracy of methods using somatic cell count and milk N-
583 acetyl-*β*-D-Glucosaminidase activity in milk to assess the bacteriological cure of bovine
584 clinical mastitis. J. Dairy Sci. 80: 2820-2825.
585 Pyörälä S. and E. Pyörälä. 1998. Efficacy of parenteral administration of three antimicrobial
586 agents in treatment of clinical mastitis in lactating cows: 487 cases (1989-1995). J. Am. Vet.
587 Med. Assoc. 212: 407-412.
588 Pyörälä, S. 2003. Indicators of inflammation in the diagnosis of mastitis. Vet. Res. 34: 565-
589 578.
590 Pyörälä, S. and S. Taponen. 2009. Coagulase-negative staphylococci – emerging mastitis
591 pathogens. Vet. Mic.134: 3-8.
592 Pyörälä, S. 2013. Treatment of bovine mastitis – Nordic principles. Page 29 in Proc. 29th
593 NKVet Mastitis Symposium, Reykjavik, Iceland.
594 Ruegg, P. 2010. The application of evidence based medicine to mastitis therapy. Pages 78-94
595 in Proc. Updates on Ruminant Production and Medicine XXVI World Buiatrics Congress,
596 Santiago, Chile.

Schukken, Y. H., H. A. Deluyker. 1995. Design of field trials for the evaluation of antibacterial products for therapy of bovine clinical mastitis. J. Vet. Pharmacol. Ther. 18: 274-83.

Serieys, F., Y. Raquet, L. Goby, H. Schmidt and G. Friton. 2005. Comparative efficacy of local and systemic antibiotic treatment in lactating cows with clinical mastitis. J. Dairy Sci. 88: 93-99.

Smith, G.W. 2010. The pharmacologic aspects of mastitis treatment. Pages 98-108 in National mastitis Council Annual Meeting Proceedings. Nat. Mastitis Counc., Inc., Madison, WI.

Sol, J., O. C. Sampimon, H. W. Barkema and Y. H. Schukken. 2000. Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. J. Dairy Sci. 83: 278-284.

Swinkels, J. M., P. Cox, Y. H. Schukken and T. J. G. H Lam. 2013. Efficacy of extended cefquinome treatmentof clinical *Staphylococcus aureus* mastitis. J. Dairy Sci. 96: 1–10

Taponen, S., K. Dredge, B. Henriksson, A. Pyyhtiä, L. Suojala, R. Junni, K. Heinonen and S. Pyörälä. 2003 a. Efficacy of intramammary treatment with procaine penicillin G vs. procaine penicillin plus neomycin in bovine clinical mastitis caused by penicillin-susceptible, gram-positive bacteria – a double blind field study. J. Vet. Pharm. Therap. 26: 193-198.

Taponen, S., A. Jantunen, E. Pyörälä and S. Pyörälä. 2003 b. Efficacy of targeted 5-day parenteral and intramammary treatment of clinical *Staphylococcus aureus* mastitis caused by penicillin-susceptible or penicillin-resistant bacterial isolate. Acta Vet. Scand. 44: 53-62.

Thomson, K., M. Rantala, M. Hautala, S. Pyörälä and L. Kaartinen. 2008. Cross-sectional prospective survey to study indication-based usage of antimicrobials in animals: results of use in cattle. BMC Vet. Res. 4:15.

620 Van den Borne B. H. P., M. Nielen, M. M. van Schaik, M. B. Melchior, T. J. G. M. Lam and
621 R. N. Zadoks. 2010. Host adaptation of bovine *Staphylococcus aureus* seems associated with
622 bacteriological cure after lactational antimicrobial treatment. J. Dairy Sci. 93: 2550-2558.
623 Ziv, G. Drug selection and use in mastitis: systemic vs. local therapy.1980. J. Am. Vet. Med.
624 Assoc. 176: 1109-1115.
625 Ziv, G., and M. Storper. 1985. Intramuscular treatment of subclinical staphylococcal mastitis
626 in lactating cows with penicillin G, methicillin and their esters. J. Vet. Pharmacol. Therap. 8:
627 276-283.

628

629 **Table 1.** The outcome of parenteral and intramammary 5-day treatment with benzylpenicillin
630 of bovine clinical mastitis (n = 140 quarters) caused by Gram-positive bacteria susceptible to
631 benzylpenicillin *in vitro*.

