IDENTIFICATION AND CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES IN RESPONSE TO *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS* IN BOVINE MAMMARY EPITHELIAL CELLS AND MAMMARY GLAND

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October 2005

A Thesis submitted to McGill University in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Your file Votre référence
ISBN: 978-0-494-24786-0
Our file Notre référence
ISBN: 978-0-494-24786-0

ABSTRACT

Bovine mammary glands respond to infection by foreign pathogens such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) through changes in gene expression. Monitoring the gene expression profiles will contribute to better understanding of the pathology of mastitis, and provide important selective markers for future animal breeding programs. Using cultured bovine mammary duct epithelial cells and somatic cells from infected bovine mammary glands, this study first examined the existence of Toll Like Receptors in these two systems. In cultured duct epithelial cells stimulated with *E. coli* LPS, both TLR 4 and 2 mRNA up regulation was detected at 2h-72h and 12h-48h respectively. For *S. aureus* LTA TLR 2 mRNA was up regulated at 48 and 72h whereas for TLR 4 mRNA expression up regulation was detected at 24, 48, and 72h in comparison to the 0h ($p<0.05$). In the case of PGN, an abundant structural component of *S. aureus*, the expression of TLR 2 mRNA was significant ($p<0.05$) at 72h whereas TLR 4 mRNA expression increased at 24, 48, and 72h. The expression of these receptors was also monitored in milk cells from cows infected with either *E. coli* or *S. aureus*. However, results obtained from the milk cells were inconclusive due to the high individual variability. Afterwards, differential gene expression profiles were monitored by the Differential Display Polymerase Chain Reaction technique in the cultured duct epithelial cells in response to *E. coli* and *S. aureus* structural components. A total of 6 candidate fragments were identified for *E. coli* LPS induction, whereas only one fragment was identified for *S. aureus* LTA induction. After LTA induction, a specific band was found to be up regulated and confirmed to be GCP-2, a chemokine involved in neutrophil recruitment. In contrast, PGN induction resulted in no change in GCP-2 levels. In different preparations of cultured duct epithelial cells both GCP-2 and IL-8 were confirmed by real time PCR to be up regulated by LTA with a significance of ($p<0.01$) when compared to the control cells. In the case of the *E. coli* identified bands, a different approach is necessary to potentially confirm the origin of these fragments. Further large scale screening of the GCP-2 and IL-8 genes in dairy cattle is necessary to test for their potential use as targets to differentiate the mastitis resistant from the mastitis prone cows.

RÉSUMÉ

Chez le bovin, les réponses aux infections par les pathogènes étrangers tels que *Escherichia coli* (*E. coli*) et *Staphylococcus aureus* (*S. aureus*) dans la glande mammaire se traduisent par des changements au niveau génétique. L'identification des profils d'expression génétiques est donc critique, d'une part pour mieux comprendre les bases du développement de la pathologie de la mammite, mais aussi pour l'identification de marqueurs sélectifs qui seront utiles pour développer de futurs programmes d'élevage bovin. Grâce à l'utilisation de cellules épithéliales, issues des tissus qui forment les conduits mammaires en culture *in vitro*, ainsi qu'a l'emploi de cellules somatic de glandes mammaires infectées, nous avons pu déterminer la présence et quantifier les taux d'expression des « Toll Like Receptors (TLR) » dans la glande mammaire. Dans les cellules épithéliales en culture, stimulées avec *E. coli* LPS, l'expression du RNA messager TLR 4 et 2 est augmentée à 2h-72h et 12h-48h respectivement par rapport au contrôle. De la même façon, l'expression du RNA messager TLR 2 est augmentée en réponse au *S. aureus* LTA, à 48h et 72h et pour TLR 4 il est augmentée à 24-72h par rapport au contrôle. Dans le cas du PGN, un autre composant abondant de *S. aureus*, le RNA messager de TLR 2 est augmentée à 72h et pour TLR 4 il est detecté à 24, 48, and 72h par rapport au contrôle. L'expression de ces récepteurs a aussi été étudiée dans les cellules somatic obtenus de glande mammaire infectée avec *E. coli* ou *S. aureus* mais n'ont pu donner de résultats concluants, probablement du au fait de la grande variabilité existant entre les differents animaux utilisés pour ces expériences. Par la suite, les profils des gènes différemment exprimés en réponse aux infections causées par *E. coli* ou *S. aureus* ont été caractérisés par la technique de « Differential Display Polymerase Chain Reaction » dans les cellules épithéliales en culture. Un total de 6 fragments potentiels a été identifié pour l'induction par *E. coli* LPS, alors qu'un seul fragment a été identifié pour l'induction par *S. aureus* LTA. La caractérisation de ce gène a révélé qu'il s'agissait de GCP-2, une chemokine impliquée dans le recrutement des neutrophiles. A l'inverse l'induction par PGN n'induit aucun changement d'expression de GCP-2. Une augmentation d'expression de GCP-2 et IL-8 en réponse à LTA et PGN a été confirmée par PCR en temps réel dans quatre échantillons de cellules issues de tissus qui forment les

conduits mammaires avec une différence significative de ($p< 0.01$). En ce qui concerne les différentes bandes identifiées pour *E. coli* il a été nécessaire d'utiliser une méthode différente pour déterminer la nature des fragments identifiés. Ces résultats sont seulement préliminaires et une analyse par criblage de ces gènes dans différentes vaches laitières sera nécessaire pour quantifier leurs niveaux expression chez les animaux enclins ou bien résistants a la mammite.

ACKNOWLEDGEMENT

I would like to express my deepest appreciation to those who generously provided their supervision and assistance. They were:

Dr. Xin Zhao, my thesis supervisor.

Dr. Dr. F. Hayes and Dr. Mustafa, my committee members.

Dr. A. Guidry, for kindly providing us with the bovine duct epithelial cell line

All the other professors (especially Dr. R. Beech), staff and students who have contributed to the completion of my thesis.

Finally, I thank my parents, brother, friends and my fiancé J. Monty for always being there by giving me the strength and encouragement needed throughout the duration of my study.

CONTRIBUTIONS TO KNOWLEDGE

1. Chapter II: This study demonstrated that bovine mammary duct epithelial cells do express TLR 4 and 2 in response to *E. coli* LPS and *S. aureus* LTA and PGN.

2. Chapter III: This study uses a molecular approach to study gene expression profiles in Bovine Mastitis. The Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT-PCR) molecular approach has allowed us to identify potential genes under *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) induced infections. Six possible genes for *E. coli* induced infections were demonstrated but unidentified whereas GCP-2(CXCL-6) a chemokine involved with neutrophil localization was identified in *S. aureus* induced infections.

FORMAT OF THESIS

This thesis has been written in the format of manuscripts submitted to scientific journals in accordance with the "**Guidelines for Thesis Preparation, C: Manuscript-based Thesis**", which states,

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

1. *a table of contents;*
2. *a brief abstract in both English and French;*
3. *an introduction which clearly states the rational and objectives of the research;*
4. *a comprehensive review of the literature (in addition to that covered in the introduction to each paper);*
5. *a final conclusion and summary;*
6. *a thorough bibliography;*

7. *Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.*

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

CONTRIBUTION OF AUTHORS

Dr. Zhao, my supervisor, is the co-author on two manuscripts (Chapter II and III) that will be submitted. Dr. Zhao contributed in a supervisory role in this research and he reviewed the manuscripts.

Authors of manuscript 1 (Chapter II): Mélanie Roy, Jai-Wei Lee, Douglas D. Bannerman, Max J. Paape, Ming-Kuei Huang and Xin Zhao.

All experiments and data analysis were carried out by Mélanie Roy. Drs. Lee, Bannerman, Paape, and Mrs. Huang contributed to part of the work described in Chapter II by providing technical support in the experimental design.

Authors of manuscript 2 (Chapter III): Mélanie Roy, Ming-Kuei Huang and Xin Zhao.

All experiments and data analysis were carried out by Mélanie Roy. Mrs. Huang contributed to part of the work described in Chapter III by providing technical support in the experimental design.

TABLE OF CONTENT

LIST OF ABREVIATION

BLAST.....Basic Local Alignment Search Tool
bp.....Base Pair
CD.....cluster of differentiation
cDNA.....copy Deoxyribonucleic Acid
cfu.....colony forming unit
CP.....capsular protein
Cy5 & Cy3.....Cytosine 5 Dye & Cytosine 3 Dye
DDRT-PCR.....Differential Display Reverse Transcription Polymerase Chain Reaction
DNA.....Deoxyribonucleic Acid
DTT.....Dithiothreitol
E. coli.....*Escherichia coli*
EST.....Expressed Sequence Tag
GCP-2.....Granulocyte Chemotactic Protein-2
Ig.....Immunoglobulin
IKK.....Inhibitory k B kinase
IL-1α & β.....Interleukin 1 alpha & beta
IL-8.....Interleukin 8
IL-6.....Interleukin 6
IRAK.....interleukin 1 receptor associated kinase
IRF.....Iron regulatory factor
LB.....Luria-Bertani
LBP.....Lipopolysaccharide Binding Protein
LPS.....Lipopolysaccharide
LTA.....Lipoteichoic acid
mRNA.....messenger Ribonucleic Acid
NADH.....Reduced form of Nicotinamide Adenine Dinucleotide
NF-κB.....Nuclear Factor kB
OD.....optical density
PAMP.....Pathogen Associated Molecular Patterns
PBS.....Phosphate Buffered Saline
PCR.....Polymerase Chain Reaction
PGN.....Peptidoglycan
PMN.....Polymorphonuclear Neutrophil
PRR.....Pathogen Recognition Receptor
ROS.....Reactive Oxygen Species
RNA.....Ribonucleic Acid
RT.....Reverse Transcriptase
S. aureus.....*Staphylococcus aureus*
SAGE.....Serial Analysis of Gene Expression
SCC.....Somatic Cell Count
SSH.....Supressive substrctive hybridization
Th.....Helper T cell

TIR.....Toll/IL-1 receptor
TLR.....Toll Like Receptor
TNF-α.....Tumor Necrosis Factor -alpha
TRAF..... Tumor Necrosis associated factor
USDA.....United States Department of Agriculture

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

CHAPTER I. GENERAL INTRODUCTION AND REVIEW OF LITERATURE

GENERAL INTRODUCTION

Mastitis continues to be the leading economic problem for the dairy industry. Significant economic losses are associated with this disease. The annual cost due to bovine mastitis for the United States and Canada are estimated to 2 billion and 200 million dollars respectively (Gill et al., 1990). These costs include loss in milk production, veterinarian, labor, culling, and especially the cost associated with the discarding of milk due to clot formation or antibiotic residues. Bovine mastitis is defined as an inflammation of the mammary gland caused by the invasion of pathogenic microorganisms through the teat end openings. There are two categories of bacterial pathogens capable of inducing bovine mastitis: contagious and environmental. Contagious pathogens include microorganisms such as *Staphylococcus aureus* and *Streptococcus dysgalactiae*, which have the ability to spread from cow to cow via milking machines, milkers' hands, farm flies and are known to produce sub-clinical cases of infection with a high reappearance rate. It is very difficult to completely eradicate contagious pathogens because of their speculated abilities to evade the host's defenses by residing within epithelial cell linings and inside macrophages. On the other hand, environmental pathogens such as *Escherichia coli* (*E. coli*), *Klebsiella,* and *Enterobacter* are present ubiquitously in the environment, especially in areas where humidity and nutrients provide optimal growth conditions (i.e. humid beddings and feces). Among all the cases of mastitis, approximately 95% are ascribed to contagious Gram-positive infections, and infections induced by environmental bacteria are responsible for less than 5%. However, the number of clinical cases diagnosed due to coliforms such as *E. coli* accounts for 24-40% of the total clinical cases, and continues to increase significantly (Eberhart, 1977; Lam et al, 1997; Paape et al., 1996). Numerous attempts have been carried out to eradicate mastitis-causing pathogens by means of hygienic management procedures, teat dipping, dry cow therapies, antibiotic treatments and vaccination. As a consequence, the incidence of contagious mastitis has been dramatically decreased. Under these circumstances, environmental pathogens have a better opportunity to inhabit in the mammary gland (Blowey and Edmondson, 1995; Burvenich et al., 2003).

