Bovine mastitis caused by *Escherichia coli*  
– clinical, bacteriological and therapeutic aspects  

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ACADEMIC DISSERTATION  

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Walter Hall, Agnes Sjöbergin katu 2, Helsinki, on 13th October 2010, at 12 noon.  

Helsinki 2010
To my Mother, my sister Päivi and my daughter Maria
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1. ABSTRACT

Bovine mastitis caused by *Escherichia coli* remains a problem in many countries despite of improvements in managing and housing of dairy cattle. Cows with compromised immune systems, especially those in early lactation, are particularly susceptible to *E. coli* intramammary infection. Mastitis caused by *E. coli* varies from mild, with local signs only, to severe systemic. The severe form of *E. coli* mastitis is associated with loss of milk production and can result in death of the cow. Intramammary infection caused by *E. coli* is often eliminated spontaneously as the defense mechanisms of the cow are able to clear the bacteria from the udder. The aims of this thesis were to investigate host response to *E. coli* mastitis in terms of characteristics of bacteria, effect of antimicrobial and non-antimicrobial treatment, and prophylactic effect of recombinant human lactoferrin expressed in milk.

Host response was studied using two consecutive intramammary challenges with *E. coli* in the same cows at a short interval. All cows became infected, but local signs were significantly milder and disappeared faster after the second challenge. The same pattern was recorded for the indicators of inflammation; the differences being statistically significant for serum and milk haptoglobin, milk serum amyloid A, and white blood cell count. Milk production returned to the pre-challenge level significantly faster after the second challenge. This study revealed a possible carry-over or immunizing effect of the previous intramammary infection by the same pathogen.

Broad-spectrum antimicrobials have been widely used for treating *E. coli* mastitis, although the results of treatment studies have been controversial. In our field study, systemic enrofloxacin treatment did not result in better bacteriological or clinical cure of acute clinical mastitis caused by *E. coli* than supportive treatment alone. Enrofloxacin treatment did not affect survival of the cows, return of quarter milk production or tissue damage of the affected quarter within three weeks post-treatment; nor did it affect the length of time the cow remained in the herd during the six-month follow-up period. The only positive effect of the enrofloxacin treatment was a higher bacteriological cure 2 days post-treatment. On the contrary, clinical cure on day 2 was lower in the enrofloxacin treated cows. Clinical cure assessed 21 days post-treatment was relatively low in both groups, which probably reflects the severe nature of acute *E. coli* mastitis. Our study did not support the use of parenteral antimicrobial treatment of clinical *E. coli* mastitis. In severe cases of coliform mastitis, antimicrobial treatment could, however, still be recommended for safety reasons, as it can increase
the elimination of bacteria and prevent possible bacteraemia. Our results indicated also that frequent milking at the acute stage of mastitis might improve the initial clinical cure of *E. coli* mastitis and decrease inflammation and tissue damage in the infected quarter.

Most *E. coli* isolates belonged to the phylogenetic group A, indicating their commensal nature. Close to 40% of the isolates had at least one virulence gene, but combinations of virulence genes varied greatly, each combination being present mainly in a single isolate. Approximately 30% of the isolates showed resistance to one or more antimicrobials tested, most commonly against ampicillin, streptomycin, tetracycline and sulphonamides. No specific virulence factor, phylogenetic group or resistance to antimicrobials was associated with persistence or severity of disease among mastitis *E. coli* isolates in our field study. These results indicate that characteristics of bacteria are not likely to affect the clinical course and outcome of *E. coli* mastitis. In 11% of mastitis cases, the same genotype of *E. coli* was isolated from the affected quarter three weeks post-treatment as originally, indicating persistence of the same genotype of *E. coli* in the quarter.

Non-antimicrobial treatments, such as lactoferrin, which has antibacterial and lipopolysaccharide neutralizing properties, could be beneficial in the treatment of *E. coli* mastitis. The efficacy of intramammary lactoferrin was compared with that of systemic enrofloxacin in an experimentally induced *E. coli* model. No significant differences were found in the clinical signs between cows treated with lactoferrin and those treated with enrofloxacin. The results from this study remained inconclusive. The prophylactic effect of lactoferrin against *E. coli* mastitis was studied in a new model of using hLf-transgenic cows, which expressed recombinant human lactoferrin in their milk. This was the first study to describe an experimentally induced *E. coli* mastitis model using transgenic cows. The high concentration of lactoferrin in the milk of the transgenic cows did not protect the cows from *E. coli* intramammary infection, and all of them became infected. No differences were noted in the bacterial growth, times to bacterial elimination, clinical signs or in any of the milk or blood inflammatory parameters, except in concentrations of haptoglobin and cortisol in the serum.

Prevention of *E. coli* mastitis relies on decreasing the infection pressure in the environment of the cows and improving the cow comfort and herd management. In *E. coli* mastitis, the treatment should be as efficient as possible, but novel therapeutic approaches are needed, as the efficacy of the current commonly used antimicrobial treatments is not satisfactory.
2. LIST OF ORIGINAL ARTICLES

This thesis is based on the following articles, referred to in the text by their Roman numerals I-V:


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3. Abbreviations

afa8 afimbrial adhesin AFA8
APP acute phase proteins
APR acute phase response
AFOS serum alanine aminofosfatase
ALAT serum alanine aminotransferase
ASAT serum aspartate aminotransferase
astA enteroaggregative heat-stable toxin
bLf bovine lactoferrin
cfu colony forming units
CMT California Mastitis Test
CNF1 cytotoxic necrotizing factor 1
CNF2 cytotoxic necrotizing factor 2
Cva colisin V plasmid
DIM days in milk
eaeA intimin
ehxA enterohemolysin
ELISA enzyme-linked immunosorbent assay
ESBL extended spectrum beta-lactamase
f17A F17 fimbria
hLf human lactoferrin
Hp haptoglobin
irp2 yersinia bactin
iss increased serum survival
iucD aerobactin
LBP lipopolysaccharide binding protein
LPS lipopolysaccharide
Lf lactoferrin
MIC minimum inhibitory concentration
NAGase N-acetyl-β-D-glucosaminidase
NSAID non-steroidal anti-inflammatory drugs
papC P-fimbria
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PCV</td>
<td>packed cell volume</td>
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<td>PC</td>
<td>postchallenge</td>
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<td>PMN</td>
<td>polymorphonuclear leucocytes</td>
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<tr>
<td>rhLf</td>
<td>recombinant human lactoferin</td>
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<tr>
<td>SAA</td>
<td>serum amyloid A</td>
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<tr>
<td>Saa</td>
<td>autoagglutinating adhesin</td>
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<tr>
<td>SCC</td>
<td>somatic cell count</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>sfaD</td>
<td>S-fimbria</td>
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<tr>
<td>stx1</td>
<td>shigatoxin 1</td>
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<td>stx2</td>
<td>shigatoxin 2</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
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<tr>
<td>Tsh</td>
<td>temperature-sensitive hemagglutinin</td>
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<tr>
<td>Vat</td>
<td>vacuolating autotransporter protein</td>
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<tr>
<td>WBC</td>
<td>white blood cell</td>
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4. INTRODUCTION

*Escherichia coli* is among the most common infectious agents isolated from severe mastitis cases in modern dairy farms (Hogan et al., 1989; Bradley et al., 2007). *E. coli* has been reported to be the most common cause of clinical mastitis in well-managed dairy herds with low milk somatic cell counts (SCC) in the United Kingdom (Bradley, 2002). In Finland, *E. coli* has been isolated in 6% of quarter milk samples taken from clinical mastitis cases, more frequently in spring and summer (Koivula et al., 2007). Clinical *E. coli* mastitis can range from mild with only local signs to severe disease with systemic clinical signs. In severe cases the outcome can be acute tissue damage and complete loss of milk production or even the death of the diseased cow (Golodetz et al., 1983; Shuster et al., 1996; Shpigel et al., 1997).

Cows in early lactation are reported to be more susceptible to *E. coli* mastitis (Hill et al., 1978; Hill et al., 1979; Pyörälä and Pyörälä, 1998). This has been shown to result from decreased neutrophil function (Kehrli et al., 1989; Mehrzad et al., 2002), delayed neutrophil migration to the mammary gland (Hill et al., 1979; Kehrli et al., 1989; Shuster et al., 1996; Vandeputte-Van Messom et al., 1993) and faster growth of bacteria in the mammary gland of early-lactating cows (Shuster et al., 1996).

Factors that are cow-dependent, like the speed of the inflammatory response, lactation stage and age of the cow, are thought to determine the severity of *E. coli* mastitis (Burvenich et al., 2003). The role of specific bacterial features, such as virulence factors, has been considered to be small. The only virulence factor of *E. coli* associated with bovine mastitis is serum resistance (Carrol and Jasper, 1977; Sanchez-Carlo et al., 1984; Fang and Pyörälä, 1996). A variety of different virulence factors, individually and in combinations, has been detected in *E. coli* isolates that cause mastitis (Nemeth et al., 1991; Nemeth et al., 1994; Lipman et al., 1995; Kaipainen et al., 2002). However, the most mastitis isolates have not possessed any of the virulence factors evaluated (Sanchez-Carlo et al., 1984; Nemeth et al., 1991; Kaipainen et al., 2002; Wenz et al., 2006).

Broad-spectrum antimicrobials are commonly used systemically or as an intramammary in the treatment of acute *E. coli* mastitis (Erskine et al., 2003). However, results of the treatment studies have been controversial. No difference was established between groups treated with or without antimicrobials in experimentally induced *E. coli* mastitis (Erskine et al., 1992; Pyörälä et al., 1994),
while other studies reported some benefits of antimicrobial treatment (Shpigel et al., 1997; Rantala et al., 2002; Poutrel et al., 2008). Parenteral administration of broad-spectrum antimicrobials has been recommended for the treatment of severe coliform mastitis, due to the risk of bacteremia (Cebra et al., 1996; Wenz et al., 2001b). Broad-spectrum antimicrobials, commonly used for mastitis treatment, are of major therapeutic importance also in human medicine, and their broad use in food-producing animals has stimulated public health concerns (Collignon et al., 2009).

Non-antimicrobial approaches for treating of *E. coli* mastitis have been studied as alternatives to antimicrobials. Non-steroidal anti-inflammatory drugs (NSAID), frequent milking and fluid therapy have been commonly recommended for supportive treatment of coliform mastitis (Radostits et al., 2007). Correction of dehydration with fluid therapy is usually indicated in severe cases. Other non-antimicrobial approaches, such as recombinant bovine soluble CD14 (Lee et al., 2003) and lactoferrin (Lf), an antibacterial glycoprotein present in milk (Komine et al., 2006; Lacasse et al., 2008), have also been tested for treating of bovine mastitis.

Prevention of *E. coli* mastitis relies on limiting the exposure of the udder to Gram-negative bacteria from the barn environment. One of the primary sources of bacterial contamination is bedding contaminated with manure. Management practices that reduce the number of bacteria in the environment of the cow generally decrease the occurrence of clinical mastitis caused by Gram-negative bacteria (Hogan and Smith 2003). Measures that have been found to be successful for decreasing mastitis caused by contagious pathogens, such as post-milking dipping of teats with antiseptic agents, have failed to protect against coliform mastitis (Pankey et al., 1984). Vaccination by Gram-negative core antigen vaccines has been used for many years in North America and is now also available in Europe. The effect of vaccination is of short-duration and does not generally protect the cow from clinical mastitis, but does decrease the severity of clinical signs (Erskine et al., 2007; Wilson et al., 2009). Genetic manipulation of dairy cows, to express recombinant immunomodulating proteins in their milk, would be one approach to mastitis prevention (Bramley et al., 2001; Oliver 2005; Wall et al., 2005).

Mastitis control in modern dairy herds relies on prevention rather than treatment. Efficient treatment of clinical cases is, nonetheless, a component of the mastitis control programme. For the dairy farmer and for the veterinary practitioner it is critical that efficient treatments for severe cases of *E. coli* mastitis become available. Treatment should be economical and pose a minimal risk for residues in milk destined for human consumption. In practice, *E. coli* mastitis is usually detected
based on clinical signs, when bacterial counts in the milk are already high and tissue damage is already evident.
5. REVIEW OF THE LITERATURE

5.1. CHARACTERISTICS OF ESCHERICHIA COLI

5.1.1. Role of lipopolysaccharide

*E. coli* is a member of the *Enterobacteriacea* family. It is Gram-negative rod and able to ferment lactose. Bacterial lipopolysaccharide (LPS), also termed endotoxin, occurs in the outer membrane of the cell wall of Gram-negative bacteria, which comprises lipid-A, a lipopolysaccharide core and polysaccharide units (O-antigens) (Cullor 1996). Lipid-A is responsible for the toxic effects of LPS (Cullor 1996). LPS is considered to be a primary virulence factor of *E. coli* and responsible for most pathophysiological reactions in *E. coli* mastitis (Hogan and Smith 2003; Burvenich et al., 2003). It is released from the bacteria following cell death due to an inflammatory response and during bacterial multiplication (Burvenich et al., 2003). The local and systemic signs are induced by LPS and the subsequent release of inflammatory mediators (Lohuis et al., 1988a; Burvenich et al., 2003). However, LPS is sufficient, but may not be an essential factor in eliciting mastitis by *E. coli* (Gonen et al., 2007), indicating that also additional bacterial factors may be involved (Shpigel et al., 2008).

LPS triggers formation of proinflammatory and inflammatory cytokines, produced predominantly by monocytes and macrophages (Henderson et al., 1996; Persson Waller et al., 2003). The cellular receptor for LPS is CD14, which mainly occurs on monocytes and macrophages, and to a lesser extent on neutrophils (Paape et al., 2003). It facilitates recognition of LPS by the TLR4 receptor. Macrophages, dendritic cells and epithelial cells are expected to express TLR4 in the mammary gland (Werling et al., 2006; Gonen et al., 2007). Cytokines, such as tumor necrosis factor alpha (TNF-α), initiate the inflammatory response, which induces the acute phase response (APR) by activating the production of acute phase proteins (APP), such as serum amyloid A (SAA), haptoglobin (Hp) and LPS-binding protein (LBP) (Bannerman et al., 2003; Bannerman et al., 2004; Eckersall et al., 2001; Hirvonen et al., 1996; Hiss et al., 2004).

5.1.2. Other virulence factors

Pathogenic *E. coli* bacteria are typically associated with a specific disease and have specific virulence factors that contribute to their ability to cause infection, i.e. to colonize, multiply and survive under particular conditions, such as in the urinary track (UPEC pathotype) or in the intestine (EHEC and EPEC pathotype) (China and Goffaux, 1999; Kaper et al., 2004). The major groups of
virulence factors of *E. coli* comprise adhesins, which help the bacteria to adhere to and colonize mucosal surfaces, and toxins, which are proteins with the ability to disturb or modify the normal function of the host cell and to help the bacteria to cross the epithelial barrier and to invade the tissue (China and Goffaux, 1999; Kaper et al., 2004). Other factors, like siderophores, which help bacteria to chelate iron, and factors that confer serum resistance, also contributed to bacterial virulence (Hirsh et al., 1993; Hogan and Smith, 2003). Most of the pathogenic *E. coli* possesses several kinds of pathogenic mechanisms and virulence factors (Kaper et al., 2004).

There is substantial genotypic variability among *E. coli* strains that cause clinical mastitis. Previous studies on *E. coli* isolated from bovine mastitis reported that most of the isolates were serum resistant (Carrol and Jasper, 1977; Nemeth et al., 1991; Fang and Pyörälä, 1996). Different virulence factors, singly and in combinations, were detected, but most strains did not carry any of the virulence genes evaluated (Nemeth et al., 1991; Nemeth 1994; Lipman et al., 1995; Kaipainen et al., 2002). No specific virulence factors or pathotypes were found for expressing special ability to induce mastitis among *E. coli* isolated from mastitis (Kaipainen et al., 2002). No association between any virulence factors and severity of mastitis has been reported (Lehtolainen et al., 2003a; Wenz et al., 2006).

**5.1.3. Antimicrobial susceptibility**

Antimicrobial susceptibility of *E. coli* isolated from mastitis has been studied, but the results vary greatly according to the methods and breakpoints used. During the national monitoring of antimicrobial resistance of pathogens isolated from animals in Finland in 2005-2006, 9% of *E. coli* isolates were resistant to streptomycin, 7% to ampicillin, 7% to sulfamethoxazole and 5% to tetracycline (FINRES-Vet 2005-2006). Resistance percentages were similar to those reported for *E. coli* isolated from clinical mastitis in Finland during 1990-1996 (Lehtolainen et al., 2003b). The proportions of resistant *E. coli* isolates in Finland were lower than those reported in the Netherlands (MARAN 2007), and much lower than those in France (AFFSA 2006).

Many of the genes coding for antimicrobial resistance, as well as some virulence factors, are located in plasmids, and can be linked (Bagg et al., 1987; Martínez and Baquero, 2002). Use of antimicrobials may therefore influence the selection of virulence factors in bacteria (Hirsh et al., 1993; Martínez and Baquero, 2002).
5.2. *E. coli* mastitis

5.2.1. Pathogenesis of acute clinical *E. coli* mastitis

Pathogens causing intramammary infections have commonly been classified as environmental or contagious pathogens. *E. coli* is considered to belong to the group of environmental pathogens, because it originates from the organic matter in the environment of the cow (Nemeth 1994; Lipman et al., 1995; Hogan and Smith, 2003). Teat-end hyperkeratosis and a dirty udder significantly increase the risk of clinical *E. coli* mastitis (Breen et al., 2009).