Pathogen	Clinical cure		Bacteriological cure	
	IM[1] n	IMM[2] n	IM[1] n	IMM[2] n
Staph. aureus (n = 8)	1/2	5/6	1/2	5/6
CNS (n = 13)	4/6	6/7	2/6	4/7
Strep. uberis (n = 66)	29/34	22/32	20/34	16/32
*Strep. agalactiae (*n = 14)	6/6	6/8	4/6	6/8
Strep. dysgalactiae (n = 19)	5/8	9/11	5/8	8/11
C. bovis (n = 6)	1/1	3/5	0/1	3/5
T. pyogenes / P.indolicus (n = 14)	3/4	8/10	1/4	2/10
Total (n = 139) (%)[3]	49/61 (80.3)	59/79 (74.7)	33/61 (54.1)	44/79 (55.7)

632 [1] Intramuscular treatment
633 [2] Intramammary treatment
634 [3] The proportion of cured udder quarters
635

636 **Table 2.** Linear regression model of associations between milk NAGase activity in the post-
637 treatment milk sample (n = 140) and route (intramammary or parenteral) of treatment in
638 clinical mastitis caused by Gram-positive bacteria.

Variable	Estimate[1]	95% CI	*P*-value	Wald test *P*-value
Treatment				
IMM (n = 79)	0			
IM (n = 61)	-0.08	-0.44; 0.26	0.688	
Bacteriological cure				
No (n =63)	0			
Yes (n = 77)	-0.58	-0.95; -0.21	0.002	
Clinical cure				
No (n = 32)	0			
Yes (n = 108)	-0.67	-1.11; -0.23	0.003	
Farm				0.000
Farm 1. (n = 17)	0			
Farm 2. (n = 11)	-0.28	-1.12; 0.55	0.507	
Farm 3. (n = 66)	-1.01	-1.67; -0.47	0.001	
Farm 4. (n = 46)	-0.13	-0.81; 0.43	0.544	
Intercept	2.532	1.851; 3.213	0.000	

639 [1] Estimates are in logarithmic scale
640

641 **Table 3.** Milk NAGase activity in milk samples from quarters with clinical or bacteriological
642 cure or no cure (n = 140) before and after 5-day parenteral or intramammary penicillin
643 treatment of clinical mastitis caused by Gram-positive bacteria.

	Median milk NAGase activity (min; max) (pmol 4-MU/min/μL	
	Before treatment	After treatment
Clinical cure		
Yes (n = 108)	24.18 (0.53; 24.49)	2.73 (0.75; 24.29)
No (n = 32)	17.17 (1.49; 24.49)	5.84 (0.59; 24.49)
Bacteriological cure		
Yes (n = 77)	17.58 (1.49; 24.49)	2.44 (0.15; 24.49)
No (n = 63)	24.49 (0.53; 24.49)	3.41 (0.16; 24.49)
Treatment		
IM (n = 61)	24.49 (1.22; 24.49)	2.32 (0.15; 24.49)
IMM (n =79)	20.53 (0.53; 24.49)	3.12 (0.16; 24.49)

644

645 **Table 4.** Individual cow composite milk somatic cell counts (CMSCCs) and proportions of
646 cows with CMSCCs <200,000 cells/mL collected during a 3-month period (21-110 days) after
647 parenteral or intramammary penicillin treatment of clinical mastitis caused by Gram-positive
648 bacteria.

Period (days) after clinical mastitis	Individual cow CMSCC (cells/mL)		Proportion of samples with CMSCC below 200,000 cells/mL	*P*-values
	Mean (±SD)	Median (min; max)	%	
21-50 days				
IM[1] (n = 59)	456,400 (± 649,800)	194,000 (17,000; 3,287,000)	50.8	0.137
IMM[2] (n = 70)	851,246 (± 1,332,200)	260,000 (5,000; 7,073,000)	39.6	
51-80 days				
IM[1] (n = 49)	408,604 (± 526,540)	210,000 (11,000; 2,384,000)	47.4	0.312
IMM[2] (n = 62)	678,700 (± 1,392,500)	1,818,000 (9,000; 6,565,000)	56.8	
81-110 days				
IM[1] (n = 46)	670,100 (± 1,175,300)	256,500 (8,000; 6,062,000)	43.2	0.456
IMM[2] (n = 53)	648,900 (± 1,251,200)	195,000 (10,000; 8,272,000)	50.6	

649 [1] Intramuscular treatment
650 [2] Intramammary treatment

CURRICULUM VITAE

First name:	Piret
Surname:	Kalmus
Date of birth:	February 20, 1971
Institution:	Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Kreutzwaldi 62, 51014 Tartu, Estonia
Position:	Lecturer