The ability of the mammary gland to mount an effective pattern of immunity in response to invading pathogens is crucial to the development of mastitis. Therefore, understanding the recognition of innate immunity toward different types of pathogens is fundamental to unveil the mystery of host-pathogen interaction. Accumulating lines of evidence indicate that mammary epithelial cells, in addition to being a protective physical barrier, are involved in the recognition of pathogens (Boudjellab et al., 1998; Boudjellab et al., 2000). It is well documented that in the cases of infections induced by *E. coli*, lipopolysaccharide (LPS), a cell wall component of all Gram-negative bacteria, is the key molecule for initiating an inflammatory response. Cells respond to LPS when it is complexed with LBP (LPS Binding Protein) via the Toll-like Receptor 4 (TLR 4) and activates a signaling cascade downstream, where the expression of specific genes is distinct to this particular infection (Hoshino et al., 1999; Takeda et al., 2003). For infections induced by *S. aureus*, lipoteichoic acid (LTA) and peptidoglycan (PGN) are the principle bacterial components which can elicit the inflammatory responses associated with the infection, and TLR 2 is the putative cellular receptor initiating the signaling cascade (Goldammer et al. 2004; Takeuchi et al., 2000; Takeda et al., 2003). Nevertheless, the involvement of TLRs in bovine mastitis has not been fully characterized. It has been demonstrated that mastitis induced *E. coli* and *S. aureus* has different gene expression profiles (Boudjellab et al., 2000; Goldammer et al. 2004; Shuster et al., 1996). Thus, it is important to investigate if the activation of different TLR receptors (TLR 4 v.s. TLR 2) as well as other genes plays a role in this scenario.

In the first study, the expression of TLR 2 and TLR 4 at the transcriptional level was investigated in milk somatic cells and cultured bovine mammary epithelial cells after the stimulation by one of the three bacterial cell wall components, including LPS, LTA, and PGN. The second study was carried out to identify genes differentially expressed in different types of infections. To that end, cultured bovine ductal epithelial cells were stimulated by individual bacterial cell wall component followed by the application of the Differential Display RT-PCR (DDRT-PCR) technique. The results from these two studies provide novel information on the involvement of TLRs in the recognition of innate immunity toward pathogens and on differentially expressed gene profiles in different types of infections in the bovine mammary gland.

REVIEW OF LITERATURE

1. Bovine Mastitis

1.1. Overview

Mastitis is defined as the inflammation of the mammary gland as a consequence of successful invasion of microorganisms through the teat end openings. Mastitis is one of the most common and costly diseases in the dairy industry. In the United States, the annual cost associated with mastitis exceeds 2 billion USD annually (Eberhart et al., 1987) and when these costs are assigned to a per-cow basis they are consistent with what the Canadian dairy industry losses yearly (Gill et al., 1990). These costs mainly result from: a) decreases in milk production b) milk discarded due to abnormal composition or from antibiotic residues c) veterinary bills d) labour and medications and e) mortality or from the upbringing of heifers to replace the ones to be culled from the herd. Approximately 70-80% of the loss due to mastitis is caused by reduced milk production (Eberhart et al., 1987) from the damaging effects the inflammation posses onto the inner lining of the mammary gland.

Bacterial microorganisms are most often associated with bovine mastitis and are classified in two distinct categories: contagious and environmental. The contagious microorganisms, including *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* (*S. agalactiae*) and *Streptococcus dysgalactiae* (*S. dysgalactiae*) are spread from cow to cow (Radostit et al., 1994) via contaminated milking apparatuses, milkers' hands, flies in the stable, including the cows' own body parts. Cases of *S. aureus* infections are most often reported amongst all other pathogens present in its category, which may be due to the fact that *S. aureus* has the ability to evade the host's defenses by hiding inside cells (Hensen et al 2000). On the other hand, *Escherichia coli* (*E. coli*), *Klebsiella*, *Enterobacter*, and *Streptococcus uberis* (*S. uberis*) constitute the major environmental pathogens existing in the cows' environment (Blowey and Edmondson, 1995). The environment in which the cow resides is optimally advantageous for environmental pathogens such as *E. coli* to populate. Temperature, humidity, and nutrient availability in the animal's feces provide these bacteria with the conditions for optimal growth. *E. coli* and *S. uberis* remain the most

virulent environmental pathogens at the present time (Bradley, 2002). Together these bacteria constitute approximately 90% of the isolates in sub-clinical diagnosis and approximately 84% in clinical diagnosis (Sandgreen, 1991).

There are three characterized types of mastitis in terms of levels of severity; clinical, sub-clinical, and chronic. Clinical mastitis is diagnosed when the production of abnormal milk becomes apparent with swelling, heat, redness, and pain of the infected quarter. Clinical mastitis can develop into more acute forms with signs of loss in appetite and weight, shivering, hyperthermia, anorexia, depression (Gruet et al., 2001) and death in severe of cases. Sub-clinical mastitis generally has no visible signs but milk production falls, and the bacteria can be detected in the milk with slight milk composition changes (Shoshani et al., 2000; Nickerson, 1993). The speculated reason for which *S. aureus* is responsible for most of the sub-clinical cases of mastitis lies in its ability to evade the hosts innate immune system by remaining undetected within the epithelial cell lining and/or inside macrophages (Gruet et al., 2001). Sub-clinical mastitis can be found in 50-70% of dairy herds, and usually departs within the following 4-6 weeks without intervention. For chronic mastitis, an inflammation exists for months and may continue from one lactation to the next. Usually chronic mastitis exists as sub-clinical forms and is most often caused by *S. aureus* that continues to induce an inflammation over time. In order to prevent spreading of chronic mastitis, it is recommended that these cows be culled from the herd.

Among all cases of mastitis, a large majority, approximately 95%, were ascribed to contagious Gram-positive causing infections, whereas less than 5 % were ascribed to environmental microorganism invasions (Eberhart et al., 1977). However, environmental mastitis-causing pathogens, for example, coliform bacteria, account for 24-40% of total clinical cases (Hogan et al., 1989). In addition, as a result of decreased incidence of contagious mastitis, the percentage of environmental mastitis among all cases has increased significantly in the past decades (Blowey and Edmondson, 1995; Burvenich et al., 2003). *E. coli* is posing a major problem because many of the current preventive measures are more efficient in controlling the spread of contagious pathogens rather than environmental pathogens.

1.2. Factors Influencing the Incidence and Aetiology of Mastitis

The severity of an infection that develops inside the udder of a cow is dependent upon the type of organism (contagious or environmental), the cow's genetic make up, the physiological status in the lactation cycle of dairy cattle, the number of somatic cells present during the infection, as well as the natural defense of the mammary gland. All of these factors play a part in determining the severity of mastitis which is entirely animal dependent.

1.2.1. Genetic Make Up of Cows

The genetic make up of animals makes the susceptibility to mastitis different from one cow to another. In the study of Bramley et al. (1981), large variations on the resistance to mastitis were observed among the animals when cows were challenged with live *E. coli* bacteria. Studies also demonstrate that the physical appearances of the teat end, including the shape (Chrystal et al., 1999) as well as the amount of keratin found in teat canals (Capuco et al., 1992) were associated with susceptibility to mastitis. It is mentioned that genetic associations concerning the severity of mastitis is determined by parameters such as the number of polymorphonuclear neutrophils (PMNs), serum concentration of the Immunoglobulins (IgG and IgA) as well as somatic cell count (SCC). These parameters vary from one cow to another and can influence their resistance to infections (Detilleux, 2002).

1.2.2. Physiological State of Cows

The susceptibility of cows to mastitis can be affected by the season, parity, and physiological stage (early-, late-, and non- lactating) (Mcdonald and Anderson 1981; Gonzalez et al. 1990; Bramley 1976). It has been demonstrated that the two periods that the cows are most susceptible to pathogens during the lactation cycle are *drying off* and *transition period.* At drying off, invading bacteria are not regularly being flushed out by

milking. Therefore, it is much easier for the pathogen to establish an infection inside the mammary gland. Moreover, milk production without milking at drying off results in milk leakage, which facilitates the invasion of bacteria through the teat canal. Recent studies also showed that a high percentage of cows fail to form a protective keratin plug in the streak canal during the early stage of drying off. During the transition period, the immune system is suppressed due to hormone changes at calving with the high energy demands from elevated milk production and reduced feed intake makes the cows more susceptible to intramammary infections (Madsen et al. 2002). In addition, nutritional deficiency may increase the susceptibility of mastitis. It has also been reported that supplementation of Vitamin A (Chew et al., 1985), Vitamin E and selenium (Ndiweni and Finch, 1996) may reduce mastitis incidence and/or severity. Although the exact signalling pathways at the molecular level remain unknown, these nutritional factors may affect the susceptibility of mastitis through their impacts on the immune responses of the animals.

1.2.3. Somatic Cell Count

The somatic cell count (SCC) plays a vital part in protecting the mammary gland against infectious diseases (Bradley, 2002; Kehrli and Shuster, 1994). Under normal conditions, the SCC should be less than 200,000 cells/ml (Miller et al., 1999) containing macrophages (35-79%), neutrophils (3-26%), lymphocytes (10-24%), and some epithelial cells (2-15%) (Lee et al., 1980; Miller et al., 1993). When mastitis occurs, a large number of neutrophils are recruited from the blood stream and migrate via diapedesis into the milk and account for more than 90% of the SCC (Kehrli and Shuster, 1994; Sordillo et al., 1997). The rate at which neutrophils are activated and recruited to the site of infection determines the severity of the incidence. Upon arrival, neutrophils exert their bactericidal functions, mainly through phagocytosis and the respiratory burst, to eliminate invading bacteria (Sordillo et al., 1997). Thus, the number and the activity of neutrophils at the site of infection are well related to the occurrence and severity of mastitis. It has been demonstrated that cows with a higher SCC are less susceptible to infections in comparison with cows with low SCC (Peeler et al. 2000; Schukken et al., 1994 and Schukken et al. 1999). Many studies indicate that impaired neutrophil activity has been associated with

high incidence of intra-mammary infection. For instance, decreased respiratory burst (Piccinini et al., 1999) and acyloxyacyl hydrolase activity (Dosogne et al., 1998) in neutrophils were suggested to increase the susceptibility of the mammary gland to invading pathogens (Dosogne et al., 1999). Weak recruitment of neutrophils was also believed to result in a higher susceptibility of mastitis (Shuster et al., 1996). Older cows usually produce more severe cases of mastitis due to the low recruitment of effector molecules with pronounced extracellular ROS production (Mehrzad et al., 2002; Burvenich et al., 2003). After the rapid influx of neutrophils, macrophages and lymphocytes are recruited into the mammary glands. The role of the macrophage is to actively participate in the removal and replacement of the neutrophils (Paape et al., 2003). Their numbers are much less elevated in comparison to neutrophils during the early stage of an infection.