Knowledge of the pathogenesis of *E. coli* mastitis mainly originates from experimental studies. Even though the subject has been under research for several decades, it is not yet well understood. Development of the intramammary infection is fast and the clinical signs develop rapidly. The required infective dose of *E. coli* can be rather low; 30-50 cfu infused into the teat canal has consistently elicited severe clinical mastitis (Hill et al., 1978; Shuster et al., 1996; Kornalijnslijper et al., 2003). However, response to the challenge varies according to the inoculum dose, the strain used and the reaction of the individual cow. Bacterial numbers increase rapidly post challenge (PC) and often reach peak concentrations in milk within 10 h to 24 hours (Shuster et al., 1996; Rantala et al., 2002; Shpigel et al., 1997; Pyörälä et al., 1994; Hirvonen et al., 1999). Cow-to-cow differences in bacterial counts in milk have been reported to be up to 15-fold 3 h post-challenge and up to more than 800-fold 6 h later (Kornalijnslijper et al., 2004). During mild infections, *E. coli* is cleared from the infected quarter in 2 to 4 days, while in moderately affected cows it takes from 3 to 8 days (Kornalijnslijper et al., 2004; Hirvonen et al., 1999). Severity of clinical signs of mastitis has been significantly related to bacterial counts in the milk; a higher number of bacteria equates with more severe clinical signs (Lohuis et al., 1990; Vandeputte-Van Messom et al., 1993; Shuster et al., 1996; Hirvonen et al., 1999).

The defense mechanisms of the cow usually eliminate the bacteria from the udder spontaneously (Hill et al., 1978). Adhesion of *E. coli* to mammary epithelium has not been considered to be essential for the development of intramammary infection, in contrast to *Staphylococcus aureus* infection (Opdebeeck et al., 1988; Anderson et al., 1977). However, recent studies of Döpfer et al. (2000 and 2001) showed that *E. coli* isolates from persistent intramammary infections were able to adhere to and invade cultured mammary epithelial cells. These *E. coli* strains invaded the cells less efficiently than *S. aureus*, but about as efficiently as *Streptococcus dysgalactiae* (Döpher et al., 2001).
Polymorphonuclear (PMN) phagocytes, including neutrophils, basophils and eosinophils, play a key role in the containment of infection (Burvenich et al., 2007). PMN are the major effector cells of the bovine innate immune system and constitute a second line of defense after the mechanical protection afforded by the teat canal (Paape et al., 2003). A rapid neutrophil migration from blood to milk is followed by a subsequent decrease in bacterial counts in the mammary gland, as a result of phagocytosis and killing of bacteria (Hill et al., 1979; Shuster et al., 1996; Burvenich et al., 2007). After experimental intramammary infusion of bacteria, it takes approximately 8 to 9 hours before PMN start to increase in the milk (Kornalijnslijper et al., 2004). Cows with moderate clinical signs have a more rapid PMN response in the infected gland compared with severe responders, which show a several hours of delay in leucocyte diapedesis into milk (Vandeputte-Van Messom et al., 1993). Hill et al. (1979) found that a slow diapedesis of neutrophils into the mammary gland is associated with the most severe cases of E. coli mastitis. This interval from ingress of the bacteria into the mammary gland before PMN influx provides time for bacteria to adapt to their new environment in the mammary gland and to replicate there. A major determinant for the severity of experimental E. coli mastitis is bacterial growth in the acute phase before the massive influx of PMN (Kornalijnslijper et al., 2004). The bacterial load in milk after the first 6 hours of infection was reported to correlate significantly with the severity of the disease (Kornalijnslijper et al., 2004).

In experimental E. coli mastitis, local signs of inflammation, such as swelling, warmth and firmness of the quarter, and changes in the appearance of the milk, usually start within 7 hours and reach their maxima in 10 to 14 hours (Vandeputte-Van Messom et al., 1993; Shuster et al., 1996). Systemic signs, which include rise in rectal temperature, decreased appetite, depression of rumen contractions, and impaired general attitude, appear together with local signs within 6 to 8 hours and peak about 9 to 12 hours PC (Lohuis et al., 1990; Pyörälä et al., 1994; Hirvonen et al., 1999; Vandeputte-Van Messom et al., 1993). Systemic signs disappear within 2 to 3 days post-inoculation (Lohuis et al., 1990; Pyörälä et al., 1994; Hirvonen et al., 1999) and local signs of the udder in one week (Hirvonen et al., 1999; Pyörälä et al., 1994). The signs of inflammation, as well as high milk SCC in the affected quarter, may persist for several weeks, or the quarter may become blind despite no growth of E. coli in the milk (Golodetz et al., 1983; Erskine et al., 1991; Pyörälä and Pyörälä, 1997).

Bacteremia was found significantly more often in cows with severe clinical signs (Wenz et al., 2001b). Leucopenia in cows with acute coliform mastitis has been described in several studies.
Cows with more severe systemic signs have been found to have more profound leucopenia, but leucopenia is not considered to be a cause of bacteraemia (Wenz et al., 2001a).

Severity of acute \textit{E. coli} mastitis differs greatly among individual animals. Clinical signs of \textit{E. coli} mastitis can differ from only mild changes in the appearance of the milk, without systemic signs, to severe clinical signs and strongly decreased milk production (Jones and Ward, 1990; Shpigel et al., 1997; Pyörälä et al., 1994; Burvenich et al., 2007). The severity of \textit{E. coli} mastitis depends on the age of the cow and on the lactation stage, i.e. older cows and cows in early lactation are more susceptible to infection (Hill et al., 1979; Hill 1981; Pyörälä and Pyörälä, 1998; Shuster et al., 1996; Mehrzad et al., 2002). In particular, the early lactating cows are more susceptible to fatal outcome from coliform mastitis, and only 30-50\% of diseased cows return to full lactation (Jones and Ward, 1990) regardless of antimicrobial and supportive therapy (Erskine et al., 1991). In early lactation the susceptibility of dairy cows to mastitis is increased, probably due to slow leukocyte recruitment to the mammary gland during the periparturient period and because of a negative energy balance and stress during early lactation (Suriyasathaporn et al., 2000). Experimentally induced endotoxin mastitis resulted in more severe response in early-lactating cows than in late-lactating cows; during early lactation the cows also had a decreased neutrophil function as compared with late-lactating cows (Lehtolainen et al., 2003c). A high number of coliform intramammary infections during the first 60 to 70 days of lactation can develop into severe mastitis and sepsis, defined as toxic mastitis (Smith et al., 1985a; Smith et al., 1985b; Hogan et al., 1989); when 25\% of the cows either die or must be culled (Burvenich et al., 2007). Septic shock is a result of overproduction of inflammatory mediators after interactions of LPS with the host immune system. Coliform mastitis tends to be milder in middle and late lactation (Burvenich et al., 2007). Severity of mastitis is a result of interaction between immune defense of the host and bacterial characteristics. Burvenich et al. (2003) concluded in their review that cow factors rather than specific features of the bacterial strain mainly determine the severity of \textit{E. coli} mastitis.

\textbf{5.2.2. Intramammary persistence of \textit{E. coli}}

Intramammary infections caused by \textit{E. coli} are often of limited duration and bacteria are cleared from the quarter spontaneously. Recurrent episodes of clinical mastitis in the same quarter caused by \textit{E. coli} have been considered to be exceptional (Lam et al., 1996). Persistent intramammary infections derived from the same \textit{E. coli} genotype were reported from 5\% to 24\% among \textit{E. coli} mastitis cases (Döpfer et al., 1999; Bradley and Green, 2001b).
Some authors have suggested that *E. coli* isolated from bovine mastitis would also have features similar to those of other animal and human pathogenic *E. coli*, which could help the bacteria to persist in the bovine mammary gland (Hill et al., 1979; Hogan et al., 1989; Lam et al., 1996; Bradley and Green, 2001b; Lehtolainen et al., 2003a; Blum et al., 2008). Lehtolainen et al. (2003a) reported that *E. coli* isolates positive for virulence factors S-fimbria, P-fimbria, CNF1 and CNF2 were significantly associated with persistent mastitis. Mastitis-associated *E. coli* strains have been reported to adhere to and invade cultured mammary epithelial cells *in vitro* (Döpfer et al., 2000). *E. coli* strains isolated from persistent intramammary infection also survived and replicated inside cultured mammary epithelial cells (Dogan et al. 2006).

**5. 3. E. COLI MASTITIS THERAPY**

**5.3.1. Antimicrobial therapy**

In *E. coli* intramammary infection, spontaneous elimination of bacteria is usually high (Hill et al., 1979; Shuster et al., 1996; Burvenich et al., 2007). The use of antimicrobial agents in treatment of *E. coli* mastitis is contentious; in several studies no beneficial effects of antimicrobial treatment were demonstrated when compared with an untreated control group, although another studies reported some advantages of antimicrobial treatment (Table 1).

The number of antimicrobials suitable for systemic treatment of coliform mastitis is limited and very few antimicrobial products have been approved specifically for this indication and even fewer have demonstrated favourable pharmacokinetic and pharmacodynamic properties (Constable et al., 2008). Broad-spectrum antimicrobials, such as fluoroquinolones, cefquinome, ceftiofur and oxytetracycline, have been used or recommended for the treatment of *E. coli* mastitis (Huxley 2004; Shpigel et al., 1997; Cebra et al., 1996; Erskine et al., 2002; Morin et al., 1998; Wenz et al., 2001b). These antimicrobials are considered to be critically important antimicrobials also in human medicine (Collignon et al., 2009). According to Ziv (1980), the ideal antibacterial for parenteral mastitis therapy would have a low MIC against the udder pathogen, have a high bioavailability from the intramuscular injection sites, be weakly basic or non-ionized in serum and sufficiently lipid soluble, have a low degree of protein binding and a long half-life in order to maintain activity in inflammatory secretions and not result in drug accumulation in specific organs. Few antimicrobials fulfil these requirements.
Table 1. Studies on the efficacy of antimicrobial treatment of clinical E. coli mastitis with information on the type of study and whether significant benefits of the treatment were found. Routes of administration: intramammary (IMM) or systemic.

<table>
<thead>
<tr>
<th>Antimicrobial and route of administration</th>
<th>Control used</th>
<th>Type of study</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin systemic</td>
<td>Erythromycin syst/ no antimicrobial syst</td>
<td>Field study</td>
<td>No benefits</td>
<td>Jones &amp; Ward 1990</td>
</tr>
<tr>
<td>Gentamicin IMM</td>
<td>No antimicrobial</td>
<td>Experimental</td>
<td>No benefits</td>
<td>Erskine et al. 1992</td>
</tr>
<tr>
<td>Amoxicillin IMM, cephapirin IMM</td>
<td>No antimicrobial</td>
<td>Field data</td>
<td>No benefits</td>
<td>Guterbock et al. 1993</td>
</tr>
<tr>
<td>Trimethoprim-sulfadiazine systemic</td>
<td>Colistine sulfate IM</td>
<td>Experimental</td>
<td>No benefits</td>
<td>Pyörälä et al. 1994</td>
</tr>
<tr>
<td>Cefquinome IMM and/or systemic</td>
<td>Ampicillin and cloxacillin IM</td>
<td>Experimental</td>
<td>Benefits</td>
<td>Shpigel et al. 1997</td>
</tr>
<tr>
<td>Cephapirin IMM and/or oxytetracycline systemic and supportive treatment</td>
<td>Supportive treatment only</td>
<td>Field data</td>
<td>Benefits</td>
<td>Morin et al. 1998</td>
</tr>
<tr>
<td>Enrofloxacin systemic</td>
<td>Procain penicillin, spiramycin, no antimicrobials</td>
<td>Field data</td>
<td>No benefits</td>
<td>Pyörälä &amp; Pyörälä 1998</td>
</tr>
<tr>
<td>Enrofloxacin systemic</td>
<td>No treatment</td>
<td>Experimental</td>
<td>Benefits</td>
<td>Hoeben et al. 2000b</td>
</tr>
<tr>
<td>Ceftiofur systemic</td>
<td>No antimicrobial</td>
<td>Field study *</td>
<td>No benefits</td>
<td>Erskine et al. 2002</td>
</tr>
<tr>
<td>Enrofloxacin systemic</td>
<td>No antimicrobial</td>
<td>Experimental</td>
<td>Benefits</td>
<td>Rantala et al. 2002</td>
</tr>
<tr>
<td>Enrofloxacin systemic</td>
<td>Cefcinuinome IM</td>
<td>Experimental and field data</td>
<td>Benefits</td>
<td>Poutrel &amp; Dellac 2004</td>
</tr>
<tr>
<td>Amoxicillin/cloxacillin IMM</td>
<td>Penethamate hydroiodide syst</td>
<td>Field data</td>
<td>No benefits</td>
<td>Sérieys et al. 2005</td>
</tr>
<tr>
<td>Danofloxacin systemic</td>
<td>No treatment</td>
<td>Experimental</td>
<td>Benefits</td>
<td>Poutrel et al. 2008</td>
</tr>
</tbody>
</table>

*coliform mastitis

According to the Finnish recommendations for the use of antimicrobials in treatment of severe E. coli mastitis in dairy cows, fluoroquinolones are the first choice and in high doses of trimethoprim-sulfonamides the second-choice (Evira 2009). Enrofloxacin is a bactericidal, concentration-
dependent fluoroquinolone antimicrobial authorized for treatment of dairy cattle in the EU. High concentrations of enrofloxacin, and particularly its active metabolite, ciprofloxacin, are achieved and maintained in the blood and milk of dairy cows (Kaartinen et al., 1995; Rantala et al., 2002). The pharmacokinetic properties of enrofloxacin are therefore favourable for the treatment of bovine mastitis. Milk does not significantly interfere with the antimicrobial activity of enrofloxacin in vitro (Fang and Pyörälä, 1996). Fluoroquinolones were also shown to have immunomodulatory capacity by increasing the killing ability of neutrophils, which may contribute to the therapeutic effect (Hoeben et al., 1997). Release of endotoxin from Gram-negative bacteria after a rapid kill by bactericidal antimicrobials is considered a risk in humans (Lepper et al., 2002), but has not been reported in the treatment of bovine *E. coli* mastitis (Pyörälä et al., 1994; Dosogne et al., 2002a).

The activity of a trimethoprim-sulfonamide combination was reduced in milk (Fang and Pyörälä, 1996), in which it is difficult to achieve and maintain therapeutic concentrations (Kaartinen et al., 1999). Phagocytosis is impaired in the mammary gland and therefore antimicrobials with a bactericidal activity would be preferable for treatment of mastitis (Constable and Morin, 2003).

Antimicrobials, to which coliforms either are or are not susceptible in vitro, have been reported to be equally efficacious in the treatment of *E. coli* mastitis in some field studies (Jones and Ward, 1990; Pyörälä and Pyörälä, 1998). Results from treatment trials using antimicrobial agents have not been consistent, and most studies have failed to show beneficial effects (Table 1). Some advantages from antimicrobial treatment have reported from experimental studies, including faster elimination of bacteria, increased survival rate of the cows, and reduction in the loss of milk production (Table1).

### 5.3.2. Non-antimicrobial therapy

#### 5.3.2.1. Anti-inflammatory therapy

Non-steroidal anti-inflammatory drugs have been recommended for supportive treatment of coliform mastitis (Radostits et al., 2007) and are widely used by veterinarians in dairy practice (Katholm and Andersen, 1992; Green et al., 1997; Huxley et al., 2004). NSAIDs have been shown to be beneficial in revealing clinical signs, reducing fever and diminishing other effects of endotoxin in *E. coli* mastitis (Shpigel et al., 1994; Pyörälä and Syväjärvi, 1987; Rantala et al., 2002).
Glucocorticoids are steroidal drugs used in treatment of severe mastitis (Erskine et al., 2003). They inhibit the production of inflammatory mediators, impair the transport of inflammatory cells to the inflammation site and decrease other inflammatory effects induced by LPS (Adams 2001). Dexamethasone-treated cows had a significantly lower rectal temperature, increased rumen motility, less udder inflammation and an improved 14-day milk yield compared with controls, when dexamethasone was given immediately after the LPS challenge (Lohuis et al., 1988b). Glucocorticoids have negative side-effects, as they suppress immune reaction, but if administered as a single dose during the early course of severe mastitis caused by coliform bacteria, the risk of adverse immune effects is considered small (Erskine et al., 2003).

5.3.2.2. Lactoferrin
Lactoferrin (Lf), an antibacterial substance in milk, acts as a bactericidal and LPS-neutralizing factor in the mammary gland (Appelmelk et al., 1994). Lf has been shown to be bacteriostatic for a variety of micro-organisms in vitro (Arnold et al., 1980; Rainard 1986; Lönnerdal and Iyer, 1995) as well as in vivo (Zagulski et al., 1986; Zagulski et al., 1989; Bhimani et al., 1999). The in vitro studies have shown inhibitory activity of Lf against E. coli (Rainard 1986; Kutila et al., 2003). This inhibitory activity is believed to result from the iron-chelating ability of Lf, making iron unavailable to bacteria (Weinberg 1978), but Lf molecules were also reported to be directly toxic to microbes and neutralize the effects of LPS (Appelmelk et al., 1994).