Education

2003-2013	Estonian University of Life Sciences Institute of Veterinary Medicine and Animal Sciences PhD studies
1998-2001	Estonian University of Life Sciences Institute of Veterinary Medicine and Animal Sciences, Master studies of Veterinary Medicine
1989-1994	Estonian University of Life Sciences, veterinary medicine studies
1978-1998	Tartu 7th High School

Work experiences:

2002- …	Department of Therapy, Institute of Veterinary Medicine and Animal Sciences, lecturer of productive animal internal medicine
1994- 2001	Department of Therapy, Institute of Veterinary Medicine and Animal Sciences, assistant of internal medicine

Administrative responsibilities:

2002-2012	Member of Education Committee of Institute of Veterinary Medicine and Animal Sciences
2005-2008	Institute of Veterinary Medicine and Animal Sciences project: Practical training reorganisation in the veterinary curriculum", project leader
2002….	Estonian University of Life Sciences, Open University, lecturer of continuing education training programs
1995- ….	Member of the Estonian Veterinary Association

Participation in reseach projects:

2013-…	Reseach project founded by the Estonian Ministry of Agriculture „Raw milk quality. A pilot study“ (8-2/T13091VLTO).
2012-…	Reseach project founded by the Estonian Reseach Council „ Transfer routes for antibiotic resistance“ (8-2/T12036VLBS).
2009-…	Reseach project founded by the Estonian Ministry of Agriculture „Monitoring of antimicrobial resistance of pathogens isolated from animals (8-2/T10043VLLT).
2009-2012	Reseach project founded by the Estonian Ministry of Education and Reseach „Measuring of host inflammatory response as a research tool in clinical veterinary science“ (8-2/T9001VLVL).
2007-2010	Reseach project founded by the University of Helsinki „Treatment of clinical mastitis with parenteral or intramammary penicillin G: a field trial.“
2004-2007	Reseach project founded by the Estonian Ministry of Education and Reseach „Endocrinological and immunological changes during the postpartum period and their association with establishment of new pregnancy, metabolic status and clinical diseases in Estonian high producing dairy cows (ETF5733).

ELULOOKIRJELDUS

Eesnimi:	Piret
Perekonnanimi:	Kalmus
Sünniaeg:	20. veebruar 1971
Töökoht:	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, Kreutzwaldi 62, 51014 Tartu, Eesti
Ametikoht:	Lektor

Haridus:

2003-2013	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, doktorantuur
1998-2001	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, magistrantuur
1989-1994	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, loomaarstiõpe
1978-1998	Tartu 7. Keskkool

Teenistuskäik:

2002- …	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, teraapia osakond, produktiivloomade sisehaiguste lektor
1994- 2001	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, teraapia osakond, kliinilis-laboratoorse diagnostika assistent

Administratiivne tegevus:

2002-2012	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, veterinaarmeditsiini eriala õppekavakomisjoni liige
2005-2008	Eesti Maaülikool, veterinaarmeditsiini ja loomakasvatuse instituut, projekti „Veterinaarmeditsiini alase õppetöö kvaliteedi tõstmine EPMÜ-s süsteemse ettevõttepraktika korraldamise kaudu“, projektijuht
2002…	Eesti Maaülikool, avatud ülikooli täiendõppekoolituste läbiviija
1995-…	Eesti Loomaarstide Ühingu liige

Osalemine uurimisprojektides:

2013-…	Põllumajandusministeeriumi poolt rahastatud projekti „Toorpiima kvaliteedialane uuring“ (8-2/T13091VLTO).
2012-….	Eesti Teadusagentuuri poolt rahastatud tervishoiuteaduste võimekuse edendamise programm TerVE projekt „Antibiootikumiresistentsuse levikuteed“ (8-2/T12036VLBS).
2009-…	Põllumajandusministeeriumi poolt rahastatud rakendusuuringu projekt „Loomade mikroobide antibiootikumiresistentsuse uuring (8-2/T10043VLLT).
2009-2012	Haridus- ja teadusministeeriumi poolt rahastatud baasfinantseering „Põletikuvastuse mõõtmise kasutamine teadusuuringutes kliinilises veterinaarmeditsiinis“ (8-2/T9001VLVL).
2007-2010	Helsinki Ülikooli poolt rahastatud kliiniline uuring „Kliinilise mastiidi ravi süsteemse ja intramammaarse penitsilliiniga“.
2004-2007	Haridus- ja Teadusministeeriumi poolt rahastatud teadusprojekt „Poegimisjärgsed endokrinoloogilised ja immuunfunktsiooni muutused Eesti kõrgetoodangulistel lehmadel, nende seos taastiinestumise, metaboolse seisundi ja kliiniliste haigestumistega“ (ETF5733).