1.2.4. Bovine Mammary Gland's Natural Defence

Bovine mammary glands are equipped with different defensive systems, including anatomical, soluble, and cellular mechanisms, to overcome invading pathogens (Sordillo et al., 1997). The anatomical system is located in the teat. The sphincter muscle, surrounding the opening of teat end, is involved in maintaining a tight closure between milkings to prevent bacterial invasion. The inner liner of the teat is covered by a layer of keratin, a waxy material produced by the ductal epithelial cells, which is capable of trapping pathogens from moving further into the mammary gland. Capuco et al. (1992) have demonstrated that removing the keratin lining increases the susceptibility of cows to bacteria. If bacteria manage to penetrate the defensive line of anatomical mechanism, they encounter attacks from soluble and cellular defensive systems. Soluble defense is composed of lactoferrin, the complement system, immunoglobulins (IgG, IgA and IgM) and cytokines. These components work synergistically with the cellular defensive system, namely the leukocytes, to neutralize bacterial toxins or to eliminate bacteria. Leukocytes are activated and recruited by cytokines and certain intermediate complex of the complement system (c5a) to kill bacteria. One of the bactericidal functions of leukocytes is phagocytosis, which is dramatically facilitated by the presence of immunoglobulines and the complement system, an immunological process called opsonization (Nickerson, 1993).

In addition, leukocytes are able to release extracellularly a number of degrading enzymes and free radicals, such as OH^-, O_2^-, and HOCl, to eliminate bacteria. However, release of these bactericidal reagents may also result in significant tissue damage to the mammary gland (Grommers et al., 1989; Hill, 1981).

1.3. Controlling Measures in the Eradication of Bovine Mastitis

Many different controlling measures have been introduced such as hygienic management procedures, antibiotic treatments, teat dipping/sealants, vaccination, and intra-mammary devises. However, the cure for bovine mastitis relies heavily on antibiotic administration. The problem with using antibiotics is that the milk may contain residuals and when not administered properly bacteria have the chance to mutate and become resistant to that particular antibiotic (Bradley. 2002). Moreover, in some cases, depending on the type of pathogens, using antibiotics alone is not sufficient to destroy the pathogen. It is the leukocytes of the innate immune system, which will be responsible for eliminating these pathogens inside of the mammary gland. Vaccination, a very innovative idea, designed specifically towards characteristic structural subunits of invading organisms, has been extensively studied in recent years. However its efficiency in field trials has not proven to be satisfactory (Yancey et al., 1999). Most mastitis vaccines fail to elicit long-term immune responses. So far only commercially available vaccines function in protecting against coliforms but in areas with a clean house management system (Yancey et al., 1999; Dosogne et al., 2002). Also the J5 vaccine designed against *E. coli* J5, a mutant strain of *E. coli* with a rough outer surface because it lacks the O antigen and consists only of lipid A and some common core polysaccharides (2-keto-3-deoxyoctonate, 2 heptose and 1 glucose residues) (Burvennish 2002), has been shown to have an effect when the cow is not pregnant, which is often too late because the mastitis infection has already developed, especially for postpartum primiparous cows. The hypothesized reason behind this observation, is that a pregnant cow will preferentially mount a Th2 immune response in order not to harm her calf, even though it is better to mount a Th1 immune response for hyper-responsiveness of polymorphonuclear neutrophil (PMN) leukocytes and macrophages in future bacterial invasions (Dosogne et al., 2002). No vaccine towards *S.*

aureus infections is currently commercially available. Another interesting discovery demonstrating potential in the reduction of mastitis occurrence in mice and cows was lysostaphin, a bactericidal enzyme (Oldham et al 1991, Bramley et al 1990). Kerr et al. (2001) developed lysostaphin-transgenic mice which demonstrated the capability of successfully preventing *S. aureus* infections. Nonetheless, the safety of applying transgenic techniques draws public concern even though it demonstrates great potential in preventing contagious bovine mastitis.

Amongst all the control measures available, the administration of antibiotics is the preferred technique. However, the use of antibiotic is becoming more and more problematic due to the introduction of resistant pathogens in the pool and antibiotic residues in the milk. In order to efficiently control mastitis, a better understanding on how the host and pathogen respond to each other might shed some light on new preventive techniques for the future mastitis control.

2. *Escherichia coli* Mastitis

Amongst all the coliform types of bovine mastitis, *E. coli* is the most prevalent in inducing infections in comparison to other Gram-negative rods such as *Klebsiella* and *Enterobacter* in bovine mammary glands. Coliform bovine mastitis accounts for approximately 24-40% of clinical cases (Eberhart, 1977; Paape et al., 1996) with the infection lasting less then seven days but in severe cases death can result from septic shock. This organism found ubiquitously in the cow's environment is becoming very problematic because of its accessibility to infect udders as well as its increased rates of incidences and in severity (Blowey and Edmondson, 1995; Burvenich et al., 2003). It even has been demonstrated that *E. coli* has the ability to persist after an infection and reappear after several months (Bradely, 2002). Ren et al., (2004) demonstrated that *E. coli* is capable of synthesizing biofilms, a polysaccharide matrix meshwork that contains water channels for the transfer of nutrients and for the removal of wastes, in order to remain undetected thus allowing the bacteria to survive. Biofilms are synthesized early-on in the infection. Upon an invasion *Escherichia coli* first transcribes genes that are involved with biofilm formation

such as type I fimbriae protein (Pratt and Kolter 1998). There is no evidence as to whether or not *E. coli* produce biofilms in milk, but the composition of milk contains plausible subunits such as intracellular matrix proteins, fibronectins, casein molecules which can be utilized to form such a complex structure to persist and eventually develop other incidences in the future. Biofilms can be an alternative to its persistence in the mammary gland since *E. coli* demonstrates no feasible evidence of adhesion or attachment to epithelial cell linings (Bramley et al., 1991). The turnover rate of *E. coli* is very quick and infections get noticed quickly when the bacterium is well established by the vital signs of the udder (redness, swelling, heat). During their multiplication, destruction and lysis, *E. coli* release an endotoxin called lipopolysaccharide (LPS) which induces the activation of defense mechanisms as well as being a toxic compound to the mammary gland epithelial cells. LPS not only causes the activation of neutrophils but also has been reported to induce upregulation of IL-1 and subsequently IL-8 genes in MAC-T cells, a non-differentiated bovine mammary epithelial cell line (Boudjellab et al., 2000). This provides evidence that epithelial cells do interact with LPS somehow to initiate an inflammatory response by expressing and secreting IL-1 and 8 cytokines which are involved with immune cell recruitment. This response is expected during an infection but the need to identify other potential candidate genes in relation to *E. coli* mastitis is crucial to develop alternative routes in the eradication and dimishments in the severity of the infection.

2.1. Host response towards *Escherichia coli* versus Lipopolysaccharide (LPS)

Bovine responds to the introduction of *E. coli* and LPS in the mammary gland by releasing cytokines such as IL-1, IL-6, and IL-8 (Shuster et al., 1996), platelet activating factors (Pearsonet al., 1993), prostaglandins (Burvenich et Peeters, 1982; Peter et al., 1990), activated compliment C5 (C5a; Shuster et al., 1997) and Nitric Oxide (Blum et al., 2000; Bouchard et al., 1999). Cow factors play a very important role in terms of the severity observed during *E. coli* infections. The level of TNF- α in the mammary gland is one of the parameters used to determine the severity of the infection (Blum et al., 2000; Hoeben et al., 1999) which is significant to *E. coli* and not *S. aureus* induced mastitis (Riollet et al., 2000). During LPS intramammary infections, the level of TNF-α present in the mammary

gland is very similar to the amount observed from naturally occurring *E. coli* incidences (Hakogi et al., 1989). LPS has also been demonstrated to elicit clinical symptoms similar to *E. coli* infections in cows during an intramammary infection study (Paape et al, 1974). All these findings demonstrate the potency of LPS as a mastitis inducing compound. LPS is more potent in mounting a quick and acute response within a shorter time span than *E. coli*. *E. coli* needs to proliferate in large enough numbers before any response can be mounted and thus it is slower in the beginning but once the response is mounted it usually lasts longer. *E. coli* infections usually results in more elevated peaks of TNF- α in comparison to LPS (Blum et al., 2000; Hoeben et al., 1999), which can be the result of other virulent factors *E. coli* secretes to increase its severity. *E. coli* infections usually last from 7-10 days whereas the effect of LPS infusion is much shorter due to host compliment activation and internalization (Burvenich et al., 2003). One advantage to use the LPS molecule is that one can induce the mammary gland with a known concentration whereas in *E. coli* infections the level of LPS is unknown unless a milk sample is obtained and analysed for its concentration (Hartman et al., 1976; Ziv et al., 1976). Although both *E. coli* and LPS are capable of initiating an inflammatory response, the method of choice to study the infection is entirely dependent on the situation or the nature of study at stake in order to mimic the best possible scenario.

2.2. Host defence to Coliform Mastitis

The faster *E. coli* is recognised by the host's immune system the faster it will be for the host to destroy the bacteria. Once established into the mammary gland an *E. coli* population doubles every 20 minutes, so the longer it takes to elicit a response to recruit the effector molecules including the neutrophils, the greater the acuteness of the infection will be. Kehrli and Harp (2001) monitored an 8-fold increase in the number of bacteria when the neutrophil recruitment was delayed by 1 hour. The bovine mammary gland is equipped with a quick immune response supported by the lymph organ situated on the very inner top of the mammary gland. Even though immune cells are at proximity, the mammary gland can still succumb to infections. The rate in which immune cells migrate to the site of infection is dependent upon cow factors such as age, lactation stage, period of parturition,

and genetic associated resistance (Burvenich et al., 2003). Opsonization of the *E. coli* by the neutrophils is crucial to eradicate the infection and this can not occur until they are activated by the release of cytokines such as IL-1, IL-6, and IL-8. Inside the mammary gland there is constant surveillance by a basal level of SCC which belong to the innate immune system, the host's primary line of defence. In addition, the epithelial cells are also involved in signalling the presence of intruders. Unfortunately, these mechanisms are much more established and studied in the leukocytes than they are in the epithelial cells (Boudjellab et al., 2000). The need to investigate the exact role of the epithelial cells in the mammary gland in terms of their input can be useful to understanding of the interaction between the host and the pathogen. The epidemiology of bovine mastitis is more due to cow factors rather than the pathogenecity of the pathogen.