Lf concentration in milk from cows with peracute E. coli mastitis was shown to be initially lower than that in cows with less severe mastitis and thus perhaps insufficient to inhibit bacterial growth (Harmon et al., 1975; Kawai et al., 1999). In theory, Lf could be beneficial in the treatment of E. coli mastitis as a non-antibiotic antibacterial and endotoxin-neutralizing alternative. Disposition kinetics of Lf after intramammary administration were reported by Kutila et al. (2002).

5.3.2.3. Frequent-milking
Frequent milking, a traditional treatment for severe mastitis, has been commonly recommended as a supportive treatment of severe mastitis in Finland. It has been recommended for use in conjunction with oxytocin to stimulate milk let-out (Radostitis et al., 2007). In theory, its efficacy is based on
removing harmful toxins and bacteria from the udder and thereby decreasing pain and inflammation. On the other hand, in removing milk, humoral anti-inflammatory agents and bactericidal proteins such as lactoferrin are also removed. In one congress report the efficacy of frequent milking was found to be better than systemic antimicrobial therapy for the treatment of severe experimental *E. coli* mastitis, but the number of the cows treated was small (Stämpfli et al., 1994). Frequent milking showed no beneficial effects in an experimental study with an *E. coli* mastitis model (Leininger et al., 2003).

### 5.3.2.4. Other approaches

Some new non-antimicrobial therapeutic approaches, such as recombinant bovine soluble CD14, the cellular receptor for LPS in macrophages and neutrophils, have been investigated in pilot experiments. CD14 was shown to reduce severity of *E. coli* mastitis in a bovine experimental model (Lee et al., 2003).

### 5.4. PREVENTION OF E. COLI MASTITIS

#### 5.4.1. Management aspects

The two main preventive measures of intramammary infection are to minimize the challenge from the contaminated environment and to maximize the cow’s own defense (Bradley and Green, 2004). Common sources of exposure to Gram-negative bacteria include manure, contaminated bedding, water, soil and feedstuffs. The management practices that reduce the number of bacteria in the environment of the cow generally decrease clinical mastitis caused by Gram-negative bacteria (Hogan and Smith, 2003). A dirty udder and injured teat skin significantly increase the risk of clinical *E. coli* mastitis (Breen et al., 2009). Post-milking dipping of the teats with antiseptic agents has proven to be unsuccessful in the control of coliform mastitis (Pankey et al., 1984), as has also dry cow therapy in general (Radostits et al., 2007). However, dry cow therapy with an antimicrobial compound efficacious against Gram-negative bacteria decreased episodes of new clinical mastitis caused by Gram-negative bacteria *post partum* (Bradley and Green, 2001a). Inadequate nutrition, stress and pregnancy can have a dramatic negative impact on the immune system of the cow (Lippolis 2008). A clean, dry, cool and comfortable environment, proper feeding and adequate supplementation of the diet with vitamins and trace elements are important for maintaining good udder health (Bradley and Green, 2004; Lippolis 2008; Pyörälä 2008).
5.4.2. Vaccination
Prophylactic immunization is used in some countries to prevent coliform mastitis. Vaccines developed using the common core antigen of coliform bacteria, administered systemically at drying-off and again 3 weeks before the calving date, have been in use in North America for many years (Hogan et al., 1992; Wilson and González, 2003; Wilson et al., 2007a). The common core antigen was recently authorized in the European Union in a combination mastitis vaccine (EMA 2009). The duration of immune response induced by vaccine was relatively short (Wilson et al., 2007a; Wilson et al., 2007b). Vaccination is able to reduce the severity of clinical signs and protect cows from culling or death and to reduce the milk loss. However, the incidence of clinical mastitis has not been reduced (Wilson et al., 2007a; Wilson et al., 2007b). In some earlier studies, a reduced risk for clinical mastitis in vaccinated cows was reported (Cullor 1991; Hogan et al., 1992). In addition to the common core antigen, iron regulated outer-membrane protein vaccines against coliform mastitis have also been investigated (Lin et al., 1999).

5.4.3. Genetic engineering
Genetic engineering is one tool suggested to increase host defense against mastitis (Bramley et al., 2001; Pyörälä 2002; Maga et al., 2005). Increasing mastitis resistance by improving the immune response through modifying activity of genes or incorporating beneficial genes from other organisms, has been proposed to have a positive impact on cow welfare and economics of milk production (Oliver 2005). The transgenic approach to enhance mastitis resistance was studied in a mouse model (Kerr et al., 2001). In the first published cow model (Wall et al., 2005), cows carrying a gene coding for an anti-staphylococcal peptide, lysostaphin, were shown to resist *S. aureus* intramammary infection. The first transgenic cows with the hLf gene were reported to express Lf in their milk at a concentration of 0.3 to 2.8 mg/ml (Brink et al., 2000; Van Berkel et al., 2002). A gene encoding human lactoferrin (hLf) in the bovine mammary gland could be a good candidate to increase resistance of dairy cows to coliform mastitis by transgenesis,
6. AIMS OF THE STUDY

The general aim of this thesis was to investigate the host response, characteristics of bacteria, and the efficacy of antimicrobial and non-antimicrobial treatments of bovine *E. coli* mastitis. The specific aims were:

1. To investigate host response in two consecutive intramammary challenges with *Escherichia coli* and to evaluate the possible carry-over effect when the same animals were used in an experimental model.

2. To compare the efficacy of the combination of systemic enrofloxacin and supportive treatment with supportive treatment only in dairy cows with clinical *E. coli* mastitis under field conditions.

3. To identify possible specific virulence genes and phylogeny types of *E. coli* associated with severity of clinical mastitis and persistence of the intramammary infection.

4. To investigate the efficacy of bovine lactoferrin as an alternative to antimicrobials in the treatment of *E. coli* mastitis.

5. To investigate the prophylactic effect of expression of human lactoferrin in the milk against *E. coli* mastitis.
7. MATERIALS AND METHODS

7.1. Animals

Seven primiparous dairy cows (three Ayrshire and four Friesian-Holstein), calved on average 3 months earlier, were used in the experimental study on the effect of two consecutive *E. coli* challenge on acute phase response (study I). Six primiparous, early-lactating dairy cows (five Ayrshire and one Finnish Landrace) were used in the study on the efficacy of lactoferrin in the treatment of *E. coli* mastitis (study IV).

In the study on the effect of recombinant human lactoferrin (rhLf) on experimental *E. coli* mastitis (study V) seven primiparous, early-lactating Friesian-Holstein transgenic cows (produced and owned by Pharming Group NV, The Netherlands) expressing human lactoferrin (hLf) in their milk were used. The control group consisted of six non-transgenic normal, early lactating Friesian-Holstein dairy cows. The cows were confirmed as being transgenic using a calf by hLf calf PCR analysis (Brink et al., 2000). The mean concentration of rhLf in the milk of these cows was 2.9 mg/ml (Hyvönen et al., 2006).

Milk somatic cell count (SCC) was < 100 000 cells/ml in all quarters of the experimental cows in studies I, IV and V prior to the experiments. The cows were clinically healthy and there was no bacterial growth in their milk samples collected before the experiments.

Lactating dairy cows with acute clinical mastitis (IDF 1999), which the veterinarian suspected to be caused by coliform bacteria in the practice areas of the Ambulatory Clinic of the University of Helsinki and the Lammi Veterinary Practice during 2003-2006, were included in the field trial (study II). The final decision to include a cow in the trial was made on the basis of the result of bacteriological examination of the pre-treatment milk sample. A total of 132 cows from 61 herds were finally enrolled in the study. Days in milk (DIM) and number of parities (1, 2 and ≥ 3) were recorded on the study form.

The Ethics Committees of the University of Kuopio and the University of Helsinki approved the experimental study protocols and The Board for Gene Technology in Finland the use of transgenic animals.
7.2. Study designs in experimental studies (study I, IV and V) and field trial (study II)

The same procedure for bacteria inoculation was used in the studies I, IV and V. One udder quarter of each cow was inoculated via the teat canal with a dose of *E. coli* of approximately 1500-1700 cfu (from 1400 to 2300 cfu), which was suspended in 10 ml of pyrogen-free saline.

The effect of two consecutive *E. coli* challenges on the acute phase response of the cow (study I) was studied by repeating the challenge after 14 days in another udder quarter of the same cow. Cows in study I were treated with a dose of flunixin meglumine at 2.2 mg/kg once at 12 hours PC (Finadyne®, Schering-Plough, Farum, Denmark).

In the experiment on the efficacy of lactoferrin in the treatment of *E. coli* mastitis (study IV) the cows were randomly allocated to two treatment groups. Treatment was started 12 hours PC. Three of the cows received 1.5 g of Lf via the intramammary route three times after milking at 12, 20 and 36 hours PC. The remaining three cows received, as a positive control treatment, enrofloxacin (Baytril®, Bayer AG, Leverkusen, Germany) at 5 mg/kg first i.v. 12 hours PC, then s.c. twice 24 hours apart (at 36 and 60 hours PC). Flunixin meglumine (Finadyne®, Schering-Plough, Farum, Denmark 2.2 mg/kg i.v.) was administered to all cows 12 hours PC, and a second dose 36 hours PC. A crossover design was used in study IV, so that each cow served as its own control: three weeks after the first challenge, a contralateral udder quarter of the same cow was inoculated.

In the field trial (study II) the cows were randomly allocated to two treatment groups, enrofloxacin and non-enrofloxacin (non-treated), using cow ID number (even and odd numbers). Cows were treated systemically with enrofloxacin (Baytril®, Bayer HealthCare AG, Germany) in the enrofloxacin group. The dosage used was 5 mg/kg administered twice at an interval of 24 h; the first dose was given intravenously by the veterinarian and the second dose subcutaneously by the herd owner. Cows in both groups received the non-steroidal anti-inflammatory drug ketoprofen either intravenously or intramuscularly at 3 mg/kg (Comforion®, Orion Oyj, Finland) or 4 mg/kg *per os* (Dolovet®, Vetcare Oy, Finland) daily for 1-3 days. Because the clinical diagnosis of *E. coli* mastitis was not accurate for the initial farm visit, experimental treatment was combined with penicillin G intramammary (Carepen® 600 mg, Vetcare Oy, Finland), once a day into the affected
quarter, until confirmation of the diagnosis. Use of frequent milking of the affected quarter was to the responsibility of the owner. Fluid therapy was used by the veterinarian when considered necessary on the basis of clinical signs. All treatments were recorded by the veterinarian on the study data collection form.

7.3. Clinical observations

Systemic and local signs of the cows were monitored throughout the experiments in studies I, IV and V; during the first two days every 4 hours and thereafter twice daily, when the cows were milked. Heart rate, rectal temperature, rumen motility, appetite and general attitude were recorded. The udder was palpated for soreness, swelling and hardness, and quarter milk samples were evaluated visually for clotting, colour changes and consistency each time the cows were milked (Pyörälä et al., 1994). The local and systemic signs were scored on a three-point scale (1 = no signs to 3 = severe signs) (Pyörälä et al., 1994). Daily milk yield was measured throughout the study.

Each cow was examined clinically by the attending veterinarian in the field trial (study II) at the time of enrolment (day 0). Clinical signs (local signs, such as swelling and pain in the udder and milk appearance, and systemic signs such as rectal temperature, general attitude and appetite) and CMT scores using the Scandinavian scoring system from 1 to 5 (Klastrup and Schmidt Madsen, 1974) were recorded on the data collection form by the veterinarian. Clinical signs were scored according to a 3-point scale (1 = no signs to 3 = severe signs), as described earlier (Pyörälä et al., 1994). On day 2 and at 3-4 weeks (defined as day 21) post-treatment, the farmer estimated milk production (normal, diminished, or a blind quarter) and clinical recovery (visible or palpable signs) of the affected quarter, carried out a CMT and recorded the results on the data collection form. Frequent milking (emptying of the infected quarter at least twice a day in addition to the regular two milkings per day) at the acute stage was also recorded.

During the field trial assessments were made of clinical and bacteriological cures on day 2 and on day 21 and return to milk production of the affected quarter on day 21 compared to quarter milk production prior to mastitis (returned, diminished, blind quarter). A cow was defined as clinically cured if no systemic signs were present, the affected quarter was free from any clinical signs, the milk appearance was normal and milk was acceptable for delivery. Growth of *E. coli* in the follow-
up milk samples on days 2 and 21 was used to assess the bacteriological cure. If bacteria other than *E. coli* were isolated in the follow-up sample on day 21, the quarter was defined as reinfected.

A cow was classified as “survived” if it remained in the herd (not dead, euthanized or culled because of *E. coli* mastitis) during the three-week post-treatment period. To evaluate the long-term presence of the cow in the herd, the farmer was asked six months later if the cow was still in the herd, after which the cow was classified as “remaining” or “not remaining”.

7.4. **Bacterial strain used in experimentally induced mastitis**

The *E. coli* strain FT238 (isolated from clinical mastitis) was used to induce experimental mastitis in studies I, IV and V. The strain was non-hemolytic, intermediately serum-resistant, and susceptible to enrofloxacin *in vitro* with a minimum inhibitory concentration (MIC) < 0.25 μg/ml (Pyörälä et al., 1994; Rantala et al., 2002). The strain was also susceptible to bovine lactoferrin (bLf) *in vitro* with a complete inhibition of the growth at a concentration of > 1.67 mg/ml (Kutila et al., 2003) as well as for hLf at a concentration of ≥ 1.5 mg/ml.

The *E. coli* strain was cultured and grown on blood agar plates overnight. A few colonies were subcultured in Iso-Sensitest Broth (ISB broth, Oxoid, Basingstoke, Hampshire, England) and incubated for 18 hours at 37°C. The broth culture was centrifuged, and the pellet was resuspended in sterile saline and diluted to an estimated concentration of 10^8 cfu/ml. The final concentration of the bacterial inoculate used in challenges was determined by culturing and colony count.

7.5. **Milk and blood samples**

7.5.1. **Sampling**

In study I, aseptic milk samples were collected from the experimental and contralateral quarter before the challenge and 12, 20, 36, 44, 60, 68, 84,108, 132 and 156 hours PC for bacteriological assessment and determination of SCC, N-acetyl-β-D-glucosaminidase (NAGase) activity, and concentration of serum amyloid A (SAA), haptoglobin (Hp) and lipopolysaccharide binding protein (LBP) in the milk. Blood samples were collected before challenge and 12, 16, 20, 24, 36, 44, 60, 68 and 156 hours PC. Serum was separated and kept frozen at -70°C for later determination of
concentrations of SAA, Hp and LBP. EDTA blood was collected for leukocyte count (WBC) and packed cell volume (PCV) determination.

In the study IV milk samples were taken aseptically from the challenged and the contralateral quarters were collected at 120 and 12 hours before the challenge and at 12, 16, 20, 36, 40, 44, 60, 84, 108, 132 and 156 hours and at 1, 2 and 3 weeks PC for bacteriological assessment, SCC, and milk NAGase activity determinations. Blood samples were collected at 12 hours before the challenge and then at 12, 16, 20, 36, 60, 84 and 180 hours PC and kept frozen at -70° C for later use.

In study V aseptic milk samples from the challenged and contralateral quarters were collected 12 hours before the challenge, immediately before the challenge, and every four hours PC during the first 24 hours. Milk samples were taken before milking at 36, 44, 60, 84, 132, 156, 180 hours and finally 14 days after the challenge. Concentrations of hLf and bLf, bacterial count, SCC, NAGase activity and concentrations of SAA, Hp and TNF-α were determined from the milk samples. Concentrations of LPS were determined from the milk samples taken 12 hours PC. Blood samples were collected at 12 hours and immediately before the challenge, and every four hours PC during the first 24 hours and then at 36, 60, 84, 168 hours (7 days) and 14 days. Serum was separated and kept frozen at –70° C for determination of concentrations of TNF-α, SAA, Hp, cortisol, urea, creatinine, albumin, total protein, alanine aminotransferase (ALAT) and alanine aminofosfatase (AFOS). EDTA blood was collected for WBC and PCV.

In the field study (study II), a 4 ml milk sample was collected aseptically from the affected quarter before treatment (day 0) and 2 days (day 2) and 3-4 weeks (day 21) post-treatment by the veterinarian or a trained owner. Follow-up milk samples taken on days 2 and 21 were stored frozen and cultured within a month.

7.5.2. Analytical methods for the milk and blood samples
SCC was measured with a Fossomatic instrument (Foss Electric, Hillerod, Denmark) in the laboratory of Valio Oy (Lapinlahti, Finland). SCC values over 30x10^6 cells/ml were recorded as 30x10^6 cells/ml in study I and over 20x10^6 cells/ml were recorded as 20x10^6 cells/ml in study V.
In study IV the concentration of LPS in the milk samples at 20 hours PC and in study V at 12 hours PC was determined using Limulus Amaebocyte Lysate (LAL) test (BioWhittaker, Walkersville, MD, USA) at the Regional Institute of Occupational Health of Kuopio, Finland. The ratio of the concentration of LPS to the bacterial count was calculated for both treatment groups in study IV.