LIST OF PUBLICATIONS

1.1. Sholarly articles indexed by Thomson Reuters Web of Science

Kalmus, P., Viltrop, A., Aasmäe,B., Kask, K., 2006. Occurrence of clinical mastitis in primiparous Estonian dairy cows with different housing conditions. Acta Vet. Scand. 48: 21.

Kalmus, P., Orro, T., Waldmann, A., Lindjärv, R., Kask, K., 2009. Effect of yeast culture on milk production and metabolic and reproductive performance of early lactation dairy cows. Acta Vet. Scand. 51: 32

Kalmus, P., Aasmäe, B., Karssin, A., Orro, T., Kask, K., 2011. Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia . Acta Vet. Scand. 53:4

Kalmus, P., Simojoki, H., Pyörälä, S., Taponen, S., Holopainen, J., Orro, T., 2013. Milk haptoglobin, milk amyloid A and NAGase activity in bovine naturally occurring clinical mastitis diagnosed with a quantitative PCR test. J. Dairy Sci. 96, 3662 - 3670.

1.3. Scholarly articles in Estonian and other peer-reviewed research journals with a local editorial board

Aasmäe, B., Kalmus, P., Tiirats, T., 2003 Antimicrobial resistance of pathogens causing clinical mastitis in dairy cows. Lüpsilehmade kliinilist mastiiti põhjustavate mikroobide antibiootikumiresistentsus. Agraarteadus: Journal of Agricultural Science: Akadeemilise Põllumajanduse Seltsi väljaanne, XIV(3), 139 - 143.

3.2. Articles/chapters in books published by the publishers not listed in Annex.

Aasmäe, B., Kalmus, P., 2012. Antimicrobial resistance of animal pathogens 2006-2009 in Estonia. In: Reseach for rural development. (Ed.) Markevica, A., Kriauciunene, Z., Karpova-Sadigova, N., Latvian University of Agriculture, Jelgava, Latvia, 181-188.

3.4. - Articles/presentations published in conference proceedings

Kalmus, P., Aasmäe, B., Viltrop, A., Kask, K., 2006. Occurrence of clinical mastitis of heifers in different housing condition in Estonian dairy herds. In: Proceedings of XXIV World Buiatric Congress 2006: XXIV World Buiatric Congress 2006, Nice, France, October, 15-19, 2006, 238.

Aasmäe, B., Kalmus, P., Kalmus, K., Häkkinen, L., 2011. Monitoring of antimicrobial resistance of animal pathogens in Estonia. In: Proceedings of XV ISAH Congress „Animal Hygiene and Suistainable Livestock Production". Vienna, Austria, July 3-7, 2011, 1435-1439.

Kalmus, P., Aasmäe, B., Häkkinen, L., Orro, T., 2013. Intramammary antibiotic usage and antimicrobial susceptibility of beetalactamasepositive *S. aureus* from clinical mastitis in Estonia 2008-2012. In the Proceeding of 29th NKVet Symposium: Mastitis -new knowledge on diagnostics and control on modern dairy farms. The Nordic Commitee for Veterinary Scientific Cooperation, Reyikjavik, Iceland, May 11-14, 2013 36.

Kalmus, P., Simojoki, H., Pyörälä, S., 2013. Vaccination with a commercial mastitis vaccine Startvac® did not affect the incidence of clinical mastitis in a large dairy herd. In the Proceeding of 29th NKVet Symposium: Mastitis -new knowledge on diagnostics and control on modern dairy farms. The Nordic Commitee for Veterinary Scientific Cooperation, Reyikjavik, Iceland, may 11-14, 2013, 37.

3.5. - Articles/presentations published in local conference proceedings

Kalmus, P., Aasmäe, B., 2010. Mikroobide antibiootikumiresistentsuse monitooring Eestis aastatel 2005.-2009. In: Terve loom ja tervislik toit, 2010, Tartu. (Ed.) Jaakma, Ü., Tartu, Estonia, 4-9.

Kalmus, P., Aasmäe, B.,2011. Ravimijääkide piima sattumise tõenäosus, ravimijääkide määramise kiirtestid. In: Terve loom ja tervislik toit 2011, Tartu. (Ed.) Jaakma, Ü., Tartu, Estonia, 85-90.

6.3. Popular science articles

Kalmus, P., 2010. Udarapõletike ravi piimaproovide järgi. In: Maamajandus, Tallinn, Estonia.

Kalmus, P., Aasmäe, B., 2010 Kuum suvi mõjutas piimakvaliteeti. In: Maamajandus, Tallinn, Estonia.