3. *Staphylococcus aureus* Mastitis

Staphylococcus aureus, a contagious Gram positive coccus, has been the most predominant contagious pathogen of bovine mastitis with a characteristic pathogenicity, and poses serious problems to the dairy industry as well as drawing public concerns. It has been estimated that 19 to 40% of cows are infected with this organism and that infected cows produce less milk as compared to non-infected cows (Natzke et al., 1972). The majority of the *S. aureus*-caused mastitis infections are subclinical with increased SCC, and result in approximately 35% of the economic losses (Fox and Hancock, 1989). *S. aureus* is a contagious pathogen present on or in infected udders and transmits usually from cow to cow during the milking (Bramley and Dodd, 1984). Even with administration of antibiotics the pathogens continue to reappear. The reason for this is the ability of *S. aureus* to evade the immune system and antibiotic treatments by hiding out in the epithelial cell linings as well as macrophages (Almeida et al., 1996; Hébert et al., 2000). Cases of antibiotic resistant *S. aureus* have been identified in addition to the incapability to completely eradicate the presence of *S. aureus* in infected cattle. *S. aureus* directly damages the mammary gland when entering the teat end openings by attaching to and multiplying on the epithelial cells at the early stage of infection (Gudding et al., 1984; Heald, 1979; Nickerson, 1993; Sordillo et al., 1989) subsequently releasing toxins which damage the cells

surrounding the pathogen. The inflammation caused by *S. aureus* when it moves up into the duct system and into the alveoli epithelial cells causes these cells to revert to a non-secretory state. During this process, *S. aureus* produce a thin polysaccharide microcapsule and/or pseudocapsule (Cifrian et al., 1994) and other factors such as protein A (Sordillo et al., 1997) that resist phagocytosis of neutrophils and macrophages by blocking the receptors on the bacterial cell required by leukocytes to identify and kill *S. aureus* (Nickerson, 1993). Most importantly, *S. aureus* has a very effective and complex pathogenic strategy that relies on the production of a large number of cell-associated and extracellular proteins, of which various cytolytic toxins and enzymes such as coagulases, proteases, lipases, catalases that act together to destroy cell membranes and to cause the degradation of ductal epithelium, the teat and gland cisterns within the quarters (Jonsson and Wadstrom, 1993; Heald, 1979). The inflammatory response initiated by *S. aureus* involves structural components such as LTA and PGN, as well as haemolytic exotoxins such as α, β, γ and δ. The toxins produce most of the damage to the epithelial cell lining which helps to promote the adherence of the pathogen (Cifrian et al., 1996). Together, all of these microbial components are recognized by the host and create an influx of PMNs to the rescue, usually causing more damage to the epithelial cells once the infection is regulated.

3.1. Host response towards *Staphylococcus aureus* versus PGN and LTA

In the case of *S. aureus* infections it is very difficult to know the exact response the host will mount. This pathogen is relatively complicated in terms of all of the possible methods it possess to evade the immune system. Most often, when the number of SCC in the milk is higher than 6 X 10^5/ml (Postle et al., 1978), *S. aureus* fails to induce mastitis. Two situations can occur; either the pathogen resides in the site of infection to cause an inflammation or remains undetected amongst the epithelial cells and invades the udder at a subsequent time. Even though *S. aureus* induces an infection, most often only sub-clinical cases are developed possibly owning to its ability to produce capsular polysaccharide (CP) and interfering with opsonization and phagocytosis from the neutrophils thus making *S. aureus* more resistant to the host immune system (Barrio et al., 2000; Hensen et al., 2000). As an alternative to using *S. aureus* for experiments, its microbial components LTA and PGN

have been demonstrated to produce various levels of inflammation. LTA is an anchored membrane component by glycolipids, while PGN is an alternating β-linked N-acetylmuramyl and N-acetylglucosaminyl glycan whose residues are cross-linked by short peptides. When LTA or PGN is infused into the mammary gland, a direct immune response is projected for a quicker eradication of the foreign components. LTA and PGN have also been demonstrated to be internalised by either immune or epithelial cells (Goldammer et al. 2004; Yao et al. 1995), which depletes the amount of components available to induce the inflammation. On the other hand, when *S. aureus* is used for inducing the inflammation, there is a constant excretion of LTA and PGN. Thus, *S. aureus* induces a distinct nature of inflammation unlike LTA and PGN. Once again depending on the type of study, both *S. aureus* and its components LTA and PGN have been used in experiments.

3.2. Host defense to Contagious Mastitis

The immune system is equipped to recognize the presence of pathogens, but in the case of *S. aureus* this task becomes daunting. Its distinct characteristics which include capsule formation it produces around itself to protect itself from getting phagocytosed (Cifrian et al., 1994) as well as its capability to evading the innate immune cells and antibiotic treatments by digesting the epithelial lining with enzymes it secretes and hiding out within these cells (Almeida et al., 1996; Hébert et al., 2000; Jonsson and Wadstrom, 1993; Heald, 1979). *S. aureus* has the potential to generate resistant strains to the antibiotic through mutations of its genome. In cases of *S. aureus* infections the quicker the response it initiates, the better chance the immune system will have for eradicating the pathogen. Unfortunately, upon first encounters, the immune system is slower in mounting a response as compared to a subsequent infection. Even though antibiotics such as penicillin are administered to control the manifestation, *S. aureus* hides out so it can reappear and infect the udder at a subsequent time. The only method demonstrated to be most effective is to cull the cows infected with *S. aureus*, which is costly for the farmer. A large amount of labor and expenses are required for the upbringing of replacement heifers. The ability for *S. aureus* to persist within the udder poses great concern because the pathogen continues to

induce inflammatory responses in the subclinical form throughout its presence inside the udder, thus making it a chronic infection.

4. Toll-Like Receptors

The immune system is composed of innate and adaptive immune responses that differ in their cell types and time of onset. The innate immune system serves as the primary line of defence in mammals in any type of invasion because of its capacity to recognize a broad spectrum of pathogens by using a repertoire of invariant receptors. Macrophage and Polymorponuclear neutrophils (PMNs) are capable of recognizing self from non-self via germ line-encoded receptors referred to as pattern-recognition receptors (PRRs) or Toll Like Receptors (TLRs). TLRs are transmembrane proteins characterized by an extracellular leucine-rich domain and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain. The identification of IL-1R receptors in humans with possible functional similarities to the Toll receptor demonstrated that through evolution these receptors were conserved for their ability to respond and initiate inflammatory and immune responses. TLRs are predominantly expressed in tissues involving immune functions, such as spleen and peripheral blood leukocytes, as well as on cells exposed to the external environment such as lung, gastrointestinal tract and the mammary glands (Zarember and Godowski, 2002; Goldammer et al. 2004). Until now, ten human and nine murine TLRs have been characterized, mapped to their appropriate chromosomal locations, including the identification of their inducing ligands (Underhill and Ozinsky, 2002; Takeda, 2003). TLRs become activated upon recognition of highly conserved structural motifs specifically expressed by microbial pathogens, so called pathogen-associated microbial patterns (PAMPs). These PAMPs include various bacterial cell wall components such as LPS, peptidoglycans and lipopeptides, as well as flagellin, bacterial DNA and viral double-stranded RNA. Stimulation of TLRs by PAMPs initiates a signalling cascade that involves a number of proteins, such as MyD88 and IRAK in the activation of the NF-κB transcription factor to upregulate cytokine mRNA expression in order to secrete pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α. Amongst the different TLRs, TLR 4 and TLR 2 have been demonstrated to be specific in recognition of Gram negative

and Gram-positive bacteria respectively through various mouse models (Hoshino et al., 1999; Takeuchi et al., 2000). LPS, the structural component of Gram-negative bacteria (*E. coli*), is recognized by the TLR 4 whereas TLR 2 has been demonstrated to recognize bacterial lipoproteins, peptidoglycan, and lipoteichoic acids (Deininger et al., 2003), which are mainly constituents of Gram-positive bacteria such as *Staphylococcus aureus* (Takeuchi and Akira, 2002).

4.1. TLR 4 Signalling Pathway

When it comes to *E. coli* infections, LPS is responsible for initiating the inflammatory response. LPS is released when *E. coli* is being phagocytosed by leukocytes such as neutrophils, and macrophages or during bacterial cell division. The LPS molecule is not only recognized by the first line of defence innate immune cells but evidence shows that the epithelial cells are also involved in its recognition (Boudjellab et al. 1998). The ability of LPS to induce an acute response of mastitis in cows results from subsequent activation of numerous endogenous inflammatory mediators, which increases in severity as the magnitude of bacterial growth increases (Burvenich et al., 2003). LPS is recognized by the Toll Like Receptor 4 and activates gene expression by the NF-kB transcription activator (Kawai et al. 2001). This conclusion was drawn from experimental analyses performed on mice. When the TLR 4 gene was mutated in C3H/HeJ and C57BL/10ScCr mice, both mutated mice became low responders to LPS (Hoshino et al., 1999; Poltorak et al., 1998). However, TLR 4 alone is not sufficient to confer LPS responsiveness. TLR 4 requires MD-2, a secreted molecule, to functionally interact with LPS (Nagai et al., 2002). Furthermore, a third protein, CD14, was shown to participate in LPS signalling (Qureshi et al., 1999). TLR 4 activates both MyD88-dependent, which directly activates NF-kB, and MyD88-independent pathways. The MyD88-independent pathway involves the (TIR) domain-containing adapter protein (TIRAP) (Horng et al., 2001). Conventional signalling mediated through the adaptor protein MyD88 demonstrated below involves the activation amongst various proteins (Complex LPS CD14 MD-2 to the TLR 4 receptor → MyD88 → IRAK→ TRAF6→ TAK1/ NIK/MKK→ IKK complex→ NF-κB→ Target genes). The transcription factor NF-κB has been characterized in the transcription of genes involved primarily with

immune specificities (see Fig 1).