Milk NAGase activity, an indicator of the degree of inflammation and tissue damage of the affected quarter, was measured using the fluorogenic method of Kitchen and co-workers (1978) with a microplate modification developed by Mattila and Sandholm (1985). In studies I, IV and V milk NAGase activity values higher than 2.8 pmol 4-MU/min/µl were recorded as 2.8 pmol 4-MU/min/µl and after adopting the revised procedure in analysis in study II, values higher than 24.49 pmol 4-MU/min/µl were recorded as 24.49 pmol 4-MU/min/µl, the upper limit for quantification.

The concentrations of SAA in milk and serum were determined using a commercial ELISA test (Tridelta Development, Wicklow, Ireland). Milk and serum samples were initially diluted 1:500 and 1:50. For very high SAA values, samples were diluted as necessary up to 1:15000 (maximum concentrations 2250 μg/ml).

Milk and serum Hp concentrations were determined using the method based on the ability of Hp to bind to hemoglobin (Makimura et al., 1982) and using tetramethylbenzidine as the substrate (Alsemgeest et al., 1994). The assay is aimed at determining of Hp in serum, but was adapted to be used for milk (Hyvönen et al., 2006). Lyophilized bovine acute phase serum was used as a standard and calibration was according to the European Union concerted action on standardization of animal acute phase protein (APP) (number QLK5-CT-1999-0153).

LBP concentrations in serum and milk were determined with a commercially available LBP ELISA kit, cross-reacting with bovine LBP (LBP ELISA for various species, Hycult Biotechnology, Uden, The Netherlands). Milk and serum samples were initially diluted 1:500 and 1:1000 respectively, and assayed following the instructions of the manufacturer. For high concentrations, milk was diluted up to 1:5000 and serum up to 1:2000. The LBP concentration was determined by extrapolation using linear regression from a standard curve of known human LBP concentrations.

In study V an ELISA for the quantification of bovine TNF-α in plasma (Carstensen et al., 2005) was modified for serum as described in Lehtolainen et al. (2004). The detection limit of the ELISA was 0.5 ng/ml for the serum. The milk samples were centrifuged and the clear supernatants were used.
for the ELISA analysis of bovine TNF-α (study V). The detection limit of the ELISA was 1.0 ng/ml for the milk.

Serum cortisol (study V) was analyzed using a radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Product Corporation, Helsinki, Finland). The results were expressed as nmol/l.

Hematological parameters (WBC and PCV) in study I, IV, and V, were determined within 24 hours after sampling using an automated multiparameter analyzer with software for animal samples (Cell-Dyn 3700 System, Abbott Diagnostic Division, Abbott Park, IL, USA). In study IV serum urea, creatinine, albumin and total protein were measured using enzymatic kinetic methods with an automatic analyzer (Kone Pro, ThermoClinical Labsystems, Espoo, Finland). For ASAT and AFOS, activities were measured using an automatic analyzer (Kone Specific, ThermoClinical Labsystems, Espoo, Finland).

**7.6. Preparation and analyses of lactoferrin (study IV and V)**

Bovine Lf used in study IV was purified from cheese whey, or concentrated cheese whey, using an expanded bed absorption chromatography method developed by Isomäki (1999). The native form of Lf was used with an iron content of approximately 8-15%. The LPS contamination in Lf was tested with the Limulus amoebocyte lycate (LAL) test using the kinetic BioWhittaker-QCL method (Walkersville, MD, USA). The LPS content of Lf did not exceed 1.7 ng/ml.

Quantitative recombinant hLf and natural bLf analyses in milk were conducted using enzyme-linked immunosorbent assays (ELISAs) in study V. hLf was measured by rhLf-specific ELISA according to the procedure recommended by Pharming, and anti-hLf was absorbed with Sepharose to remove cross-reacting antibodies (van Berkel et al., 1996). bLf levels were measured using a bovine Lf ELISA quantification kit (Bethyl Laboratories, Inc. Montgomery, USA). The cross-reactivity of bLf with hLf was tested with bLf and hLf standards (Sigma, St. Louis, USA). The level of detection was 0.008 mg/ml.
7.7. Bacteriological methods

Bacterial counts in the milk samples in experimental studies (study I, IV and V) were determined by preparation of 10-fold dilution series of milk in sterile saline. Bacteria were cultured on blood agar at 37º C for 24 hours using serial dilutions and counted using a routine plate count method.

In the field trial (study II) a sample of 0.01 ml of milk was cultured on blood agar (6-10% sheep blood) (Tammertutka, Tampere, Finland). Isolates were identified as *E. coli* by colony morphology, Gram-stain and typical growth on eosin methylene blue (EMB) agar (Hogan et al., 1999). The diagnosis was confirmed by the API 20E test (bioMérieux, Marcy l’Etoile, France) at the Finnish Food Safety Authority Evira (Helsinki, Finland). Follow-up milk samples taken on days 2 and 21 were stored frozen and cultured within a month.

*E. coli* isolates from pre- and post-treatment samples at 3 weeks served as the material in study III. A total of 144 isolates originated from the pre-treatment samples and 10 from the follow-up samples 3 weeks post-treatment. Antimicrobial susceptibility of the initial *E. coli* isolates (study II and III) was tested using the VetMIC™ microdilution method (SVA Uppsala, Sweden) (CLSI 2004) using the epidemiological cut-offs of EUCAST (2008), except for ciprofloxacin, for which a cut-off > 0.06 \( \mu \text{g/ml} \) was used. Susceptibilities to ampicillin, streptomycin, tetracycline, sulfamethoxazol, trimethoprim, kanamycin, kephotaxim, nalidixin acid, ciprofloxacin, gentamicin, florfenicol and ceftiofur were investigated. Isolates of *E. coli* from pre- and post-treatment samples were genotyped using pulsed-field gel electrophoresis (PFGE) at the Finnish Food Safety Authority Evira (Kuopio, Finland). DNA was extracted, digested with XbaI and subjected to PFGE essentially according to the CDC/PulseNet protocol for *E. coli* O157:H7 (CDC 2008). *Salmonella* Braenderup strain H9812 DNA digested with XbaI was used as a DNA size standard. The PFGE patterns were analyzed for similarity by visual comparison. Strains were considered distinct if the pulsotypes were not identical.

The polymerase chain reaction (PCR) was used to analyze the genes of colisin V plasmid (*cva*), vacuolating autotransporter protein (*vat*), temperature-sensitive hemagglutinin (*tsh*), aerobactin (*iucD*), P-fimbria (*papC*), yersinia bactin (*irp2*), increased serum survival (*iss*) and enteroaggregative heat-stable toxin (*astA*) from the isolates (Ewers et al., 2005). PCR described by
Paton and Paton (2002) was used to detect genes of shigatoxin 1 and 2 (stx1 and stx2), intimin (eaeA), enterohemolysin (ehxA) and autoagglutinating adhesin (saa) and that by van Bost et al. (2003) to detect S-fimbria (sfaD), F17 fimbria (f17A) and afimbrial adhesin AFA8 (afa8E). The gene for cytotoxic necrotizing factor 2 (cnf2) was analyzed as described earlier (Kaipainen et al., 2002). Isolates were assigned to a phylogeny group using the PCR method of Clermont et al. (2000).

7.8. Statistical analyses

Linear random-intercept models were used in study I to explore time trend differences between challenge times in milk production data, milk SCC, milk NAGase, WBC and all APP measurements. Bacterial counts in milk and local and systemic sign differences between challenges were tested using generalized linear mixed models in which a Poisson distribution was used for response variables. The cow was included as a random factor. Overall time trend differences between challenges were tested with an F-test. Due to different intervals between sampling, isotropic spatial exponential correlation structures were used for modeling serial correlations of repeated measurements within cows. Logarithmic transformation of milk SCC, milk NAGase and APPs in milk and serum was used. The nlme-package (Pinheiro et al. 2006) with statistical software R 2.5.0 (R Development Core Team 2006) was used for fitting linear random-intercept models and generalized linear mixed models were fitted using the GLIMMIX procedure (2006) software with the SAS/STAT 9.1 (SAS Institute Inc., Cary, NC, USA).

In study II, statistical analyses were carried out using Stata Intercooler version 9.0 (Stata Corporation, Texas, USA). The unit for the analysis used was the cow. Logistic and linear regression models were used as described below. The clustering effect of a herd was estimated with logistic and linear mixed models. Continuous variables (DIM and milk NAGase activities) were examined for linearity with the outcomes. To improve linearity, NAGase activity on day 0 and 21 was logarithmically (natural logarithmic) transformed; NAGase activity on day 2 was bimodally distributed and therefore, for the purposes of the analysis the activity at this time analyzed dichotomously. Dichotomous variables were clinical signs (mild/severe), enrofloxacin treatment (yes/no), amount of bacterial growth on day 0 (< 10 cfu/μl / ≥ 10 cfu/μl), fluid therapy given (yes/no), use of frequent milking (yes/no) and reinfection on day 21 (yes/no). Parity was used as a categorical variable (parities 1, 2 and ≥ 3). The variables were subjected first to univariable and then
multivariable analyses. Variables with a P-value of less than 0.25 in a univariable analysis were entered into the multivariable analysis. Collinearity was tested with a pair-wise correlation test. Enrofloxacin treatment was always kept in the final model. Possible confounding factors (DIM, frequent milking, fluid therapy and parity) were tested separately with all models and were kept in the analysis if they affected the results. No significant correlations or interactions were found among any of the variables studied. A multivariable logistic regression model was used for the outcomes other than NAGase activity. For outcome of NAGase activity on day 21, a multiple linear regression model was used. Full and nested models were compared with a likelihood ratio test. A simple logistic regression model was used to test the effect of survival of a cow, because of the small number of dead or killed cows on day 21. Model fit was assessed with the assumptions of normality and homogeneity of variances, which were evaluated by checking the residuals. The two-way sample size calculation for the statistical analyses in final models allowed detection of differences of \( \geq 25\% \) between treatment groups for all outcomes evaluated; the power of analyses was 0.80.

Three logistic regression models were used in study III. Parity (1, 2, \( \geq 3 \) times), phylogeny groups (A, B1, B2, D) and DIM (1 week ante partum to 3 weeks post partum, \( \geq 3 \) weeks to 120 days pp, \( > 120 \) days pp) were measured on a nominal scale and used as categorical variables. Dichotomous variables were virulence genes, phylogeny groups and antimicrobial susceptibilities, and additionally the following variables: clinical signs on day 0, NAGase activity on day 0 and antimicrobial treatment. Each of the virulence genes and antimicrobial resistance traits were included in the statistical models only if data consisted of at least 10 positive findings. Multivariable logistic regression models were used for the outcomes (clinical signs on day 0, milk NAGase activity on day 0, bacterial persistence on day 21). The clustering effect of a herd was estimated with a logistic mixed model. The herd was included as a random effect with models of clinical signs on day 0 and bacterial persistence. The variables were subjected first to univariate, then multivariable analyses. Variables with a P-value of less than 0.15 in a univariate analysis were retained in the multivariable analysis after testing the variables for correlation with pair-wise correlation test. Possible confounding factors were kept in the analysis when there was a likely effect on the results. Significant correlations (\( > 0.65 \)) and interactions were taken account of in a final model as the most significant variable was chosen to representative of the group. In the logistic regression model of milk NAGase activity on day 0, full and nested models were compared with a likelihood ratio test. Model fit was assessed with the assumptions of normality and homogeneity of variances, which were evaluated by checking the residuals.
In study IV, repeated measures analysis of variance with treatment and sampling time as within-factors was used to test for differences for variables (SCC, NAGase activity, bacterial counts, systemic and local clinical signs, and total and quarter milk yields) between the two treatments. The significance of effects was evaluated by using Greenhouse-Geisser adjusted P-values. Possible carry-over effects were assessed using $t$-test procedures (Jones and Kenward 1989). Statistical significance of the ratio of LPS to bacterial count was determined using a Wilcoxon Signed Ranks Test.

In study V, the effects of time post-challenge on concentrations of measured parameters and on clinical signs were analyzed statistically using mixed-model analysis of variance (SPSS 11.0, SPSS Inc., Chicago, IL, USA).

For all tests, $P < 0.05$ was considered significant.
8. RESULTS

8.1. The effect of two consecutive experimental E. coli challenges (study I)

After both challenges all cows became infected and developed clinical mastitis within 12 hours of inoculation. All cows showed systemic and local inflammatory response after challenges. Systemic response began within 12 hours and was moderate in all cows. Systemic signs disappeared in cows after both challenges by 36 hours PC. Local signs were detected at the end of the experimental period of 6 days PC after the first challenge, but disappeared by 2.5 days PC after the second challenge. In both challenges cows developed a similar systemic response, but their local responses varied more. After the second challenge, local signs were significantly milder (P < 0.05), but no statistically significant differences were noted in systemic signs (Figure 1).

Figure 1. Mean scores for systemic and local signs in two consecutive E. coli challenges at an interval of 2 weeks. Values are mean scores for six cows with standard error of the mean (SEM) represented by vertical bars.
The total daily milk yield during the course of the experiment was significantly higher after the second challenge (Figure 2). Bacterial counts in the milk of the challenged quarters peaked 12 hours PC at both challenge times. Bacteria were still isolated in low numbers from one cow (80 cfu/ml) 6 days PC after the first challenge, but after the second challenge they were totally eliminated in all cows within 68 hours. Overall bacterial counts were lower at the second challenge (Figure 3).

![Experimental quarter milk yield](image1)

**Experimental quarter milk yield**

- ○ First
- ● Second

![Total milk yield](image2)

**Total milk yield**

- ○ First
- ● Second

**Figure 2.** Mean total daily milk yield and milk yield of the experimentally infected quarter in two consecutive E. coli challenges at an interval of 2 weeks. The values are means for six cows with SEM represented by vertical bars.
**Figure 3.** Mean somatic cell counts and bacterial counts in milk in two consecutive E. coli challenges at an interval of 2 weeks. Values are means for six cows with SEM represented by vertical bars.

After the first challenge, milk NAGase activity remained elevated over the experimental period of 6 days, but returned to the baseline value within 6 days after the second challenge. After the second challenge, milk SAA and Hp concentrations decreased significantly faster (Figure 4). LBP was still increased 6 days after the first challenge, but had reached the pre-challenge level by that time after the second challenge (Figure 4).
The concentrations of SAA and Hp in serum were lower in the second challenge, but the difference was only statistically significant for Hp (Figure 5). Serum LBP increased rapidly after the first and second challenge; again concentrations were lower after the second challenge. Leucopenia was noted at both challenges (Figure 5). Leucocyte counts were higher after the second challenge and the difference was statistically significant (Figure 5).
Figure 5. Concentrations of SAA, LBP and Hp in serum and blood leucocyte counts in two consecutive E. coli challenges at an interval of 2 weeks. Values are means for six cows with SEM represented by vertical bars.