Kalmus, P., 2011. Piimaproovide uurimise olulisus, vajadus ja mõttekus. In: Maamajandus, Tallinn, Estonia.

Aasmäe B., Kalmus, P., 2011. Antibiootikumid-nii sõbrad kui vaenlased. In: Eesti Loomaarstlik Ringvaade 4, Tallinn. (Ed.) Aland, A., Tallinn, Estonia, 15-19.

Aasmäe, B., Kalmus, P., Onoper, A., Lehtla, A., Häkkinen, L., Birkenfeldt, M., 2012. Soovitused antibiootikumide mõistlikuks kasutamiseks eri loomaliikide bakteriaalsete infektsioonide ravis. In: Eesti Loomaarstlik Ringvaade4,Tallinn. (Ed.) Gerz, A., Tallinn, Estonia, 18-24.

Raaperi, K., Kalmus, P., 2013. Abortide sagedasemad põhjused ja diagnoosimine veistel. In: Eesti Loomaarstlik Ringvaade1, Tallinn. (Ed.) Gerz, A., Tallinn, Estonia, 16-19.

Meremäe, K., Roasto, M., Kalmus, P., Viltrop, A., Kramarenko, T., 2013. Toorpiima ohutusest põhjalikumalt. In: Eesti Loomaarstlik Ringvaade2, Tallinn. (Ed.) Gerz, A., Talinn, Estonia, 22-29.

Kalmus, P., 2013. Udaratervis robotlüpsiga lautades. In: Maamajandus, Tallinn, Estonia.

VIIS VIIMAST KAITSMIST

ARNE KÜÜT

CHARACTERISTICS OF BIOETHANOL FUEL OBATAINED FROM LIGNOCELLULOSE BIOMASS IN INTERNAL COMBUSTION RECIPROCATING ENGINES WITH SPARK- AND COMPRESSIONIGNITION
LIGNOTSELLULOOSSEST BIOMASSIST SAADAVATE BIOETANOOLKÜTUSTE KARAKTERISTIKUD SÄDE- JA SURVESÜÜTEGA SISEPÕLEMISMOOTORITES
Professor **Jüri Olt**
8. november 2013

LEILA MAINLA

CHANGES IN THE BIOCHEMICAL COMPOSITION OF APPLE (*Malus domestica* Borkh.) FRUITS DEPENDING ON ROOTSTOCK AND CALCIUM TREATMENT
MUUTUSED ÕUNTE BIOKEEMILISES KOOSTISES SÕLTUVALT AED-ÕUNAPUU (*Malus domestica* Borkh.) POOKEALUSEST JA KALTSIUMIGA VÄETAMISEST
Professor **Kadri Karp**, dotsent **Ulvi Moor**
15. november 2013

FLOORTJE VODDE

MICROSITES AND TREE REGENERATION DYNAMICS: PROLONGED STORM EFFECTS IN HEMIBOREAL MIXED FOREST
TORMIKAHJUSTUSTE KÄIGUS TEKKINUD MIKROALADE DÜNAAMIKA JA HÄIRINGUJÄRGNE PUURINDE UUENEMINE HEMIBOREAALSES SEGAMETSAS
Professor **Kalev Jõgiste**, professor **Frits Mohren** (*Wageningen University, The Netherlands*)
16. detsember 2013

MEELIS SEEDRE

DISTURBANCE EFFECTS ON BOREAL FOREST ECOSYSTEM CARBON DYNAMICS
HÄIRINGUREŽIIMI MÕJU BOREAALSE METSAÖKOSÜSTEEMI SÜSINIKUVOOGUDELE
Professor **Kalev Jõgiste**, prof. **Han Chen** (*Lakehead University, Canada*)
16. detsember 2013

HEDI HARZIA

ASSOCIATIONS BETWEEN METABOLIC PROFILE AND COAGULATION ABILITY OF BOVINE MILK, EFFECT OF FEEDING AND LACTATION STAGE
LEHMAPIIMA METABOOLSE PROFIILI JA LAAPUMISE VAHELISED SEOSED, SÖÖTMISE JA LAKTATSIOONIPERIOODI MÕJU
Prof. *emer.* **Olav Kärt**, prof. **Ursel Soomets** (Tartu Ülikool), vanemteadur **Kalle Kilk** (Tartu Ülikool)
20. detsember 2013

ISBN 978-9949-536-13-9 (trükis)
ISBN 978-9949-536-14-6 (pdf)

roheline trükis

Trükitud taastoodetud paberile looduslike trükivärvidega © Ecoprint