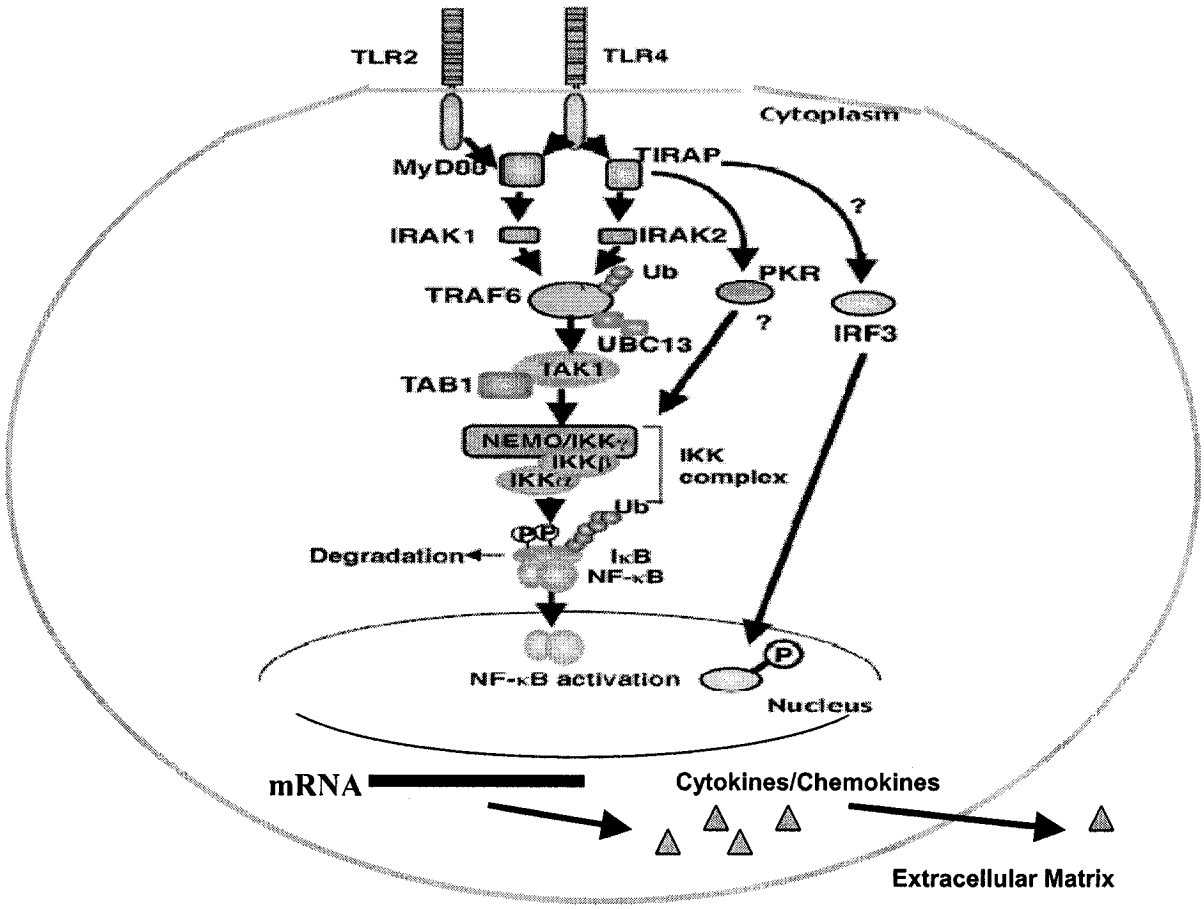

Fig. 1. Overview of TLR 2 and 4 signaling pathways. The presence of bacterial components triggers the sequential activation of a signaling cascade leading to the nuclear translocation of NF-κB. LPS activates both MyD88- dependent and -independent pathway. The MyD88-independent pathway activates TIRAP/MAL and induces nuclear translocation of IRF-3. Adapted from Takeuchi and Akira (2002) with modifications.

4.2. Toll- Like Receptor 2

In the presence of *S. aureus,* TLR 2 is activated (Takeda et al., 2003). The expression of TLR 2 mRNA has been observed in certain tissues such as lung, spleen, and peripheral

blood leukocytes which is consistent with the location and function of these tissues (Zarember and Godowski 2002). On the other hand, TLR 2 knock out mice are unresponsive to the pathogen and its structural subunits (Takeuchi et al. 2000). Wang et al. (2003) demonstrated that LTA stimulated the expression of various genes via the NF-κB transcription factor in human lung airway epithelial cells. It is plausible that the mammary gland epithelial cells would respond to LTA in a similar manner. In the case of PGN, it is believed that other TLRs are involved in its recognition, where TLR6 functions with TLR 2 (Ozinsky et al. 2000; Wyllie et al. 2000). The TLR 2 signalling pathway initiates the activation of the MyD88 protein as well which subsequently activates the NF-κB transcription factor (Takeuchi and Akira, 2002) (Figure. 1). NF-kB is well known for initiating the expression of cytokine related genes.

4.3. Differential Gene Expression Profiles Stimulated by *Escherichia coli*, Lipopolysaccharide (LPS), *Staphylococcus aureus*, PGN and LTA in the Bovine Mammary Gland

The teats of the mammary gland provide an opening for infection which most often occurs due to *E. coli* and *S. aureus* invasions. In cattle, the mammary gland possesses multiple barriers to discourage the propagation of these pathogens but unfortunately, they sometimes still manage to surmount these barriers. After successful bypass of the teat's initial barrier the bacteria are free to move into the duct cistern and cause the most damage. The recognition of these bacteria either via the innate immune system or by the epithelial cells, initiates an inflammatory response. Inflammations are well know to involve the innate immune system but it has also been shown that either epithelial or endothelial cells in open systems of the body are involved in the recognition of pathogen and subsequently express genes to provide a reinforcement in recruiting neutrophils. The MAC-T cells, a non differentiated bovine epithelial cell line, has previously been used as an *in vitro* model to study the response of these epithelial cells upon LPS stimulation and have subsequently been shown to express IL-8, a cytokine involved in the recruitment of neutrophils (Boudjellab et al., 2000). This result is interesting but what interests us the most is if other genes get upregulated or downregulated as a consequence of these bacterial invasions.

Both *E. coli* and *S. aureus* pathogens as well as their structural components LPS, LTA and PGN have been used to study their effects on different tissues or cells located at different locations in the body and have been show to have an effect by the genes they expressed (Kumar et al., 2004; Boudjellab et al., 1998; Boudjellab et al., 2000). Upon *E. coli* or LPS stimulation, epithelial cells were reported to express cytokines such as IL-1 α and β, IL-8 and TNF-α . Whereas for *S. aureus* stimulation, similar type cytokines are expressed once again, as well as a β-Defensin 5 gene, an early bactericidal effector molecule (Table 1). Our interest lies in the epithelial cells located within the mammary gland of cattle. Epithelial cells are joined together by tight junctions which allow for cell-to-cell communication without the possibility for intruders to pass through unless there is a lesion. As previously mentioned TLRs allow for the recognition of specific pathogen via their specific PAMPs, thus allowing only the cells possessing these receptors to be involved in the recognition of pathogen. These receptors have already been previously demonstrated to be expressed in epithelial cells (Kumar et al., 2004) but for the mammary epithelial cells their existence needs to be addressed as a possible intracellular signaling mechanism in pathogen recognition.

Immunogenic Components / Tissue Cells	*E. coli*	LPS	*S. aureus*	LTA	PGN
Oral Epithelial	No information	IL-8 after being primed with IFN-γ (Uehara et al., 2002)	No information	IL-8 after being primed with IFN-γ (Uehara et al., 2002)	IL-8 after being primed with IFN-γ (Uehara et al., 2002)
Mammary Epithelial	Bcl-2, Bax and Bcl-x (Long et al., 2001) β-defensin 5 (Goldammer	IL-1 and IL-8 in MAC-T cells (Boudjellab et al., 2000)	β-defensin 1 in women (Jia et al., 2001) β-defensin 5 in cattle (Goldammer et	CXCL-6 (Under study in bovine)	Under study

	et al., 2004)		al., 2004)		
T84 Colonic and HeLa cervical epithelial	IL-8 and TNF- α (Eckmann et al., 1993)	IL-8 (Schuerer-Maly et al., 1994; Eckmann et al., 1993)	No information	No information	No information
Biliary Epithelial	TNF-α Harada et al., 2003)	TNF-α, NFκB (Harada et al., 2003) MUC2 & MUC5AC (Zen et al., 2002)	No information	TNF-α Harada et al., 2003)	No information
Pulmonary Epithelial	No information	IL-6 and IL-8 (Guillot et al., 2004)	Nitric oxide release, prostanoid generation, and IL-8 in alveolar cells (Rose et al., 2002)	Cyclooxygenase-2 (Lin et al., 2001) and hBD2 (Wang et al., 2003)	No information

Table 1. Genes identified under the presence of either *E. coli* and its structural component LPS or *S. aureus* and its structural components LTA and PGN in various tissue.

The majority of the genes identified so far under a bacterial infection of bovine mammary glands relate to the immune system such as cytokines and chemokines which function in the recruitment of additional effector molecules. The identification of these cytokines is necessary in providing information concerning the role of each cytokine during the inflammatory response but it would be even more interesting to identify other genes which also participate in the signalling and proper functioning of the cells during an infection. The identification of the alteration of gene expression profiles in a specific physiological or pathological condition may lead to clarification of the signalling events at the molecular level. In the case of mastitis, exploring the changes in gene expression in response to bacterial infection in the mammary gland may result in understanding the molecular and physiological mechanisms of the pathogenesis of this disease. Consequently, the genes involved in mastitis development and host defense can be identified. This will be

beneficial for the development of new approaches to controlling mastitis control and reducing the damaging effects of mastitis. Identification of the genes, which are differentially expressed, may also help us find genetic marker(s) with physiological significance in screening the cows for mastitis susceptibility. However, changes in a large number of genes, rather than a few individual genes are expected in response to a particular stimulus. Therefore, it is important to seek a powerful approach to identify as many genes as possible, which are differentially expressed in a different physiological condition.

5. Molecular Techniques used to Identify Differentially Expressed Genes

There is a growing interest in identifying the gene expression profiles in body cells such as epithelial cells in response to exposure of pathogens. Until now, cytokine related genes have been identified during infection. In order to be able to identify other genes being expressed in response to pathogens, several molecular techniques have been developed to perform such a task. Four common techniques are Suppressive Subtractive Hybridization (SSH), micro-array, Serial Analysis of Gene Expression (SAGE) and Differential Display-PCR (DD-PCR).

5.1. Suppressive Subtractive Hybridisation Analysis

The method of suppressive subtraction was developed in 1985. This method is now well developed and can easily identify differentially expressed genes between two sample populations (Diatchenko et al., 1999). The idea behind this method involves isolating mRNA from two cell populations of interest. One of the mRNA sample, usually the control or driver, is converted to cDNA (complementary DNA) by Reverse Transcriptase (RT). The single stranded cDNA will be allowed to hybridize in a subtractive manner with the mRNA from the second sample population, which is believed to contain differentially expressed genes (tester). The mRNA that did not hybridize with the driver is believed to be the ones that are differentially expressed. By using this technique, the genes preferentially displayed in human ovaries or testes were identified (Jin et al., 1997). Additionally, the differential expression of ESTs (expressed sequence tags) in estrogen-receptor positive

mammary epithelial cell lines was detected by this method (Kuang et al., 1998). The suppressive subtraction hybridisation assay is an efficient assay in selecting genes with dramatic differences. But when it comes to the subtle or moderately expressed genes this method is somewhat inefficient.

5.2. Micro-array analysis

The micro-array technique demonstrates great potential in identifying subtle gene expression (Cummings and Relman, 2000). The conventional method of labelling one condition with the Cy5 dye and the other with the Cy3 is now being replaced by more resonance controlled dyes (Invitrogen), which can correlate more than one effect. Micro-array can compare the different gene expression patterns among three or more samples. The micro-array technique invented in 1995 has been applied extensively. A 15,000-mouse developmental cDNA micro-array was used to profile the genome-wide expression of mid-gestation placenta and embryo (Tanaka et al., 2000) gene differences. Micro-arrays have also been extensively used in the transcriptional analysis of cancer and other diseases, as well as in the stress response and aging process (Epstein and Butow, 2000). A chip specific to the bovine mammary gland gene library exists (USDA) but the costs associated with the use of this technique pose some questioning towards the cost efficiency of obtaining results. This technique is proficient in identify many genes at one time but is restricted to the identification amongst already known genes, since the micro-array technique only allows detection of the expression levels of the genes or ESTs on the particular chip. Furthermore, the high variation from one chip to the next and complex of statistical analysis to further accept a value have limited its application. Also, subsequent conformation is necessary to confirm the initial results from microarray studies.

5.3. Serial Analysis of Gene Expression (SAGE)

Serial analysis of gene expression (SAGE) was developed in 1995 to allow for a rapid, detailed analysis of thousands of transcripts. Compared to the suppressive subtraction, this

technique provides broader information on gene expression profiles, and it also facilitates comparison between more than two groups of samples (Velculescu et al., 1995). The data obtained from this method are more quantitative as compared to the micro-array. SAGE allows the detection of genes or ESTs with unknown sequences. The frequency of each gene/EST expression can be calculated and expressed as a quantitative result. Nevertheless, SAGE only detects the expression of 10 bp tags, and the PCR amplification and automatic sequencing may result in false sequence data, which might affect the accuracy in calculating the frequencies of gene expression. To our knowledge, the SAGE library has not been established on any farm animal species.