8.2. The efficacy of enrofloxacin in the treatment of E. coli mastitis (study II)

A total of 132 cows (mean 2.2 cows per herd) with confirmed growth of E. coli in the pre-treatment milk sample were included in the study (Table 2). Fewer E. coli bacteria than 10 cfu/μl milk were isolated in 8.4% of the pre-treatment samples. The enrofloxacin-treated group comprised 64 cows (48.5%) and the non-treated group 68 (51.5%) cows. According to clinical signs on day 0, 80.1% (n = 105) of the cows had moderate to severe signs (severity score ≥ 2) and 19.9% (n = 26) mild signs (score ≤ 1.5). All cows receiving fluid therapy (n = 40), had moderate to severe disease.
### Table 2. Descriptive statistics and univariable results of naturally occurring acute E. coli mastitis

<table>
<thead>
<tr>
<th>Dichotomous variables</th>
<th>Level</th>
<th>Frequency (%)</th>
<th>Enrofloxacin treated (%)</th>
<th>Non-treated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriological cure on d 2 (^{a,b,f})</td>
<td>Not-cured</td>
<td>72 (72.0)</td>
<td>31 (64.6)</td>
<td>41 (78.8)</td>
</tr>
<tr>
<td></td>
<td>Cured</td>
<td>28 (28.0)</td>
<td>17 (35.4)</td>
<td>11 (21.2)</td>
</tr>
<tr>
<td>Clinical signs d 0 (^{a,b,c,d,e,f})</td>
<td>Mild</td>
<td>26 (19.9)</td>
<td>9 (14.1)</td>
<td>17 (25.4)</td>
</tr>
<tr>
<td></td>
<td>Severe</td>
<td>105 (80.1)</td>
<td>55 (85.9)</td>
<td>50 (74.6)</td>
</tr>
<tr>
<td>Clinical signs d 2 (^{c,d,e,f})</td>
<td>Mild</td>
<td>69 (62.2)</td>
<td>28 (48.3)</td>
<td>41 (77.4)</td>
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<tr>
<td></td>
<td>Severe</td>
<td>42 (37.8)</td>
<td>30 (51.7)</td>
<td>12 (22.6)</td>
</tr>
<tr>
<td>Clinical cure on d 2 (^{c,e,f})</td>
<td>Not-cured</td>
<td>109 (85.8)</td>
<td>57 (91.9)</td>
<td>52 (80.0)</td>
</tr>
<tr>
<td></td>
<td>Cured</td>
<td>18 (14.2)</td>
<td>5 (8.1)</td>
<td>13 (20.0)</td>
</tr>
<tr>
<td>Frequent milking (^{b,e})</td>
<td>No</td>
<td>54 (67.5)</td>
<td>25 (59.5)</td>
<td>29 (76.3)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>26 (32.5)</td>
<td>17 (40.5)</td>
<td>9 (23.7)</td>
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<tr>
<td>Fluid therapy (^{a,b,c,d,e,f})</td>
<td>No</td>
<td>90 (69.2)</td>
<td>38 (61.3)</td>
<td>52 (76.5)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>40 (30.8)</td>
<td>24 (38.7)</td>
<td>16 (23.5)</td>
</tr>
<tr>
<td>Treatment (^{a,b,d,e})</td>
<td></td>
<td>132</td>
<td>64 (48.5)</td>
<td>68 (51.5)</td>
</tr>
<tr>
<td>Growth of E. coli (^{b,f})</td>
<td>&lt; 10 cfu/μL</td>
<td>11 (8.4)</td>
<td>4 (6.3)</td>
<td>7 (10.3)</td>
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<tr>
<td></td>
<td>≥ 10 cfu/μL</td>
<td>120 (91.6)</td>
<td>59 (93.7)</td>
<td>61 (89.7)</td>
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<tr>
<td>Reinfection on d 21</td>
<td>No</td>
<td>53 (69.7)</td>
<td>28 (70.0)</td>
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<td>Yes</td>
<td>23 (30.3)</td>
<td>12 (30.0)</td>
<td>11 (30.6)</td>
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<table>
<thead>
<tr>
<th>Categorical variable</th>
<th>Level</th>
<th>Frequency (%)</th>
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</thead>
<tbody>
<tr>
<td>Parity (^{a,e})</td>
<td>1</td>
<td>26 (19.7)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>46 (34.9)</td>
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<tr>
<td></td>
<td>≥3</td>
<td>60 (45.4)</td>
</tr>
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<table>
<thead>
<tr>
<th>Continuous variables</th>
<th>Frequency</th>
<th>Mean (SE)</th>
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<tbody>
<tr>
<td>Days in milk (^{c,e})</td>
<td>100</td>
<td>125 (10.7)</td>
</tr>
<tr>
<td>Enrofloxacin treated</td>
<td>51 (51.0)</td>
<td>110.4 (14.0)</td>
</tr>
<tr>
<td>Non-treated</td>
<td>49 (49.0)</td>
<td>140.2 (16.3)</td>
</tr>
<tr>
<td>NAGase activity d 0 (^{a,c,d,e,f})</td>
<td>112</td>
<td>16.21(0.72)</td>
</tr>
<tr>
<td>Enrofloxacin treated</td>
<td>52 (46.4)</td>
<td>17.9 (1.0)</td>
</tr>
<tr>
<td>Non-treated</td>
<td>60 (53.6)</td>
<td>14.8 (1.0)</td>
</tr>
<tr>
<td>NAGase activity day 2 (^{a,c})</td>
<td>94</td>
<td>21.1 (0.7)</td>
</tr>
<tr>
<td>Enrofloxacin treated</td>
<td>46 (49.0)</td>
<td>24.0 (0.3)</td>
</tr>
<tr>
<td>Non-treated</td>
<td>48 (51.0)</td>
<td>18.3 (1.3)</td>
</tr>
</tbody>
</table>

\(^{a}\) Variables with a P < 0.25 in the univariable analyses of bacteriological cure on d 2

\(^{b}\) Variables with a P < 0.25 in the univariable analyses of clinical cure on d 2

\(^{c}\) Variables with a P < 0.25 in the univariable analyses of clinical cure on d 21

\(^{d}\) Variables with a P < 0.25 in the univariable analyses of NAGase activity on d 21

\(^{e}\) Variables with a P < 0.25 in the univariable analyses of returning of quarter milk production
On day 2, *E. coli* (n = 72), other coliforms (n = 2) and other bacteria (n = 5) were isolated in 72%, 2% and 5% of the samples, respectively (n = 100; 32 missing data), while no bacterial growth was detected in 21% of the samples. In the enrofloxacin group, number of quarters positive for *E. coli* on day 2 was significantly lower than in the non-treated group (P = 0.002, odds ratio for bacteriological cure 3.32; Table 2). On day 21, *E. coli*, other coliforms, other bacteria and mixed growth were isolated 14.5%, 4.8%, 22.9% and 4.8% of the samples, respectively (n = 83, 49 missing data). In 53.0% of the samples (44 out of 83) no bacterial growth was detected. Persistent *E. coli* infection, i.e., re-isolation of the original *E. coli* pulsotype in the post-treatment sample on day 21, was detected in 9 quarters (11.3% of the 80 cases sampled at both occasions), 4 of them in the enrofloxacin and 5 in the non-treated group.

More severe signs on day 0 increased the risk of having *E. coli* growth in the milk sample on day 2 (P = 0.02, odds ratio for cure 0.22). None of the tested factors affected bacteriological cure on day 21 (n = 79 in the analyses).

Enrofloxacin treatment decreased clinical cure on day 2 (P = 0.016, odds ratio 0.05), but use of frequent milking improved it (P = 0.005, odds ratio 39.82). Clinical cure was significantly more frequent on day 2 in cows that had milder systemic signs on day 0 (P = 0.002, odds ratio 0.25). Clinical cure on day 2 improved clinical cure on day 21 (P = 0.04, odds ratio 10.12). Bacteriological and clinical cure on day 21 in enrofloxacin and non-treated groups is depicted in Figure 6.

Milk NAGase activity on day 21 was associated with severity of the clinical signs on day 0; the more severe signs, the higher NAGase activity on day 21 (P = 0.007). Only frequent milking was related to decreased milk NAGase activity on day 21 (P = 0.04). Enrofloxacin treatment did not decrease mammary gland tissue damage estimated using determination of milk NAGase activity (mean 10.37 pmol 4-MU/min/μl in the treated quarters and 7.71 pmol 4-MU/min/μl in the non-treated quarters).
Quarter milk production returned to the pre-infection level in 31 (29.3%) cows and diminished in 70 (includes 31 cows with the blind quarter) (69.3%) cases (n = 101, 31 missing data). In the enrofloxacin group, quarter milk production returned in 21.8% (n = 12) of the cows and in the non-treated group in 37.3% (n = 19). Enrofloxacin did not affect quarter milk production. Diminished quarter milk production or a blind quarter due to *E. coli* mastitis was found in 78.2% of the treated and in 62.8% of the non-treated cows. More severe clinical signs on day 0 impaired the return of quarter milk production (P = 0.02, odd ratio 16.2). The other variables had no effect on milk production.

Three cows in the enrofloxacin group and five in the non-treated group (n = 132) did not survive (died or were euthanized) by day 21 post-treatment. All these cows were classified as severe cases on day 0. Of all cows, 31 (23.5%) (18 in the enrofloxacin group and 13 in the non-treated group; no significant difference) had left the herd during the six-month follow-up (Table 2). Enrofloxacin had no effect on remaining of the cows in the herd 6 months after treatment (71.9% of treated and 80.6% of non-treated).
8.3. Characteristics of E. coli isolated from clinical mastitis (study III)

The majority of initial 144 isolates, 119 (82.6 %), were found belong to phylogeny group A, followed by group D 16 (11.1%), group B1 7 (4.9 %) and B2 2 (1.4%). Of the 144 E. coli isolates, 56 (38.9%) had at least one virulence factor gene detected by PCR (Table 3). The combinations of virulence genes varied greatly, and each combination was present mostly in one isolate only (Table 3). Genes irp2, papC, iucD and iss were the most common. Genes iucD, cva, iss and papC correlated together ((rφ=0.56-0.74). Only astA, eaeA, irp2 and genes for F17 were present alone; all other genes were detected in different combinations. Genes svg, stx1, stx2, cnf1 and hlyA were not found in any of the isolates.

Of the initial 144 isolates, 40 (27.8%) showed resistance to one or more of the antimicrobials tested. In total 20.1% of isolates showed resistance to more than two antimicrobials. The most frequent resistances were for ampicillin (18.6%), streptomycin (16.4%), tetracycline (15.7%) and sulfametoxazol (13.6%). No resistance was found to gentamicin, florphenicol or ceftiofur (Table 4). Resistances to streptomycin, ampicillin, sulfametoxazol and trimethoprim were correlated with each other (rφ = 0.65-0.88). The virulence gene iucD was found to correlate with resistances to streptomycin, ampicillin, sulfametoxazol and trimethoprim (rφ = 0.60-0.81). None of phylogeny groups, virulence genes or resistances to antimicrobials had any association with the clinical signs of mastitis or milk NAGase activities.

The same pulsotype of E. coli was isolated after 3 weeks from ten quarters of 85 sampled quarters (11.8%) reflecting the persistence of E. coli intramammary infection. Persistent isolates belonged to the phylogenetic group A, except one in group D. One of the persistent strains was resistant to seven different antimicrobials, six were susceptible to all antimicrobials evaluated. Four persistent strains were negative for all virulence genes studied.

The clustering effect of the herd significantly affected the bacterial persistence (P = 0.03). The same pulsotype caused persistent mastitis in two different cows during 3 years on the same farm. This strain caused acute clinical mastitis episodes with severe clinical signs. None of the virulence factors, phylogeny groups or antimicrobial resistance traits was associated with the persistence of E. coli intramammary infection.
Table 3. Combinations of virulence factor genes detected among initial isolates of E. coli.

<table>
<thead>
<tr>
<th>Virulence genes and their combinations</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>astA eaeA</td>
<td>1</td>
</tr>
<tr>
<td>astA irp2</td>
<td>2</td>
</tr>
<tr>
<td>astA iucD</td>
<td>1</td>
</tr>
<tr>
<td>sfaD papC irp2 vat</td>
<td>1</td>
</tr>
<tr>
<td>sfaD papC irp2</td>
<td>1</td>
</tr>
<tr>
<td>irp2 iss vat</td>
<td>1</td>
</tr>
<tr>
<td>tsh cva iucD papC irp2 iss</td>
<td>1</td>
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Table 4. Distributions of MICs for *E. coli* from bovine mastitis (*n* = 144). MICs equal to or lower than the lowest concentration tested are given as the lowest concentration. Numbers of resistant isolates for each antimicrobial tested are indicated with gray fields.

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8.4. The efficacy of lactoferrin in the treatment of experimental E. coli mastitis (study IV)

In this experimental model, all cows were infected and developed clinical mastitis, which tended to be milder for the second challenge. Differences in systemic and local signs (Figure 7) between Lf- and enrofloxacin treated cows were not statistically significant.

Bacterial counts in milk decreased more rapidly in enrofloxacin- than Lf-treated cows, the difference almost reaching significance ($P = 0.054$) (Figure 7). After the second challenge, three cows had very low bacterial counts in the milk before the first enrofloxacin treatment at the first sampling 12 hours PC. The average bacterial counts are shown in Figure 7.

![Figure 7](image)

**Figure 7.** Scores for mean (± SEM) local signs, total daily milk production, milk NAGase activity of the infected quarter and milk bacteriological counts for the lactoferrin (Lf) and enrofloxacin (Enro) treated cows with experimentally induced E. coli mastitis. Challenges were carried out 3 weeks apart in six cows.
The corresponding ratios of the concentration of LPS to the number of bacteria in milk for the enrofloxacin- and Lf-treated cows were $3.7 \times 10^{-1}$ and $2.6 \times 10^{-4}$, the difference being statistically significant ($P = 0.028$). Milk NAGase activity remained high for a significantly longer time in cows treated with Lf than in milk of enrofloxacin-treated cows ($P = 0.046$) (Figure 7).

8.5. Experimental E. coli mastitis in rhLf-transgenic cows (study V)

All cows in the rhLf-group (transgenic) and in the control group became infected and developed clinical mastitis within 8-12 hours PC. The transgenic cows developed significantly less severe clinical signs compared with the control cows ($P = 0.020$). The transgenic group recovered significantly faster than the control group ($P = 0.008$). All transgenic cows and five cows in the control group showed mild to moderate systemic signs, and only one cow in the control group showed a severe reaction. Systemic signs of all cows in the transgenic group returned to normal within 24 hours, while the recovery of the control cows was slower and lasted over 48 hours. The local signs of the infected quarters and changes in the appearance of milk disappeared within 7 days PC in both groups (Figure 8). Expression levels of rhLf remained relatively constant during the experiment and rhLf concentrations in the milk ranged in the milk from 2.35 to 2.89 mg/ml, while bLf showed marked increase in both groups (Figure 9).

Milk bacterial counts, concentrations of SAA and Hp, SCC and NAGase activities did not differ statistically between the groups (Figure 10). All cows showed local and systemic TNF-α response. A concentrations of TNF-α in the milk were higher than concentrations in serum in both groups. The difference between the groups was not significant. All cows showed increased serum cortisol concentrations (Figure 11). The difference in serum cortisol concentrations between the groups was significant at 8, 36 and 168 hours PC ($P = 0.003$, $P = 0.031$, $P = 0.048$).

The mean values of serum SAA and Hp are shown in Figure 11. The difference between the groups was significant only at 8 ($P = 0.035$) and 168 hours PC ($P = 0.029$). No differences were noted in serum concentrations of AFOS, ALAT, total protein, urea, creatinine, and albumine, or in the WBC or PVC values between the groups.
Figure 8. Rectal temperature (a) and local signs (b) in transgenic (◊) and control (■) cows in experimental E. coli mastitis. Data are presented as means ± SEM for six transgenic and five control cows.
Figure 9. Mean concentrations of rhLf (♦) and bLf (◊) in the milk in challenged udder quarters of transgenic cows and of bLf (■) in milk from control cows in experimental E. coli mastitis. Data are presented as means ± SEM for six transgenic and five control cows.

Figure 10. Mean (a) bacterial counts, (b) milk SAA levels, (c) milk SCC, and (d) Hp concentrations in milk from transgenic (◊) and control (■) cows during the experiment. Data are presented as means ± SEM for six transgenic and five control cows.
Figure 11. Mean serum (a) TNF-α, (b) cortisol, (c) SAA, and (d) Hp concentrations in transgenic (◊) and control (■) cows in experimental E. coli mastitis. Data are presented as means ± SEM for six transgenic and five control cows. The P values indicated in panel b are 0.003 (*), 0.031 (**), and 0.048 (***) for cortisol, and those in panel d are 0.035 (*) and 0.029 (**) for serum Hp.
9. DISCUSSION

9.1. Pathogenesis of E. coli mastitis

Host responses and the clinical course of experimentally induced E. coli mastitis have been described in many previous studies (Hill et al., 1978; Pyörälä et al., 1994; Shuster et al., 1996; Shpigel et al., 1997; Hirvonen et al., 1999; Hoeben et al., 2000a; Rantala et al., 2002). E. coli intramammary infection typically results in acute clinical mastitis. The course of the disease varies from mild with only local signs in the udder to very severe or even fatal. Local signs and possible general signs, such as fever, increased heart rate, depressed rumen motility, inappetite and depression, gradually become apparent. The first clinical signs are detected in the affected quarter 8 h post-challenge and general signs by 12 h (Lohuis et al., 1990; Hirvonen et al., 1999; Hoeben et al., 2000a; Kornalijnslijper et al., 2004). Milk SCC dramatically increases in the mastitic quarter. Milk production decreases or is completely lost in the affected quarter, and in severe cases milk production ceases in all four quarters (Vandeputte-Van Messom et al., 1993; Kornalijnslijper et al., 2004). In mild or moderate cases, general signs disappear within two days and local signs by one week (Hill et al., 1978; Lohuis et al., 1990; Hirvonen et al., 1999; Hoeben et al., 2000a; Kornalijnslijper et al., 2004). In severe cases the cow may become recumbent and die (Hirvonen et al., 1999; Rantala et al., 2002).

This thesis is based on the results of three separate studies using an experimentally induced E. coli mastitis model (study I, IV and V). The development of infection and clinical course of the disease were quite similar in all the studies, and also similar to the previously described studies. The majority of the cows had mild or moderate mastitis and recovered within a week after the E. coli challenge.

In the study using S. aureus as the challenge organism, repeated intramammary inoculation at an interval of four weeks in the same cows revealed possible carry-over effects of the previous intramammary infection by the same pathogen (Eckersall et al., 2006), but only a tendency towards elevated concentrations of APP was recorded. Hirvonen et al. (1999) did not find stronger systemic acute phase responses after a second intramammary challenge with E. coli within a shorter interval of three weeks. In study I of this thesis, using two consecutive intramammary challenges with E. coli two weeks apart, all cows at both times became infected and developed local and systemic
inflammatory reactions, but after the second challenge local signs were significantly milder and disappeared faster. Milk production returned to the pre-challenge level significantly faster after the second challenge. The same pattern was noted for the indicators of inflammation evaluated, indicating a clear carry-over effect from the first challenge. In another experimental challenge study included in this thesis (study IV) we also detected rapid elimination of bacteria after the second challenge in three cows, which similarly reflected a carry-over effect. In that study, the interval between challenges was three weeks.

In previous studies using an experimentally induced *E. coli* mastitis model and 3 week interval, the disease was slightly milder after the second challenge, but the differences were not statistically significant (Pyörälä et al., 1994; Rantala et al., 2002; Kutila et al., 2004). Sensitization of the mammary gland after the first contact with a moderate dose of LPS was observed in the study with repeated challenges with LPS at a 24 h interval (Rainard and Paape, 1997). They did not detect systemic signs after the first LPS challenge, which was suggested to be due to too small an amount of LPS to trigger a systemic response, but after the second infusion, 24 hours later, the systemic signs were apparent. Relatively large doses of live *E. coli* were used in our study with a much longer interval (2 weeks), which resulted in significant changes in inflammatory response.

Recognition of LPS is an important event in the activation of the innate immune response to Gram-negative bacteria. LPS directly interacts with neutrophils through CD14, which is expressed on cell surfaces (Paape et al., 2003). The effective removal of the bacteria by neutrophils is important for the elimination of the infection. If delayed, infection can lead to development of endotoxin shock (Burvenich et al., 2003). Some immunization effect could have occurred in our study, which resulted in a faster host response and milder disease, as well as rapid elimination of bacteria from the infected gland after prior inoculation of a different quarter. In developing immunological memory for improving host defense, B-cells change from production of IgM to other antibody isotypes, in particular IgG1 and IgG2 (Burton and Erskine 2003). Vaccination with a core antigen (*E. coli* J5) promoted production of more IgG2 at early stages of the intramammary infection, which probably increased phagocytosis by PMN and elimination of bacteria (Wilson et al., 2007b). Repeated infections caused by the same bacterial species could trigger a vaccination effect, i.e. result in faster elimination of bacteria and decreased severity of the clinical signs because of the immunological memory of the cow. However, Schukken et al. (2009) did not find evidence for this in their field study on repeated cases of mastitis caused by the same bacterial group. The milk loss reflecting clinical signs was the same during the first, second and third episode of mastitis caused
by Gram-negative bacteria during the same lactation period. The mean interval between mastitis episodes in that study was about two months. If some vaccination effect ever was present, it had waned by that time. The duration of the effect of the common core vaccine has been reported to be approximately 2.5 months (Wilson et al., 2009). The vaccine antigens should provide protection against all Gram-negative mastitis causing species. Schukken et al. (2009) grouped the bacteria into Gram-negative and Gram-positive classes, without going to species level, which may have affected their results. In our study, the consecutive challenges were done with the same bacterial strain. A protective effect was apparent, but according to other experimental studies (Hirvonen et al. 1999; Kutila et al. 2004; Rantala et al., 2002) it would last about 3 weeks and not longer. The route of immunization affects the development of the immune response of the host, and intramammary challenge certainly differs from systemic administration of antigens.

Only few studies have reported concentrations of acute phase proteins in the milk during experimentally induced E. coli mastitis. In the study of Jacobsen et al. (2005), with a lower dose (50 cfu) of E. coli, concentrations of SAA in plasma were at a similar level, but those in the milk were 5-times as high as recorded in our study. Milk concentrations of SAA were highest in cows with severe mastitis but did not differ between those with moderate or mild signs (Jacobsen et al., 2005). Concentrations of mammary-derived SAA in milk were many times higher than concentrations of SAA in serum in their study and in ours. SAA has been suggested to have an important role in the modulation of the host response during infection (Patel et al., 1998; Vallon et al., 2001). SAA is able to bind outer membrane protein A of E. coli, which may also contribute to the recognition of Gram-negative bacteria by the host (Hari-Dass et al., 2005). Rapid mammary SAA response is probably involved in the innate local protection against pathogens invading the udder.

In our two experimental studies (study I and V), concentrations of Hp in the milk were similar. In the study of Hiss et al. (2004), using LPS challenge, the concentrations of Hp in milk increased by the end of the 12 h follow-up period and were less than half the concentrations recorded here. In our studies and in the cited study where an ELISA assay was used (Hiss et al., 2004), the concentrations of Hp in milk were approximately half those in serum. The Hp assay used in our studies has not been validated for milk and the results should be interpreted with caution. The local production of Hp seems not to be so pronounced as that of SAA. Hp binds harmful molecules produced after tissue damage, such as hemoglobin, which then becomes inaccessible to bacteria and limits their growth (Baumann et al., 1994). With this function, Hp may complete the actions of Lf (Hiss et al., 2004) and may thus play a role in host defense against E. coli mastitis.
LBP is a relatively new acute phase protein in the cow, and Bannerman et al. (2003) first reported concentrations of LBP in milk. Concentrations of LBP in milk and plasma have been shown to increase after intramammary challenge with LPS (Bannerman et al., 2003) and *E. coli* (Bannerman et al., 2004; Vangroenweghe et al., 2004). Concentrations of LBP in blood and milk found in our study (study I) are higher than reported in the previous studies using *E. coli* challenge models (Bannerman et al., 2004; Vangroenweghe et al., 2004). In our study (study I), concentrations in the milk were higher than those in blood, contrary to the findings of Bannerman et al. (2004). Challenge models and other methods used may be different, which could partly explain differences between results from different studies. LBP is a hepatocyte-derived protein that binds LPS, enhances LPS-CD14-complex formation and increases the sensitivity of the host’s innate response to Gram-negative bacteria (Wright et al., 1990; Burvenich et al., 2007; Le Roy et al., 2001). It is possible that LBP is also produced locally by the mammary epithelial cells, as also suggested by Bannerman et al. (2003), which would explain the high concentrations in the milk recorded in our study.

Lf is believed to act as a LPS-binding protein during the inflammatory activation of macrophages (Na et al., 2004). Lf competes with LBP for binding to LPS and might interfere with the interaction of LPS with CD14 (Legrand et al., 2004). LBP and CD14 are known to enhance LPS-induced host cell activation and to facilitate detoxification of LPS, preventing this response from becoming excessive and harmful to the host itself (Bannerman et al., 2003). The LPS-neutralizing activity of Lf may depend on the presence and concentration of other LPS-binding proteins.

The concentrations of SAA and Hp in serum and milk in this *E. coli* infection model were much higher than those reported in experiments using Gram-positive pathogens (Eckersall et al., 2006), indicating the strong inflammation induced by *E. coli*. We used one dose of anti-inflammatory medication at 12 h PC in our study (study I), which may slightly affect the inflammatory response, but given at both challenges, allows comparison of the two subsequent challenges. Use of acute phase proteins as early markers of mastitis has been suggested. They would also be useful parameters to monitor the severity of mastitis, to be used in studies on pathogenesis and effect of treatments.

Pathogenesis of *E. coli* mastitis and immunological mechanisms of the mammary gland deserve more research. Experimental models are available, and they do not necessarily have to be bovine
(Gonen et al., 2007). Repeated experimental intramammary induction of the same animals with \textit{E. coli} bacteria has been used as a model in crossover studies to reduce the individual variation between cows, but cow models are expensive and group size is limited. If cow-models are used for crossover studies, the significant differences between the consecutive challenges seen here suggest that in these studies the interval between challenges should be longer than 2 weeks.

**9.2. \textit{E. coli} mastitis therapy**

**9.2.1. Antimicrobial treatment**

The role of antimicrobials in the treatment of \textit{E. coli} mastitis is still open to debate. Clinical signs are mainly caused by LPS and treatment should be targeted towards these effects. On the other hand growing bacterial populations produce increased amounts of LPS, and consequently, infection contributes to the effects of LPS. Spontaneous elimination of intramammary infection with \textit{E. coli} is generally high (Anderson 1989; Erskine 1991). In some field studies on clinical mastitis caused by coliform or \textit{E. coli} bacteria, antimicrobial treatment has offered some advantages (Morin et al., 1998; Poutrel and Dellac, 2004), but many have shown no beneficial effects (Jones and Ward, 1990; Guterbock et al., 1993; Pyörälä and Pyörälä, 1998; Erskine et al., 2002; Sérieys et al., 2005). In some field studies equal cure rates of \textit{E. coli} mastitis have been the same using antimicrobials active or inactive against coliforms \textit{in vitro} (Jones and Ward, 1990; Pyörälä and Pyörälä, 1998). Most, if not all, published field trials have not been designed as prospective studies to determine the efficacy of defined antimicrobial treatment in \textit{E. coli} mastitis, but have used retrospective data. They lack an untreated control group, and mostly do not include a species-specific diagnosis. Susceptibility testing of the causal bacteria to the antimicrobial used has been tested only seldom, and when performed, the methods have not been properly described. Without knowledge on the susceptibility of the causal bacteria to the antimicrobial agent used, a critical assessment of the treatment outcome is difficult. In experimental studies in which the conditions can be controlled, some advantage from antimicrobial treatment over no treatment has been shown, such as faster elimination of bacteria, prevention of death of the cows, or reduction of the loss of milk production (Shpigel et al., 1997; Hoeben et al., 2000; Rantala et al., 2002; Poutrel et al., 2008).

In severe coliform mastitis, a substantial proportion of the cows, about 30\%, has been reported to develop bacteremia (Cebra et al., 1996; Wenz et al., 2001b). In the earlier study of Powers et al. (1986) only \textit{Bacillus sp} was isolated in cultured blood samples from cows with acute coliform mastitis and attributed to skin contamination. In the study of Pyörälä et al. (1994) on experimental
E. coli mastitis, no bacteremia was detected. Based on the results from the field studies, systemic administration of antimicrobials has been recommended for the treatment of coliform mastitis (Cebra et al., 1996; Wenz et al., 2001b; Erskine et al., 2003). The field studies on bacteremia in E. coli mastitis, as well as the recommendations based on them, originate from the United States, and their applicability to other conditions requires confirmation. Systemic administration of antimicrobial used is the only route to treat bacteremia (Constable et al., 2008). Another advantage of the systemic route is that antimicrobials penetrate better into the udder tissue, in particular in clinical mastitis, when the udder can be swollen and hard (Ziv 1980).

The selection of antimicrobials available for systemic treatment of coliform mastitis in dairy cows differs among countries. For example, in the United States, fluoroquinolones are not approved for treating dairy cows. Few antimicrobials have favourable pharmacokinetic and pharmacodynamic properties for treatment of acute clinical mastitis (Erskine et al., 2003; Constable et al., 2008). Broad-spectrum antimicrobials, such as fluoroquinolones, ceftiofur, and oxytetracycline, have been tested and recommended for treatment of E. coli mastitis (Shpigel et al., 1997; Cebra et al., 1996; Erskine et al., 2002; Morin et al., 1998; Wenz et al., 2001b).

Fluoroquinolones are recommended for treatment of severe E. coli mastitis in dairy cows in Finland as the first choice and high doses of trimethoprim-sulphamethoxazole as an alternative (Evira 2009). Milk disturbs the activity of trimethoprim-sulphonamides (Fang and Pyörälä, 1996), and therapeutic concentrations of this combination are difficult to achieve and maintain in the milk (Kaartinen et al., 1999). Phagocytic activity is decreased in the infected mammary gland and bactericidal antimicrobial agents would be preferable over bacteriostatic agents, the efficacy of which depends to a greater extent on the level of host defense (Kehrli and Harp, 2001). The trimethoprim-sulphamamide combination can be bactericidal in some concentration ranges, but probably not in the bovine udder where pharmacodynamic conditions are far from optimal (Pyörälä et al., 1994; Wagner and Erskine, 2006).

Oxytetracycline is an antimicrobial substance used for treatment of E. coli mastitis (Morin et al., 1998). The efficacy of oxytetracycline in the treatment of E. coli mastitis has not been proven (Constable and Morin, 2003). Antibacterial activity of tetracycline is reduced in milk because of chelate formation of tetracycline with casein (Fang and Pyörälä, 1996). In a recent study the increase of MIC of E. coli isolated from mastitis to oxytetracycline was reported to be over 16-fold in raw milk as compared with conventional growth media (Kuang et al., 2009). The doses suggested
for treatment of coliform mastitis (5-20 mg/kg) are too low to achieve and maintain therapeutic concentrations in the milk when only the free part of the drug is active. The breakpoint concentration for susceptibility of *E. coli* to tetracycline is 8 μg/ml (FINRES-Vet 2005-2006), which is hardly reached even in serum, and only after i.v. administration (Pharmaca Fennica Veterinaria 2010). The concentration of oxytetracyclin in milk after administration of 10 mg/kg is about 1.9 μg/ml 10 h after systemic administration, which is far from the therapeutic concentration (Erskine et al., 2003).

A third generation cephalosporine, ceftiofur, is authorized for treatment of respiratory disease in cattle with a dose of 1 mg/kg (Pharmaca Fennica Veterinaria 2010). It diffuses poorly to milk and does not have a withdrawal time for milk (Prescott 2006). Ceftiofur administered at 3mg/kg every 12 h failed to eliminate bacteria in experimental *E. coli* mastitis (Erskine et al., 1995). Ceftiofur was not detectable in milk of healthy cows, but could be detected in milk of challenged cows, with a maximum concentration of 0.28 μg/ml (Erskine et al., 1995). This concentration could be, in theory, be enough for susceptible *E. coli* bacteria. For naturally occurring coliform mastitis, systemic ceftiofur therapy with a dose of 2.2 mg/kg/day intramuscularly for five days, was shown to favourably influence the clinical outcome of severe cases of coliform mastitis (Erskine et al., 2002). Using ceftiofur for treatment of mastitis is off-label and should be avoided; the dose used is also higher than the label dose and the zero withdrawal time for milk is then not applicable.

The fluoroquinolones danofloxacin and enrofloxacin are authorized for lactating cattle in the European Union, and are also used for treatment of coliform mastitis (Poutrel and Della, 2004; Poutrel at al., 2008; Constable et al., 2008). Mastitis is not an approved indication for danofloxacin in most EU countries; also the common label dose, 1.25 mg/kg, appears low for mastitis treatment (Pharmaca Fennica Veterinaria 2010). In Sweden, danofloxacin has recently been approved for treatment of coliform mastitis at a higher dose level, 6 mg/kg (FASS 2010). Enrofloxacin is one of the few antimicrobial drugs, that have been recommended for the treatment of coliform mastitis, due to its favorable pharmacokinetic and pharmacodynamic properties (Constable et al., 2008). Enrofloxacin has reported to increase the killing capacity of neutrophils (Hoeben et al., 1997), which may contribute to its therapeutic effect. However, the influence of fluoroquinolones on PMN activity and function is controversial (Labro 2000). The antimicrobial activity of enrofloxacin is not reduced in milk (Fang and Pyörälä, 1996). All isolates in our field study (study II) were susceptible *in vitro* to ciprofloxacin, which is the active metabolite of enrofloxacin. High ciprofloxacin concentrations are reached in blood and milk (Kaartinen et al., 1995; Rantala et al., 2002). Here we
used the higher label dose of 5 mg/ml, which should maintain the therapeutic concentrations in the milk (Rantala et al., 2002). Despite this, in our field study systemic enrofloxacin treatment did not result in better bacteriological or clinical cure of acute clinical mastitis caused by *E. coli* than supportive treatment alone. Neither did enrofloxacin treatment affect survival of the cows, returning of quarter milk production or tissue damage of the infected quarter. The only positive effect of the enrofloxacin treatment was the better bacteriological cure on day 2 post-treatment, although, clinical cure on day 2 was significantly lower. The clinical relevance of these findings on day 2 appears minor, as the final outcomes from mastitis assessed 3 weeks post-treatment did not differ. For the farmer, the outcome of the greatest economic importance is the return of the quarter to producing milk acceptable for delivery.

Faster elimination of bacteria from the quarter was reported in cows treated with enrofloxacin than in non-treated cows in an experimental *E. coli* mastitis model (Rantala et al., 2002). Furthermore, in experimentally induced *E. coli* mastitis, enrofloxacin treatment significantly decreased the milk loss determined 2 days post-treatment and alleviated local signs in the affected quarter after 24 hours post-treatment (Hoeben et al., 2000b). Poutrel et al. (2008) tested another fluoroquinolone, danofloxacin, also using an experimental *E. coli* model and reported that the initial clearance of bacteria from the quarters was faster in the danofloxacin group, but the final bacteriological cure was similar as in the untreated group. Our results from the field study (study II) on the better bacteriological cure on day 2 thus agree with these experimental studies, but the clinical cure on day 2 was significantly lower in the enrofloxacin-treated than in non-treated cows, and the clinical cure on day 21 was slightly inferior compared with that for the untreated cows. In theory, this could due to release of endotoxin after the more efficient killing of bacteria by enrofloxacin. In humans, the release of endotoxin from dead Gram-negative bacteria after antimicrobial treatment is considered a risk for endotoxin shock (Lepper et al., 2002), a finding that supports this theory. However, Dosogne et al. (2002a) showed that concentrations of endotoxin in the plasma did not differ in enrofloxacin-treated and untreated cows. In support of this, no differences in the concentrations of endotoxin in the milk were found for cows with experimentally induced *E. coli* mastitis treated with antimicrobials or left untreated (Pyörälä et al., 1994). Therefore, the theory of excessive release of endotoxin in the treated group is not supported. Clinical cure on day 21 (Figure 6) was rather low in both treatment groups, which probably reflects the severe nature of *E. coli* mastitis. Katholm & Andersen (1998) reported similar proportions of clinical and bacteriological cure from a Danish field study on *E. coli* mastitis with no antimicrobial treatment, but no control group was included in their study. Contrary to our findings, danofloxacin reduced decrease in the milk production and
lowered SCC and udder inflammation scores assessed 3 weeks post-treatment (Poutrel et al., 2008). Enrofloxacin did not affect returning of quarter milk production evaluated at 3 weeks post-treatment in our study, Discrepant results may be explained by the different nature of the experimental and field studies.