5. 4. Differential Display-PCR (DD-PCR)

The Differential Display technique invented in 1992 by Drs. Arthur Pardee and Peng Liang is considered one of today's methods of choice for identifying differentially expressed genes (Figure 2). This technique allows for rapid, accurate and sensitive detection of altered gene expression between cells of different nature (Liang and Pardee, 1992). The mRNA Differential Display technology works by systematic amplification of the 3' terminal portions of mRNAs with short arbitrary primers (13mers) and anchored oligo dT (AAGC-T_{11}M, M = G, A, C) primers for subsequent analysis of product resolution of the fragments on a DNA sequencing gel. The more arbitrary primers you use the higher the probability of obtaining more differentially expressed genes (Table 2).

# Of Arbitrary Primers n	Reactions	Probability Of Detection $P = 1 - (0.96)^n$
20	60	56%
30	90	71%
40	120	80%
80	240	96%

Table 2. The number of arbitrary 13mers needed in combinations with all three one-base anchored primers to detect a given fraction of mRNA by differential display

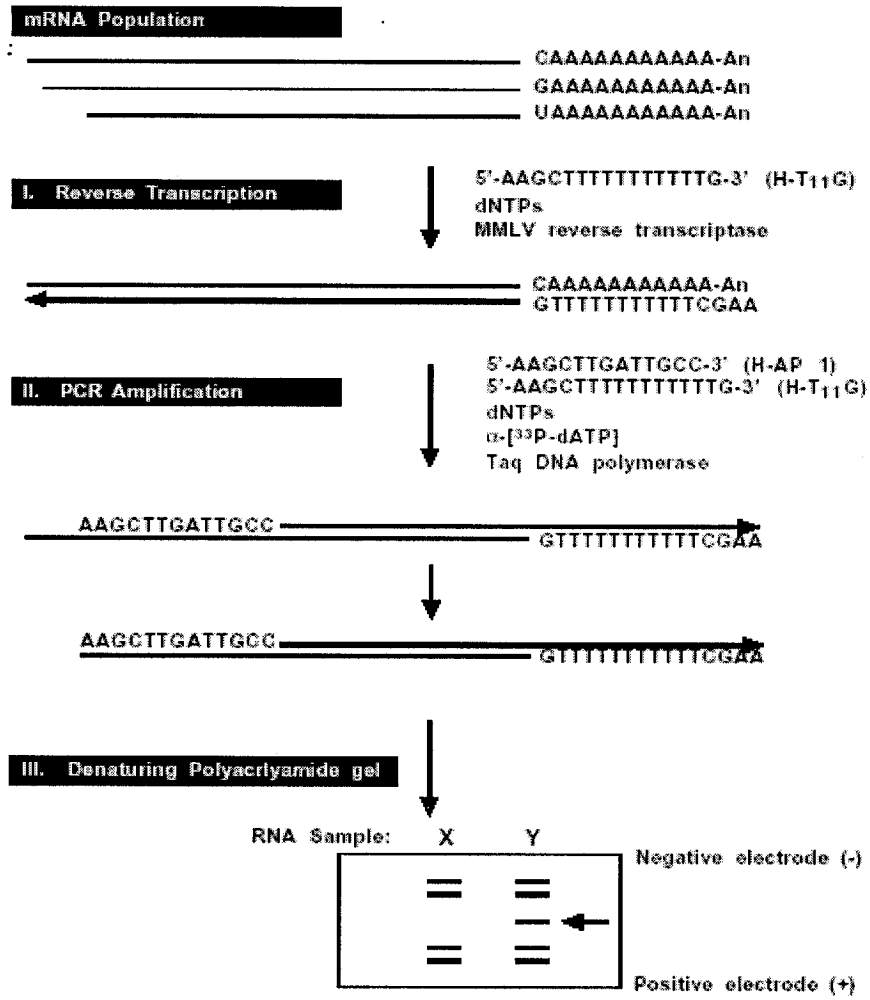

Figure 2- Schematic representation of DD-PCR. **Reference:** www.genhunter.com

By using anchored primers designed to bind to the 5' boundary of the poly-A tails for the reverse transcription, as well as the upstream arbitrary primer, followed by PCR amplification, DD-PCR allows for mRNA sub-populations to be visualized using a denaturing polyacrylamide electrophoresis apparatus. The direct side-by-side comparison of most of the mRNAs between or among related sample populations allows us to detect differentially expressed genes, Because of its simplicity, sensitivity, and reproducibility, the mRNA Differential Display method is finding wide-ranging and rapid applications in

developmental biology, cancer research, neuroscience, pathology, endocrinology, plant physiology, and many other fields.

In summary, using molecular gene identification techniques to examine the gene expression profiles during *S. aureus* and *E. coli* mastitis, will allow for a better understanding of the molecular and physiological mechanisms involved in the pathogenesis of this disease. Even though above-mentioned molecular techniques are capable of identifying differentially expressed genes, further conformational analysis is often required in order to determine its validity. Real-time PCR is the conformational technique most often used because of its efficiency in quantifying and identifying low levels of mRNA expression.

Molecular Technique	Advantages	Disadvantages
SSH	Quick, reproducible, cost efficient.	Difficulties in identifying differences in gene expression for similar cell types
Microarray	Possible to identify gene differences for the whole genome at one time	Costly, large variation, need to perform extensive statistical analysis on the results. Can only identify differences amongst known genes.
SAGE	Provides broader information on gene expression profiles, get quantitative values, facilitates comparison among more than two groups of samples	Time consuming, accuracy may be biased due to the 10bp tags produced
DD-PCR	Quick, cheap, reproducible, accurate, sensitive, technique preferred by researchers	Possibility of false positive identification, easily biased by external factors intrinsic or extrinsic in nature, need to be very careful and pay attention to detail

Table 3. Advantages and disadvantages of Molecular Tools available for identifying differentially expressed genes.

6. Hypothesis and Objectives of project

Our hypothesis for this study was that the bovine milk somatic cells and the mammary epithelial cells express various genes when challenged by *Escherichia coli* and *Staphylococcus aureus* pathogens or their structural components LPS, LTA, and PGN.

The primary objective of this study was to identify differentially expressed genes in bovine mammary glands when stimulated with *Escherichia coli, Staphylococcus aureus* and their microbial immunogenic components Lipopolysaccharide (LPS), Lipoteichoic Acid (LTA) and Peptidoglycan (PGN). Specifically, the expression of the respective Toll Like Receptors 4 and 2 under *Escherichia coli* and *Staphylococcus aureus* infections was first identified in the milk somatic cells and in cultured mammary duct epithelial cells. Then, bovine mammary duct epithelial primary cell line were stimulated with LPS, LTA

and PGN and alteration of gene expression in these cells were identified by the Differential Display PCR and confirmed by real-time PCR.

REFERENCES

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

Barrio B., Vangroenweghe F., Dosogne H., Burvenich C. 2000. Decreased neutrophil bactericidal activity during phagocytosis of slime-producing Staphylococcus aureus strain. Vet. Res. 31: 603-609.

Blowey R, Edmondson P. 1995. Mastitis - causes, epidemiology and control. In: Mastitis control in dairy herds: an illustrated and practical guide. Farming Press, Ipswich, UK. Pp27-45.

Blum J.W., Dosogne H., Hoeben D., Vangroenweghe F., Hammon H.M., Bruckmaier R.M., Burvenich C. 2000. Tumor necrosis factor-alpha and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows, Domest. Anim. Endocrinol. 19: 223–235.

Bouchard L., Blais S., Desrosiers C., Zhao X., Lacasse P. 1999. Nitric oxide production during endotoxin-induced mastitis in the cow, J. Dairy Sci. 82: 2574–2581.

Boudjellab, N., H.S. Chan-Tang, X. Li and X. Zhao. 1998. Bovine mammary epithelial cells secrete interleukin-8 in response to lipopolysaccharide stimulation. Am. J. Vet. Res. 59:1563-1567.

Boudjellab, N., H.S. Chan-Tang and X. Zhao. 2000. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp. Physiol. Biochem. 127:191-199.

Bradley A.J., 2002. Bovine mastitis: An evolving disease. Vet. J. 164: 116-128.

Bramley AJ. 1976. Variations in the susceptibility of lactating and non-lactating bovine udders to infection when infused with Escherichia coli. J. Dairy Res. 43: 205-11.

Bramley AJ, Godinho KS, Grindal RJ. 1981. Evidence of penetration of the bovine teat duct by Escherichia coli in the interval between milkings. J. Dairy Res. 48: 379-386.

Bramley AJ, Dodd FH. 1984. Reviews of the progress of dairy science: mastitis control-progress and prospects. J. Dairy Res. 51: 481-512.

Bramley A.J., Foster R., 1990. Effects of lysostaphin on *Staphylococcus aureus* infections of the mouse mammary gland. Res. Vet. Sci. 49, 120-121.

Bramley A.J. 1991. Scientific summary of the Ghent meeting, in: Burvenich C., Vandeputte-Van Messom G., Hill A.W. (Eds.), New insights into the pathogenesis of mastitis, Flem.Vet. J.: 235–238.

Burvenich C., Peeters G. 1982. Effect of prostaglandin synthetase inhibitors on mammary blood flow during experimentally induced mastitis in lactating goats, Arch. Int. Pharmacodyn. Thér. 258 :128–137.

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

Capuco AV, Bright SA, Pankey JW, Wood DL, Miller RH, Bitman J. 1992. Increased susceptibility to intramammary infection following removal of teat canal keratin. J. Dairy Sci.75: 2126-2130.

Chew BP, Zamora CS, Leudecke LO. 1985. Effect of vitamin A deficiency on mammary gland development and susceptibility to mastitis through intramammary infusion with Staphylococcus aureus in mice. Am. J. Vet. Res. 46: 287-293.

Chrystal MA, Seykora AJ, Hansen LB. 1999. Heritabilities of teat end shape and teat diameter and their relationships with somatic cell score. J Dairy Sci. 82: 2017-22.

Cifrian E., Guidry A. J., O'Brien C.N., Nickerson S.C., Marquardt W.W. 1994. Adherence of Staphylococcus aureus to cultured bovine mammary epithelial cells. J. Dairy Sci. 77:970-983.

Cifrian E., Guidry A. J., Bramley A.J. 1996. Effect of β-toxin on Staphylococcus aureus cytotoxicity, proliferation and adherence to bovine mammary epithelial cells. Vet. Microbiol. 48:187-198.

Cummings Craig A., Relman David A. 2000. Using DNA Microaarays to Study Host-Microbe Interactions. Emer. Infec. Dise. 6:513-525.

Detilleux J.C.2002. Genetic factors affecting susceptibility of dairy cows to udder pathogens. Vet. Immunol. Immunopathol. 88:103–110.

Diatchenko Luda, Lukyanov S, Lau Yun-Fai Chris, Siebert Paul D. 1999. Suppression Subtractive Hybridization: A Versatile Method for Identifying Differentially Expressed Genes. Met. Enzym. 303:349-380.