Milk SCC can remain high for a long time after *E. coli* mastitis (Pyörälä and Pyörälä, 1997) and such quarters often develop new infections caused by other bacteria. In our study (study II) 30% of quarters were infected by other bacteria three weeks after *E. coli* mastitis developed. This could be because mastitic milk has been shown to be a better growth medium for mastitis bacteria than milk from healthy quarters (Mattila et al., 1984).

The proportion of died or euthanized cows after coliform mastitis was reported to be approximately 25% in the United States (Cebra et al., 1996; Wenz et al., 2001b). This was much higher than our corresponding figure of 6%. The follow-up period in the former U.S. study was 4 weeks compared to our 3 weeks, and they included all cows culled during the study period. Furthermore, the U.S. study was carried out in a teaching hospital clinic, which could indicate that many cows with severe disease were included in the material, explaining the high fatality rate. Factors including cow breed, milk yield, management, environment and herd size may also explain the differences. *E. coli* mastitis is known to cause a long-lasting decrease in milk production of the affected quarter (Golodetz et al., 1983), milk SCC remains easily high (Pyörälä and Pyörälä, 1997) and milk is not accepted for marketing. The proportion of cows removed from the herd within 6 months in our study was 23.5%, indicating a high risk for a cow to be culled after *E. coli* mastitis. Bar et al. (2008) showed that clinical mastitis increased the odds of a cow dying or being sold for slaughter, thereby supporting our findings. In our study, remaining in the herd and DIM were significantly associated, indicating that a cow was more likely to be kept in the herd if it was in late lactation and approaching drying-off. This was in contrast to findings that culling rates after clinical mastitis were increased in later-lactating cows (Gröhn et al., 2005; Bar et al., 2008). Those studies were carried out in the United States, possibly reflecting management differences for large dairy herds. In the smaller, family-owned herds of Finland, cows are managed more individually, which may explain differences in culling policies.

Our results indicate that antimicrobial treatment is not needed in acute clinical mastitis caused by *E. coli* if the clinical signs are mild or moderate. Enrofloxacin treatment did not affect the outcome from mastitis, not even in severe cases. This means that at least in mild and moderate acute clinical
mastitis, routine use of broad-spectrum antimicrobials is not necessary, and the treatment can be targeted towards the common Gram-positive mastitis bacteria (Constable 2008). Rapid diagnostic tools to differentiate among coliform and other types of mastitis causing agents would be useful. The only cow-side test developed for this purpose, based on detection of endotoxin (Waage et al., 1994), is no longer available. Targeting the antimicrobial treatment of mastitis towards Gram-positive bacteria and using a non-antimicrobial approach for coliform mastitis has been suggested as a routine procedure for herds (Bennedsgaard et al., 2010; Lago et al., 2009).

Antimicrobial therapy can reduce the number of bacteria at the acute stage of infection in severe mastitis and provide time for the immune system to act. In the study using a severe experimental *E. coli* mastitis model by Rantala et al. (2002), enrofloxacin treatment appeared to prevent death of the cows. In peracute or severe acute *E. coli* mastitis, the potential risk of increasing bacterial counts in the milk and development of bacteremia support the use of antimicrobials to save the life of the cow. If the number of *E. coli* bacteria in the milk is very high, the prognosis for the cow is poor (Katholm and Andersen, 1992; Vandeputte-Van Messom et al., 1993; Katholm and Andersen, 1998). The bacterial count in the initial sample could represent a valuable tool for a cattle practitioner in to evaluate the prognosis of the cow and possible need for antimicrobial treatment could be (Vandeputte-Van Messom et al., 1993; Hirvonen et al., 1999; Katholm and Andersen, 1998). Katholm (2003) has suggested that there is no need for antimicrobial treatment in *E. coli* mastitis if the bacterial count in the milk is low, i.e. only single colonies and no carpet growth is detected on the agar plate. Nevertheless, in cases of severe acute mastitis, it is recommended to start treatment with antimicrobials on the basis of the clinical signs before the bacteriological diagnosis. In severe cases of *E. coli* mastitis, tissue damage may, however, be too grave for antimicrobial treatment to offer any significant benefit (Hill 1984; Vandeputte-Van Messom et al., 1993).

Fluoroquinolones are important antimicrobials for the treatment of severe and invasive infections in humans and animals. They are of special interest for public and animal health (Collignon 2009). The development of the antimicrobial resistance among some species of bacteria has been alarming (White 2006). Fluoroquinolones should only be used for severe infections and when their efficacy has been confirmed (EMEA 2006). Antimicrobial residues in the tissues and milk of the treated cows are one reason for concern about antimicrobial therapy. Fluoroquinolone residues are not necessarily detected by the current tests for antimicrobial residues in the milk (Pyörälä 2009). Mastitis treatments should be evidence-based, i.e. the efficacy of all treatments should be
demonstrated by scientific studies, and this is particularly critical for reserve antibiotics (Cockcroft and Holmes, 2003).

9.2.2. Non-antimicrobial therapy
Non-steroidal anti-inflammatory drugs (NSAID) are commonly used and recommended for treatment of coliform mastitis (Katholm and Andersen, 1992; Green et al., 1997; Erskine et al., 2003; Huxley 2004; Leslie et al., 2010). NSAIDs possess anti-inflammatory, antipyretic, and analgesic properties (Smith 2005). As discussed earlier, in cases of mild and moderate \textit{E. coli} mastitis the emphasis of treatment should be focused on supportive therapy, and not on antimicrobial treatment. In severe cases of mastitis, the presumptive diagnosis of coliform mastitis can be based on clinical signs, systemic disease signs in particular, such as rectal temperature, rumen contraction rate, hydration status and signs of depression (Wenz et al., 2001a). Correction of dehydration using fluid therapy is usually, if not always, indicated in severe cases of coliform mastitis with orally administered or i.v. administered hyper- or isotonic saline (Smith 2005). Mastitis is a painful condition and cow welfare and relief of pain should be taken into account (Leslie et al., 2010). The efficacy of NSAIDs has been shown in revealing clinical signs and diminishing effects of endotoxin (Shpigel et al., 1994; Pyörälä and Syväjärvi, 1987; Vangroenweghe et al., 2005; Banting et al., 2008). Several NSAIDs are authorized for dairy cows in the EU for the indication of treatment of mastitis. Meloxicam, for example, has been reported to have a longer-acting effect, approximately 48 h, of reduction of pain in the quarter and decreasing in heart and respiratory rates in an endotoxin mastitis model (Banting et al., 2000).

Systemic administration of glucocorticoids has previously been used and recommended for treatment of coliform mastitis (Anderson and Hunt, 1989; Erskine et al., 2003). They are considered to decrease the effects of endotoxemia and reduce the clinical signs, but a negative effect is that they suppress the neutrophil migration to the infection site (Diez-Fraile et al., 2002; Erskine et al., 2003; Burton and Erskine, 2003). If these drugs are used, it should be only for severe cases of mastitis (Erskine et al., 2003). NSAIDs are considered more effective than glucocorticoids in alleviating clinical signs associated with endotoxemia in cattle (Smith 2005), although controlled studies comparing these two classes of substances in mastitis models are very few (Wagner et al. 2004).
Frequent milking is a traditional treatment for mastitis and is commonly recommended as a supportive treatment for clinical mastitis (Radostits et al., 2007). Studies on its efficacy in treating severe experimental *E. coli* mastitis have furnished contradictory results, as both advantageous effect and no benefits have been shown (Stämpfli et al., 1994; Leininger et al., 2003). The results of experimentally induced *E. coli* mastitis model indicated that frequent milking as a treatment did not improve outcome nor did it appear to have negative effect (Leininger et al., 2003). Results from our field data (study II) indicated that frequent milking at the acute stage of mastitis can improve the initial clinical cure of *E. coli* mastitis. It is possible that frequent milking decreased inflammation and limited tissue damage in the infected quarter. In our study, the number of observations was quite small, which reduces the statistical power. Frequent milking is laborious and seldom applicable in the modern large dairy herds, but if done, can offer some benefits for the individual cow.

Several other non-antimicrobial approaches to mastitis treatment have been investigated. For example, herbal remedies and homeopathy are without effect (Hektoen et al., 2004; Tikofsky and Zadoks, 2005). Antimicrobial proteins, such as nisin and lysostaphin, cytokines, and recombinant soluble CD14, have been investigated under experimental conditions for the treatment of acute mastitis, but have provided no evidence of practical benefits (Sears et al., 1995; Bramley and Foster, 1990; Lee et al., 2003). Lactoferrin is one of the non-antimicrobial substances tested against mastitis bacteria *in vitro* and *in vivo*. Synergistic effects of the combinations of Lf and antimicrobials have been reported for Gram-positive bacteria (Sanchez and Watts, 1999; Diarra et al., 2002; Komine et al., 2006; Lacasse et al., 2008). The research in this treatment area has concentrated on staphylococcal mastitis. Early studies showed that Lf inhibited bacterial growth by the acquisition of essential iron from the environment of the bacteria, whereas more recent findings have indicated wider interactions with bacterial cells (Bellamy et al., 1992; Lönnerdal and Iyer, 1995; Legrand et al., 2004). When Lf affects Gram-negative bacteria, there is a concomitant release of LPS, after which Lf can inhibit its detrimental effects (Yamauchi et al., 1993). An earlier attempt to use a LPS-binding substance to treat coliform mastitis was made by Ziv and Schultz (1983), who tested polymyxin B, an antibiotic with the capacity to neutralize LPS, in an experimental LPS mastitis model. No significant beneficial effects were found when polymyxin B was administered shortly after LPS. In experimental *E. coli* mastitis model Pyörälä et al. (1994) compared intramammary treatment containing colistin, which also has a LPS neutralizing effect, with systemic trimethoprim-sulphonamide treatment or no-treatment, and found no significant differences in any clinical variables or the concentrations of LPS in the milk of infected quarters.
In our study (study IV) using intramammary Lf for treatment of *E. coli* mastitis, we decided to retain the advantage of a non-antibiotic approach and did not combine Lf with antimicrobials. Bovine Lf is known to inhibit the growth of *E. coli* isolated from mastitis *in vitro* (Kutila et al. 2003) and the disposition kinetics of Lf after intramammary administration were previously determined (Kutila et al., 2002). With a dose of 1g Lf, high concentrations of Lf are maintained in milk for several hours; the mean elimination half-life was 2.2 hours (Kutila et al., 2002). With an appropriate dose, Lf could support the clearance of bacteria from the udder and simultaneously block the effects of LPS by inhibiting the production of pro-inflammatory cytokines. Enrofloxacin was selected for our experiment as the positive control, because it was recommended for a treatment of coliform mastitis and approved for lactating cattle in the EU. For animal-welfare reasons, we decided to use supportive treatment for all cows. It would have been optimal to use an untreated control group, which would have revealed the net effect of the intramammary Lf infusion. In the treatment of clinical *E. coli* mastitis, treatment is targeted at inhibiting bacterial growth, to reduce the exposure of the quarter to LPS. Furthermore, the effect of the LPS release should be neutralized to reduce the severity of the clinical signs (Erskine et al., 1991).

We did not observed any significant differences in the clinical signs between cows treated with Lf and those receiving enrofloxacin. The NAGase activity, indicating inflammation in the quarter, decreased significantly faster in the group treated with enrofloxacin and a trend of faster elimination of bacteria from the challenged quarters was also seen.

Zhang and co-workers (1999) studied a human Lf-derived 33-mer synthetic peptide (Lf-33) as a potential treatment for LPS-induced septic shock. In a mouse model, administration of Lf-33 simultaneously with LPS significantly reduced mortality compared with LPS alone. In our study, LPS concentrations in milk were about 1400 times lower in relation to the number of bacteria in the Lf-treated cows than in the enrofloxacin-treated cows, which may indicate a LPS-neutralizing effect of the exogenous Lf. Another explanation could be that enrofloxacin killed bacteria more efficiently, resulting in a higher release of LPS from the bacterial cells and consequently a higher ratio of LPS to bacterial count. Three cows treated with Lf at the first challenge had lower bacterial counts at the second exposure compared with those treated with enrofloxacin. A rapid immunoresponse of the host may have eliminated most of the bacteria from the udder before the treatment with enrofloxacin was started at the second challenge. One explanation for the more rapid
immunoresponse may be the initial exposure to the bacteria during the first challenge or a possible immunostimulatory effect of Lf.

Definite conclusions cannot be drawn as the study material was limited and an untreated control group was not included. Despite of the inconclusive results furnished by our experiment, we believe that Lf has potential as a non-antibiotic mastitis treatment of bovine mastitis. More studies using in vivo models are needed to explore this. Lactoferrin has antimicrobial activity against a variety species, and broad immunomodulatory properties, which make it interesting for treating inflammatory and infectious diseases.

9.3. Virulence of mastitis E. coli isolates

Major virulence factor groups of E. coli are adhesins and toxins. Adhesins, such as Afa adhesin, intimin, Dr adhesin, P- and S-fimbriae, CFAs, Saa, OmpA, Curli, aerobactin, yersiniabactin, Chu, flagellin and LPS, help bacteria to adhere, colonize and act as fitness factors (Kaper et al., 2004; Smith et al., 2007). Toxins, such as enterotoxins, Shiga toxin, Stx, CDT, Tsh, Stc, Map, IpaA, HlyA, Ehx, CNF1, CNF2 and Sta, are targeted at different cellular functions and act in cell lysis (Kaper et al., 2004; Smith et al., 2007). The genes for virulence factors can be present in the genome of bacteria or in mobile genetic elements, including transposons, plasmids, bacteriophages and pathogenicity islands (Kaper et al., 2004; Smith et al., 2007). E. coli do not produce virulence factors continuously, but only when specific signals are received from the environment (China and Goffaux, 1999).

Different virulence factor genes and their combinations were present in 39% of our E. coli isolates collected from our field study. Typically only one isolate represented each combination of genes, reflecting the high genotypic variability among E. coli isolates causing bovine mastitis. Our results agree with those from earlier studies, but the panel of the studied virulence factors differed among the studies (Nemeth et al., 1991; Nemeth et al., 1994; Kaipainen et al., 2002; Bean et al., 2004; Wenz et al., 2006). In our study the virulence factors detected, phylogeny groups or antimicrobial resistance traits of E. coli were not associated with the clinical signs of the cow at the acute stage of the disease, nor with the recovery from mastitis. These results are consistent with the previous findings, suggesting that severity of E. coli mastitis is not associated with any known specific virulence factor (Lehtolainen et al., 2003a; Wenz et al., 2006; Dyer et al., 2007). In our material,
over 60% of the isolates were devoid of the studied virulence genes. In an earlier Finnish study (Kaipainen et al., 2002), approximately 50% of the isolates did not possess any virulence genes. In another study on *E. coli* isolates from bovine mastitis, 57.5% were without any virulence genes (Bean et al., 2004). The genes studied in those two studies differed from those included in our panel. In a recent study of Silva et al. (2009) on *E. coli* isolates from the uterus of puerperal dairy cows, only four virulence factor genes (*hlyE*, *hlyA*, *iuc*, and *eaeA*) in various combinations were detected in the isolates. None of the examined virulence genes was associated with infection in the uterus (Silva et al., 2009).

The majority of the *E. coli* isolates in our study belonged to the phylogeny group A. Group A consists mainly of strains that are commensally orientated and not pathogenic for a healthy host (Clermont et al., 2000). They have been reported to have few virulence factors. Pathogenic intestinal *E. coli* strains occurred in groups A, B1 and D (Picard et al., 1999; Russo and Johnson, 2000), whereas pathogenic extraintestinal *E. coli* belong mainly to groups B2 and D (Clermont et al., 2000). In the study of Silva et al. (2009), *E. coli* isolates originating from the bovine uterus belonged mainly to phylogenetic groups A and B1, and had a low potential virulence. Dogan et al. (2006) reported in their study with three persistent and three transient *E. coli* mastitis strains, that all belonged to phylogeny group A and lacked virulence genes of invasive *E. coli*. This supports our conclusion that the mastitis-causing *E. coli* bacteria are typical commensals.

In our field study the same genotype of *E. coli* was isolated three weeks post-treatment from 11% of quarters as was isolated initially from the same quarter, indicating persistence of the same strain of *E. coli* in the quarter. This figure is much lower than the 20.5% reported in a UK study by Bradley and Green (2001b), but close to 12.4% reported in an earlier Finnish study (Lehtolainen et al., 2003a). The British study included only six herds compared with our 65 herds, which may have affected the results. In our study, post-treatment samples were stored frozen, which can influence growth of *E. coli* (Schukken et al., 1989) and may decrease the frequency of re-isolation. No bacterial feature – phylogeny group, virulence factor or resistance trait – was associated with persistence of the *E. coli* intramammary infection in the udder. Contradictory results were presented in a previous Finnish study of Lehtolainen et al. (2003a), where the genes *cnf1*, *cnf2*, *sfaD* and *papC* were significantly associated with re-isolation of *E. coli* post-treatment. In their study, the persistence of the same strain was not confirmed by PFGE. We found only three strains with virulence genes for CNF2 and none of them was persistent. In our data P-fimbria was detected in 16% and S-fimbria in 2% of the isolates and neither of them was linked to persistence of the
intramammary infection. Recurrent *E. coli* mastitis has been reported to have a tendency towards milder clinical signs than seen in the non-recurrent cases, which was suggested to favour the intramammary infection becoming chronic (Bradley and Green, 2001). In the earlier Finnish study, no differences were found in the severity of clinical signs of intramammary infections, which remained persistent as compared with those that were eliminated (Lehtolainen et al., 2003a), in agreement with our findings.

Commensal strains of *E. coli* commonly lack the specialized virulence traits that are present in pathogenic strains (Russo and Johnson, 2000). Dogan et al. (2006) found that *E. coli* isolated from persistent intramammary infection survived and also replicated intracellularly *in vitro*. However, intracellular multiplication of *E. coli* in the mammary epithelial cells has not yet been reported *in vivo*. Shpigel et al. (2008) suggested that *E. coli* associated with persistent bovine intramammary infection could have some unknown bacterial factors for pathogenity. Our results did not support their suggestion of a specific mammary-pathogenic *E. coli*. Our conclusion was that mastitis-causing *E. coli* bacteria, at least in this study material (study III), appeared to be typical commensals. However, we found one *E. coli* pulstype that caused persistent mastitis in two individual cows at a 3 year interval in one herd. In the study of Blum et al. (2008), comprising one herd, isolates of *E. coli* were suggested to form a subset of the general environmental *E. coli* population. The authors speculated that these isolates could better multiply in the mammary gland and were genetically distinct from most environmental strains. It is possible that some *E. coli* strains could have some other mechanisms, not covered in the published studies, which influence their ability to cause mastitis, in particular persistent intramammary infections. One possible mechanism is suggested to be biofilm formation of *E. coli* bacteria in chronic or recurrent type of mastitis (Melchior et al., 2006). This ability of *E. coli* was not found to be associated with bovine uterine infections (Silva et al., 2009). More research is needed with new markers for virulence to further explore the existence of possible mammary pathogenic *E. coli*.

Among the virulence factor genes detected in our study, only *iucD* was associated with antimicrobial resistance to streptomycin, ampicillin, sulfametoxazol and trimethoprim. The gene *iucD* codes for aerobactin, which is involved in the iron-chelating ability of *E. coli* and may be located either on a chromosome or on a plasmid, where also many of the antimicrobial resistance genes are reside (Bagg et al., 1987; Hirsh et al., 1993; Johnson et al., 2002). The gene *iucD* was also associated with *iss* (gene for increased resistance to serum), which might also be located on a plasmid (Johnson et al., 2002). Plasmids are major factors for the dissemination of both antibiotic
resistance and virulence determinants among bacterial populations. The presence of virulence and antibiotic resistance determinants in the same genetic element will produce co-selection of both types of determinant. For bacteria carrying those linked determinants, the selection of an infective population will evolve towards antibiotic resistance, and antibiotic selective pressure will select the virulence trait (Martínez and Baquero, 2002). In our study antimicrobial resistance traits of the *E. coli* isolates were not associated with any clinical variables or recovery from mastitis.

In the present study (study III), 18.6% of *E. coli* isolates from mastitis were resistant to ampicillin, 16.4% to streptomycin, 15.7% to tetracycline and 13.6% to sulfamethoxazol. The proportions of resistance to the other antimicrobials were lower. Only one isolate showed resistance to ciprofloxacin. The prevalences of antimicrobial resistances of *E. coli* isolated from mastitis seen here were higher than in the national resistance surveillance programme (FINRES-Vet 2005-2006), in addition 20% of our material were multi-resistant as compared with only 6% in the national surveillance data (FINRES-Vet 2005-2006). Our data originates from limited geographical area, which may partly explain the differences when compared to national data. In a Swedish report, in which the same cut-off values were used, resistance proportions of *E. coli* isolates were generally only half of those recorded for our material (Bengtsson et al., 2009). The most frequent resistance of mastitis *E. coli* isolates was found against antimicrobials used for years in dairy cows, including ampicillin, streptomycin, sulphonamide and tetracycline. In addition, resistances to most of these antimicrobials often were associated, indicating that they appear in the same isolates, also seen by Lehtolainen et al. (2003b). Resistances to the traditional antimicrobials of *E. coli* mastitis isolates found here were more numerous than reported in the Netherlands (MARAN 2007), but taking into account cut-offs of the Dutch study, the concentrations and the resistance figures were mainly similar to ours. Antimicrobial resistance can only be compared if the same cut-offs have been used (Schwarz et al., 2010). The cut-off values used in the resistance monitoring systems, e.g. for enrofloxacin, can differ by as much as 3 dilution steps, from 0.25μg to 2μg, and data cannot be compared (SVARM 2008; MARAN 2007). The development of antimicrobial resistance among *E. coli* isolated in animals has generally increased (White 2006). Resistance against fluoroquinolones is still rare among *E. coli* isolated from bovine mastitis, being only 0-2% (FINRES-Vet 2005-2006; MARAN 2007; AFFSA 2008). Resistance against fluoroquinolones among enterobacteria is caused by mutations in the gyrase or topoisomerase genes (Robicsek et al., 2006).

In our material 1% of the isolates was resistant to ceftazidime, but none to ceftiofur. In other studies resistance of bovine *E. coli* isolates to third of fourth generations cephalosporins has also been low
Resistance to cephalosporins in *E. coli* is primarily caused by production of extended spectrum beta-lactamases (ESBL) coding for resistance not only to aminopenicillins, but also to cephalosporins. Genes coding for ESBLs have been increasingly reported in *Enterobacteria*, including *E. coli* isolated from food producing animals (Batchelor et al., 2005; White 2006; SAGAM 2009), which is a reason for concern. Detection of the first *E. coli* isolates from bovine mastitis producing ESBL was recently reported (Locatelli et al., 2009). Resistance genes for fluoroquinolone resistance and production of ESBL are often encoded on plasmids or other mobile genetic elements and often linked to other resistance genes (Robicsek et al., 2006; White 2006; SAGAM 2009).

Resistance among mastitis causing *E. coli* may reflect the general use of antimicrobials in the herd, as these bacteria are mostly commensals. The use of antimicrobials in the Finnish dairy herds has been strictly controlled and all antimicrobials are provided under veterinary prescription. Use of narrow-spectrum antimicrobials such as penicillin G is recommended, which should reduce the selection pressure to evolve resistance (Evira 2009). Routine antimicrobial treatment is not recommended for *E. coli* mastitis in Finland (Evira 2009).

### 9.4. Prevention of E. coli mastitis

The main principles to prevent mastitis and maintain good udder health are a comfortable, clean and dry environment for dairy cows, good-quality feeding and fresh drinking water, good milking hygiene, and proper herd management (Hogan and Smith, 2003; Pyörälä 2008; Corbett 2009). A contaminated environment is the main source of *E. coli* mastitis (Nemeth 1994; Lipman et al., 1995). Keeping stalls and yards, cow traffic areas, and lying areas clean generally reduces contamination of the udder and consequently environmental mastitis (Hogan and Smith, 2003). Dry cow management and proper maintenance of calving units significantly affects the susceptibility of cows to puerperal mastitis. This is important because *E. coli* intramammary infections are most common and severe around calving and during early lactation (Bradley and Green, 2000; Green et al., 2007). Inadequate nutrition, pregnancy and stress can influence the immune system of the cow by depressing the function of immune cells (Lippolis et al., 2008). Feed should contain the recommended levels of vitamin and trace elements. Vitamins E and A, selenium, copper and zinc have a role in defense of the cow against intramammary infections (Godden et al., 2006).
Prophylactic immunization using vaccines has been used for preventing coliform mastitis. Vaccines based on the core antigen of coliform bacteria (*E. coli* J5), have been in use in the North America for two decades (Cullor et al., 1991; Wilson and González, 2003). This type of vaccine is now also available in the EU (EMA 2009). The common core vaccine is administered systemically at the drying-off and again 3 weeks before the calving date. The effect of vaccination lasts about 2.5 months, but does not protect the cow from clinical mastitis (Wilson et al., 2007a; Wilson et al., 2007b). Smith et al. (1999) showed that subcutaneous combined with intramammary immunization with *E. coli* J5 bacterin produced enhanced antibody titers in milk and serum, but did not reduce clinical signs after subsequent challenge with *E. coli*. Vaccination does not reduce the incidence of clinical mastitis, but reduces clinical signs of coliform mastitis, culling, cow death and milk losses (Wilson et al. 2007a; Wilson et al. 2007b). The mechanism of action of J5 vaccine is still unclear (Wilson et al. 2007b). One hypothesis for the potential mechanism of action of *E. coli* vaccine is an enhanced PMN diapedesis caused by mammary gland hyper-responsiveness (Dosogne et al. 2002b). Recently it was suggested that the positive effect of *E. coli* vaccination is associated with a memory antibody response of IgG1 and IgG2 isotypes (Wilson 2007b). Iron regulated outer membrane protein *E. coli* vaccines have been investigated *in vitro* (Lin et al. 1999), but no practical applications have been developed. It can be concluded that the only current mastitis vaccines with evidence of clinical efficacy are the common core vaccines used against coliform mastitis. A decision on the use of vaccination against coliform mastitis should be made for each herd, based on the mastitis situation in the herd and a cost-benefit analysis. In Finland coliform mastitis is rarely such a problem that introduction of the vaccination program would be economical, as the herds are relatively small and are mostly housed in stanchion barns.

Genetic engineering of the dairy cow has been proposed by several authors to increase host defense against mastitis (Bramley et al., 2001; Pyörälä 2002; Maga et al., 2005; Oliver 2005). Resistance of the cows to mastitis could be increased by improving the immune response through modifying genes or by transferring genes from the other organisms (Oliver 2005). The first published study on the transgenic approach to enhance mastitis resistance was a mouse model against staphylococcal intramammary infection (Kerr et al., 2001). In the first published bovine model (Wall et al., 2005), cows carrying a gene coding lysostaphin were shown to resist *S. aureus* intramammary infection.

In this thesis (study V) we investigated whether expression of hLf in the bovine mammary gland would increase the resistance of dairy cows to *E. coli* mastitis. Our study was the first describing an experimentally induced mastitis model using rhLf-transgenic dairy cows. The high concentration of
Lf in the milk of the transgenic cows did not protect the udder from *E. coli* intramammary infection, and all of the cows became infected. No differences were noted in the bacterial growth, times to bacterial elimination, local signs, peak concentrations of LPS in the milk, or any of the milk or blood inflammatory parameters, except in concentrations of Hp and cortisol levels in the serum. However, the systemic signs were significantly milder in the transgenic group.

Recombinant hLf present in milk of transgenic animals is structurally and functionally similar to natural hLf (Nuijens et al., 1993), but despite sequence homology, differences in their activity may exist, as has been shown for bovine and human lactoferricin (Farnaud et al., 2004). The human Lf gene is under the control of bovine αS1-casein promoter (Brink et al., 2000). During intramammary infection αS1-casein decreases in the milk, but the expression of casein does not change significantly (Schmitz et al., 2004), neither does the expression of rhLf. The regulation of bLf differs from that of the other milk proteins, such as casein (Schanbacher et al., 1993). The concentration of bLf in milk is related to the number of neutrophils in milk because bLf is released from the specific granules of neutrophils (Hagiwara et al., 2003). In mastitic milk, the differences were found in antibacterial activities of bLf compared with healthy milk (Komine et al., 2005). In our study (study V), the level of rhLf was relatively constant during intramammary infection, which suggests that rhLf may be largely independent of the regulation of bLf during the inflammation.

Kutila et al. (2003) found that *E. coli* isolates, including our experimental strain, were inhibited by Lf at concentration of 1.67 mg/ml *in vitro* and the bacterial killing effect occurred at a relatively high initial concentration of bacteria (5000 cfu/ml). In our study (study V), bacterial counts in the milk of the groups did not differ significantly, which could mean that the level of Lf in milk was insufficient for a bacteriostatic effect, despite the susceptibility of the *E. coli* strain to Lf. The concentration of Lf mRNA in the cisternal region and in ducts near the teat is higher than in the epithelial ducts of the mammary parenchyma and location may affect the prophylactic capacity of Lf against bacterial invasion via the teat (Molenaar et al., 1996).

Part of the immunomodulatory function of Lf is to bind LPS and down-regulate LPS-induced cytokines (Legrand et al., 2004). Lf is one of the proteins with high-affinity LPS binding, but it does not completely neutralize LPS activity because lipid A can remain active even after a Lf-LPS-complex has been formed (Na et al., 2004). Our finding of reduced clinical signs for transgenic animals may indicate that elevated Lf inhibited the activity of LPS to some extent, but not totally. Lf is also believed to act like LPS-binding protein during the inflammatory activation of
macrophages (Na et al., 2004) and may compete with LPS-binding protein (LBP) for binding to LPS (Legrand et al., 2005). The LPS-neutralizing activity of Lf may depend on the presence and concentration of other LPS-binding proteins.

In studies of experimentally induced LPS and *E. coli* mastitis, the increase in concentrations of cortisol in the blood was temporary in cows with mild and moderate responses (Hirvonen et al., 1999; Jackson et al., 1990), but remained elevated in cows with the most severe reaction (Hirvonen et al., 1999). In our study, concentration of cortisol in serum increased earlier and remained high for a longer time in the control cows than in the transgenic cows, indicating that the controls developed a stronger inflammatory reaction.

The lower concentrations of acute phase proteins, SAA and Hp, in the transgenic cows indicated a milder response to infection. SAA and Hp peaked earlier in the milk than in the serum in both groups, which probably resulted from the rapid local production of these proteins in the mammary gland as described before (Hiss et al., 2004), and as recorded in study I. Haptoglobin may complement the actions of Lf (Hiss et al., 2004). It helps restrict the infection by limiting the free iron available for *E. coli* bacteria, which is also a function of Lf, by binding harmful molecules such as hemoglobin produced after tissue damage (Baumann et al., 1994).

The rise of serum TNF-α reflects the severity of the systemic signs (Blum et al., 2000; Hirvonen et al., 1999). Concentrations of TNF-α were generally higher in the transgenic cows, but the difference between the groups was not significant. Lf may modulate immune responses by inhibiting cytokine activity and this inhibition has been shown to be concentration dependent (Crouch et al., 1992; Haversen et al., 2002). Blum et al. (2000) reported that peak TNF-α concentrations in plasma were up to 20-fold lower than maximal concentrations reached in milk in acute mastitis induced by *E. coli* infection and endotoxin. In our study, serum TNF-α concentration in the transgenic group was almost 100-fold and in the control group 30-fold lower than maximal concentrations in the milk, probably due the higher Lf concentrations and its LPS-neutralizing effect resulting inhibited TNF-α response in the milk of transgenic cows.

The bovine hLf-transgenic model did not provide protection against *E. coli* mastitis in dairy cows, in contrast to lysostaphin-transgenic cows, which resisted intramammary *S. aureus* infection (Wall et al., 2005). The differences between these two infection models may partly explain the inconsistent results. The inflammatory response of the mammary gland to *E. coli* is characterized by
an acute rapid onset of infection, whereas *S. aureus* tends to cause slow-developing chronic infection (Bannermann et al., 2004). The protective effect of additional Lf in milk could be enhanced under natural conditions, where the infection generally develops more gradually. Lf reduced the severity of the inflammatory reaction, which could be seen in the systemic signs and in the serum cortisol and haptoglobin concentrations. However, Lf may not be a likely candidate for genetic engineering to enhance mastitis resistance of dairy cattle, but increasing the innate immunity of the dairy cows using such techniques cannot be completely dismissed. The ethical aspects of this kind of modification of the genome of the dairy cow need discussion as consumers have concerns regarding food produced via gene manipulation. It is more likely that specific genetic traits linked with mastitis resistance are identified (Griesbeck-Zilch et al., 2009; Alain et al., 2009) and used for breeding dairy cows to enhance their resistance against mastitis in the future.
10. CONCLUSIONS

1. Two consecutive experimental *E. coli* intramammary infections in dairy cows 2 weeks apart caused a significant immunological carry-over effect. The interval between two experimental challenges should be longer than 2 weeks if the same cows are used in crossover trials.

2. Systemic enrofloxacin treatment of acute clinical mastitis caused by *E. coli* was not more efficacious than supportive treatment only.

3. Frequent milking at the acute stage of *E. coli* mastitis was beneficial and may reduce tissue damage in the udder.

4. *E. coli* isolated in mastitis were mainly of commensal phylogeny type and had no specific virulence factors tested here related to clinical signs or persistence of the infection in the mammary gland.

5. *E. coli* isolated from mastitis in southern Finland were susceptible to most antimicrobials tested. The highest prevalences of resistance were found for ampicillin, streptomycin, oxytetracycline and sulfametoxazol.

6. Lactoferrin administered via the intramammary route was less efficacious than systemic enrofloxacin for treatment of experimental *E. coli* mastitis.

7. hLf-transgenic dairy cows, expressing recombinant human lactoferrin in their milk, were not protected against experimental *E. coli* intramammary infection, but their response was milder as compared with that of normal cows.
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