Deininger S., Stadelmaier A, von Aulock S, Morath S, Schmidt RR, Hartung T. 2003. Definition of structural prerequisites for lipoteichoic acid-inducible cytokine induction by synthetic derivatives. J Immunol. 170: 4134-4138

Dosogne H, Capuco AV, Paape MJ, Roets E, Burvenich C, Fenwick B. 1998. Reduction of acyloxyacyl hydrolase activity in circulating neutrophils from cows after parturition. J. Dairy Sci. 81: 672-677.

Dosogne H., Burvenich C., Freeman A.E., Kehrli M.E.J., Detilleux J., Sulón J., BeckersJ.-F., Hoeben D. 1999. Pregnancy-associated glycoproteinand decreased polymorphonuclear leukocyte function in early post-partum dairycows, Vet. Immunol. Immunopathol. 67: 47–54.

Dosogne H, Vangroenweghe F, Burvenich C. 2002. Potential mechanism of action of J5 vaccine in protection against severe bovine coliform mastitis. Vet. Res. 33:1-12.

Eberhart RJ. 1977. Coliform mastitis. J. A. V. M. A. 170: 1160-63.

Eberhart RJ, Harmon RJ, Jasper DE, Natzke RP. 1987. Introduction-the mastitis problem. In: Current concepts of bovine mastitis. National Mastitis Council, Arlington, VA, USA. pp6-8.

Eberhart RJ, Natzke RP, Newbould FHS, Nonnecke B, Thompson P. 1979. Coliform mastitis - a review. J. Dairy Sci. 62: 1-22.

Eckmann L, Kagnoff MF, Fierer J. 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun. 61:4569-4574.

Epstein CB, Butow RA. 2000. Microarray technology-enhanced versatility, persistent challenge. Curr. Opinion Biotech. 11: 36-41.

Fox L. K. and Hancock D. 1989. Effect of segregation on prevention of intramammary infections by Staphylococcus aureus. J. Dairy Sci. 72:540-544.

Gill R., Howard W. H., Leslie K. E., Lissemore K. 1990. Economics of mastitis control. J. Dairy Sci. 73:3340-3348.

Goldammer T., Zerbe H., Molenaar A., Schuberth H. J., Brunner R. M., Kata S. R. , Seyfert H. M. 2004. Mastitis Increases Mammary mRNA Abundance of β-Defensin 5, Toll-Like-Receptor 2 (TLR 2) and TLR 4 but not TLR 9 in Cattle. Clin. Diag. Lab. Immuno. 11:174-185.

Gonzalez R. N., Jasper D. E., Kronlund N. C., Farver T. B., Cullor J. S., Bushnell R. B., Dellinger J. D. 1990. Clinical mastitis in two California dairy herds participating in contagious mastitis control programs. J. Dairy Sci. 73: 648-660.

Grimminger F., Rose F., Sibelius U., Meinhardt M., Potzsch B., Spriestersbach R., Bhakdi S., Suttorp N., Seeger W.1997. Human endothelial cell activation and mediator release in response to the bacterial exotoxins Escherichia coli hemolysin and staphylococcal alpha-toxin. J.Immuno. 159:1909-1916.

Grommers F. J., Van de Geer D., Van der Vliet H., Henricks P. A., Nijkamp F. P. 1989. Polymorphonuclear leucocyte function: relationship between induced migration into the bovine mammary gland and in vitro cell activity.Vet. Immunol. Immunopathol. 23: 75-83.

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

Gudding R, McDonald JS, Cheville NF. 1984. Pathogenesis of Staphylococcus aureus mastitis: bacteriologic, histologic, and ultrastructural pathologic findings. Am. J. Vet.Res.45:2525-2531.

Guidry AJ. 1985. Mastitis and immune system of the mammary gland. In: Lactation. Larson B. L. (ed.) The Iowa State University Press. Ames, Iowa, USA. pp229-262

.

Guidry A. J., Pearson R E., Paape M. J., Williams W. F. 1980. Relationship among leukocyte phagocytosis, milk immunoglobulins, and susceptibility to intramammary infection. Am. J. Vet. Res. 41: 997-1001.

Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, Si-Tahar M. 2004.Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR 4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR 4. J Biol Chem. 279:2712-2718.

Hakogi E., Tamura H., Tanaka S., Kohata A., Shimada Y., Tabuchi K., Endotoxin levels in milk and plasma of mastitis-affected cows measured with a chromogenic limulus test. Vet. Microbiol. 20 (1989) 267–274.

Harada K, Ohira S, Isse K, Ozaki S, Zen Y, Sato Y, Nakanuma Y. 2003. Lipopolysaccharide activates nuclear factor-kappaB through toll-like receptors and related molecules in cultured biliary epithelial cells. Lab Invest. 83:1657-1667.

Hartman I., Ziv G., Saran A.1976. Application of the Limulus amoebocyte lysate test to the detection of gram-negative bacterial endotoxins in normal and mastitic milk, Res. Vet. Sci. 20:342–343.

Heald C. W. 1979. Morphometric study of experimentally induced Staphylococcus bovis mastitis in the cow. Am. J. Vet. Res. 40:1294-1298.

Hébert A.,Sayasith K., Sénéchal S., Dubreuil P., Lagacé J. 2000. Demonstration of intracellular *Staphylococcus aureus* in bovine mastitis alveolar cells and macrophages isolated from naturally infected cow milk. FEMS Microbiol. Letters. 193:57-62.

Hensen S. M., Pavicic M. J., Lohuis J. A., de Hoog J. A., Poutrel B. 2000. Location of *Staphylococcus aureus* within the experimentally infected bovine udder and the expression of capsular polysaccharide type 5 in situ. J. Dairy Sci. 83:1966-1976.

Hill A. W. 1981. Factors influencing the outcome of *Escherichia coli* mastitis in cows. Res. Vet. Sci. 31:107-112.

Hoeben D., Burvenich C., Eppard P.J., Hard D.L. 1999. Effect of recombinant bovine somatotropin on milk production and composition in bovine *Streptococcus uberis* mastitis, J. Dairy Sci. 82:1671–1683.

Hoeben D., Burvenich C., Trevisi E., Bertoni G., Hamann J., Bruckmaier R.M., Blum J. 2000. Role of endotoxin and TNF-α in the pathogenesis of experimentally induced coliform mastitis in periparturient cows, J. Dairy Res. 67:503–514.

Hogan JS, Smith KL, Hoblet KH, Schoenberger PS, Todhunter DA, Hueston WD, Pritchard DE, Bowman GL, Heider LE, Brockett BL, Conrad HR. 1989. Field survey of clinical mastitis in low somatic cell count herds. J. Dairy Sci. 72: 1547-1556.

Horng T., Barton G. M., Medzhitov R. 2001. TIRAP: an adapter molecule in the Toll signaling pathway. Nat. Immunol. 9:835-841.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. 1999. Cutting edge: Toll-like receptor 4 (TLR 4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR 4 as the Lps gene product.J Immunol. 162:3749-3752.

Jia H. P., Starner T, Ackermann M, Kirby P, Tack B. F., and McCray P. B. J. 2001. Abundant human beta-defensin-1 expression in milk and mammary gland epithelium. J. Pediatr. 138:109–112.

Jin H, Cheng X, Diatchenko L, Siebert PD, Huang CC. 1997. Differential screening of a subtracted cDNA library: A method to search for genes preferentially expressed in multiple tissues. Biotech. 23: 1084-1086.

Jonsson P., Wadstrom T. 1993. Staphylococcus. In: Pathogenesis of bacterial infections in animals. 2nd Ed. Gyles C. L. and Thoen C. O. (eds). pp21-35.

Kawai T., Takeuchi O, Fujita T, Inoue J, Muhlradt P. F., Sato S, Hoshino K, Akira S. 2001. Lipopolysaccaride Stimulates the MYD88-Independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a sunset of lipopolysaccharide-iducible genes. J. Immunol. 167:5887-5894.

Kehrli M. E., Shuster D. E. 1994. Factors affecting milk somatic cells and their role in health of the bovine mammary gland. J Dairy Sci. 77:619-627.

Kehrli M. E., Harp J. A. 2001. Immunity in the mammary gland. Vet. Clin. North. Am. Food. Anim. Pract. 3:495-516.

Kerr D. E., Plaut K., Bramley A. J., Williamson C. M., Lax A. J., Moore K., Wells K. D., Wall R. J. 2001. Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. Nat. Biotechnol. 19: 66-70.

Kuang WW, Thompson DA, Hoch RV, Weigel RJ. 1998. Differential screening and suppression subtractive hybridization identified genes differentially expressed in an estrogen receptor-positive breast carcinoma cell line. Nucleic Acids Res. 26:1116-1123.

Kumar A., Zhang J. Yu F. S. 2004. Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci. 10:3513-22

Lam T.J.G.M., Van Vliet J.H., Schukken Y.H., Grommers F.J., Van Velden-Russcher A., Barkema H.W., Brand A. 1997. The effect of discontinuation of postmilking teat disinfection in low SCC herds. 1. Incidence of clinical mastitis, Vet. Q. 19:41–47.

Lee C. W., Wooding F. B. P., Kemp P. 1980. Identification, properties, and differential counts of cell populations using electron microscopy of dry cow secretion, colostrums, and milk from normal cows. J. Dairy Sci. 47:39-50.

Liang P., Pardee A. B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Sci. 257:967-971.

Lin C. H., Kuan I. H., Lee H. M., Lee W. S., Sheu J. R., Ho Y. S., Wang C. H., Kuo H. P. 2001. Induction of cyclooxygenase-2 protein by lipoteichoic acid from Staphylococcus aureus in human pulmonary epithelial cells: involvement of a nuclear factor-kappa B-dependent pathway.Br J Pharmacol. 134:543-552.

Long E, Capuco A. V., Wood D. L., Sonstegard T, Tomita G, Paape M. J., Zhao X. 2001. Escherichia coli induces apoptosis and proliferation of mammary cells. Cell Death Differ. 8:808-816.

Madsen S. A., Weber P. S., Burton J. L. 2002. Altered expression of cellular genes in neutrophils of periparturient dairy cows.Vet Immunol Immunopathol. 86:159-75.

McDonald JS, Anderson AJ. 1981. Experimental infection of bovine mammary glands with Streptococcus agalactiae during the nonlactating period. Am. J. Vet. Res. 42: 462-464.

Mehrzad J., Duchateau L., Pyörälä S., Burvenich C. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation J. Dairy Sci. 85:3268–3276.

Miller J.K., Brezinska-Slebodzinska E., Madsen F. C. 1993. Oxidative stress, antioxidants and animal function. J. Dairy Sci. 76:2812-2823.

Miller R.H., Norman H. D., Wiggans G. R., Wright J. R. National survey of herd average somatic cell counts on DHI test days. National Mastitis Council Annual Meeting Proceedings, 1999, p161-162.

Nagai Y., Akashi S., Nagafuku M., Ogata M., Iwakura Y., Akira S., Kitamura T., Kosugi A., Kimoto M., Miyake K. 2002. Essential role of MD-2 in LPS responsiveness and TLR 4 distribution. Nat Immunol. 3:667-672.

Natzke R. P., Everett R. W., Guthrie R. S., Keown J. F., Meek A. M., Merrill W. G., Roberts S. J., Schmidt G. H. 1972. Mastitis control program: effect on milk production. J Dairy Sci. 55:1256-60.

Ndiweni N, Finch JM. 1996. Effects of in vitro supplementation with alpha-tocopherol and selenium on bovine neutrophil functions: implications for resistance to mastitis. Vet. Immunol. Immunopathol. 51: 67-78.

Nickerson SC. 1993. Preventing new Staphylococcus aureus mastitis infections. Vet. Med. 4:368-374.

Nickerson SC. 1993. Eliminating chronic Staphylococcus aureus mastitis. Vet. Med.4:375-381.

Oldham E. R., Daley M. J. 1991. Lysostaphin: use of a recombinant bactericidal enzyme as a mastitis therapeutic.J. Dairy Sci. 74, 4175-4182.

Ozinsky A, Underhill D. M., Fontenot J. D., Hajjar A. M., Smith K. D., Wilson C. B., Schroeder L, Aderem A. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.Proc Natl Acad Sci U S A. 97:13766-71.

Paape M. J., Schultze W. D., Desjardins C., Miller R. H. 1974 Plama corticosteriods, circulating leukocyte and milk somatic cell responses to *Escherichia coli* endotoxin-induced mastitis. Proc. Soc. Exp. Biol. Med. 145:533-539.

Paape MJ, Lillius E-M, Wiitanen PA, Kontio MP, Miller RH. 1996. Intramammary defense against infections induced by *Escherichia coli* in cows. Am. J. Vet. Res. 57: 477-482.

Paape M.J., Bannerman D.D., Zhao X., Lee J.-W. 2003. The bovine neutrophil: structure and function in blood and milk, Vet. Res. 3:597–627.

Perrson K., Larrson I., Sandgren C. H.1993. Effects of certain inflammatory mediators on bovine neutrophil migration in vivo and in vitro. Vet. Immunol. Immunopathol. 37:99-112.

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

Peter A.T., Clark P.W., Van Roekel D.E., Luker C.W., Gaines J.D., Bosu W.T. 1990. Temporal changes in metabolites of prostanoidsin milk of heifers after intramammary infusion of *Escherichia coli* organisms, Prost. 39:451–457.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29:2002-2007.

Piccinini R, Bronzo V, Moroni P, Luzzago C, Zecconi A. 1999. Study on the relationship between milk immune factors and Staphylococcus aureus intramammary infections in dairy cows. J. Dairy Res. 66: 501-510.

Poltorak A., He X., Smirnova I., Liu M.Y., Van Huffel C., Du X., Birdwell D., Alejos E., Silva M., Galanos C., Freudenberg M., Ricciardi-Castagnoli P., Layton B., Beutler B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr 4 gene. Sci. 282:2085-2088.

Postle D. S., Roguinsky M., Poutrel B. 1978. Induced staphylococcal infections in the bovine mammary gland. Am. J. Vet. Res. 39:29-35.

Pratt L.A., Kolter R.1998. Genetic analysis of Escherichia coli biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 2:285-293.

Radostitis O. M., Blood D. C., Gay C. C. 1994. Mastitis. In: Veterinary medicine: A text book of the diseases of cattle, sheep, pigs, goats and horses. 8th Ed. Bailliére Tindall, London, UK. Pp563-527.

Rainard P, Poutrel B. 1984. Non-random distribution of udder infections among cows. 49. Evaluation of some contributing factors. Ann. Rech. Vet. 15: 119-27.

Ren D., Bedzyk L. A., Thomas S. M., Ye R. W., Wood T. K. 2004. Gene expression in Escherichia coli biofilms. Appl. Microbiol. Biotechnol. 64: 515–524.

Riollet C., Rainard P., Poutrel B.2000. Differential induction of complement fragment C5a and inflammatory cytokines during intramammary infections with *Escherichia coli* and *Staphylococcus aureus*, Clin. Diagn. Lab. Immunol. 7:161–167.

Rose F, Dahlem G, Guthmann B, Grimminger F, Maus U, Hanze J, Duemmer N, Grandel U, Seeger W, Ghofrani H. A.. 2002. Mediator generation and signaling events in alveolar epithelial cells attacked by S. aureus alpha-toxin. Am J Physiol Lung Cell Mol Physiol.. 282: 207-214.

Sandgren. C. H., Nordling K., Bjork I. 1991. Isolation and phagocytic properties of neutrophils and other phagocytes from nonmastitic bovine milk. J. Dairy Sci. 74:2965-2975.

Schuerer-Maly CC, Eckmann L, Kagnoff MF, Falco MT, Maly FE. 1994. Colonic epithelial cell lines as a source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharide. Immunol. 81:85-91.

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

Schukken YH, Mallard BA, Dekkers JC, Leslie KE, Stear MJ. 1994. Genetic impact on the risk of intramammary infection following Staphylococcus aureus challenge. J. Dairy Sci. 77: 639-647.

Shuster DE, Lee EK, Kehrli ME. 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. Am. J. Vet. Res. 57: 1569-1575.

Shuster D.E., Kehrli Marcus E. JR, Rainard P, Paape M. 1997. Complement Fragment C5a and Inflammatory Cytokines in Neutrophil Recruitment during Intrammary Infection with *Escherichia coli*. Infec. Immun. 65:3286-3292

.

Soderquist B, Kallman J, Holmberg H, Vikerfors T, Kihlstrom E. 1998. Secretion of IL-6, IL-8 and G-CSF by human endothelial cells in vitro in response to Staphylococcus aureus and staphylococcal exotoxins. APMIS. 106:1157-1164.

Sordillo LM, Doymaz MZ, Oliver SP. 1989. Morphological study of chronic Staphylococcus aureus mastitis in the lactating bovine mammary gland. Res. Vet. Sci. 47:247-252.

Sordillo LM, Nickerson SC. 1989. Pathology of Staphylococcus aureus mastitis during lactogenesis: relationships with bovine mammary structure and function. J. Dairy Sci. 72:228-240.

Sordillo LM, Shafer-Weaver K, Derosa D. 1997. Immunobiology of the mammary gland. J. Dairy Sci. 80:1851-1865.

Takeda K., Kaisho T., Akira S. 2003. Toll-Like Receptors. Ann. Rev.Immunol. 21:335-376.

Tanaka TS, Jaradat SA, Lim MK, Kargul GJ, Wang X, Grahovac MJ, Pantano S, Sano Y, Piao Y, Nagaraja R, Doi H, Wood WH III, Becker KG, Ko MSH. 2000. Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. Proc. Natl. Acad. Sci. USA. 91: 9127-9132.

Taniguchi M, Miura K., Iwao H., Yamanaka S. 2001. Quantitative assessment of DNA microarrays - comparison with northern blot analyses. Gen. 71:34-39.

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. 1999. Differential Roles of TLR 2 and TLR 4 in Recognition of Gram–Negative and Gram-Positive Bacterial Cell Wall components. Immunity. 11:443-451.

Takeuchi O, Hoshino K, Akira S. 2000. Cutting Edge: TLR 2-Defficient and MyD88-Deficient Mice Are Highly Suceptible to Staphylococcus aureus Infection. J. Immuno. 165:5392-5396.

Takeuchi O. and Akira S. 2001. Toll-like receptors; their physiological role and signal transduction system. Rev.Inter.Immunopharm. 1:625-635.

Takeuchi O. and Akira S. 2002. Genetic approaches to the study of Toll-like receptor function. Micro. Infec. 9:887-895.

Uehara A, Sugawara S, Takada H. 2002. Priming of human oral epithelial cells by interferon-gamma to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. J Med Microbiol. 51:626-634.

Underhill DM. and A. Ozinsky, 2002. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol, 14:103-110

Velculescu V. E., Zhang L, Bogelstein B, Kinzler K. W. 1995. Serial analysis of gene expression. Sci. 270: 484-487.

Wang X., Zhang Z., Louboutin J. P., Moser C., Weiner D. J., Wilson J. M. 2003. Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. FASEB J.17:1727-1729.

Wyllie D. H., Kiss-Toth E, Visintin A, Smith S. C., Boussouf S, Segal D. M., Duff G. W., Dower S. K. 2000. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J Immunol. 165:7125-32.

Yancey RJ. 1999. Vaccines and diagnostic methods for bovine mastitis: fact and fiction. Adv. Vet. Med. 41: 257-273.

Yao L, Bengualid V, Lowy FD, Gibbons JJ, Hatcher VB, Berman JW. 1995. Internalization of Staphylococcus aureus by endothelial cells induces cytokine gene expression. Infect. Immun.63:1835-1839.

Zarember K. A. and Godowski P.J. 2002. Tissue expression of human Toll-Like Receptors and differential regulation of Toll-Like Receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J. Immuno.168: 554–561.

Zen Y, Harada K, Sasaki M, Tsuneyama K, Katayanagi K, Yamamoto Y, Nakanuma Y. 2002. Lipopolysaccharide induces overexpression of MUC2 and MUC5AC in cultured biliary epithelial cells: possible key phenomenon of hepatolithiasis. Am J Pathol. 161:1475-1484.

Ziv G., Hartman I., Bogin E., Abidar J., Saran A. 1976. Endotoxin in blood and milk of cows during experimental *Escherichia coli* endotoxin mastitis, Ther. 6:343–347.

CHAPTER II. QUANTIFICATION OF TOLL LIKE RECEPTOR (TLR) 4 AND TLR 2 mRNA EXPRESSION IN CULTURED BOVINE DUCT EPITHELIAL CELLS UNDER LPS LTA AND PGN STIMULATION AND IN COW MILK SOMATIC CELLS DURING INTRA-MAMMARY CHALLENGE WITH *ESCHERICHIA COLI*, *STAPHYLOCOCCUS AUREUS* BY REAL-TIME PCR

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ABSTRACT

The presence and function of TLRs in the innate immune cells has been well characterized. However, the immune response in the mammary epithelial cells under bacterial invasion has remained largely unexplored with respect to TLR presence and function. Pathogen recognition in tissues such as endothelial and epithelial cells composing the inner linings of the mammary gland is expected to possess a defence mechanism to signal for reinforcements especially in bovine who are constantly under invasion. *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) are the most virulent pathogens, causing mastitis in cattle. Monitoring the role of TLR 4 and 2 mRNA levels during the infection will shed some light as to how these epithelial cells of the mammary gland are involved in pathogen recognition.

After identifying the appropriate stimulation concentration for each of the microbial components LPS, PGN and LTA in the duct epithelial cells, the mRNA levels of TLR 4 and 2 were monitored. For the LPS stimulated cells the expression of both the TLR 4 and TLR 2 mRNA expression was significantly increased ($p<0.05$) for 2h-72h and 12h-48h respectively when compared to the 0h. In the case of PGN, the expression of TLR 2 mRNA is most significant ($p<0.05$) at 72h whereas TLR 4 mRNA expression increases at the 24, 48, and 72h. In terms of LTA, another structural component of *S. aureus* and more potent than PGN, mRNA expression levels were increased at 48 and 72h for TLR 2 and 24, 48, and 72h for TLR 4 in comparison to the 0h ($p<0.05$). The milk somatic cells were also monitored for TLR 4 and 2 mRNA expression. Due to the high variability observed from each cow the results were not conclusive.

INTRODUCTION

When it comes to infections, all multi cellular organisms possess an immune system capable of protection against various pathogens. The immune system is composed of two immune responses: innate and adaptive that differs in their cell types and time of onset. The innate immune system serves as the primary line of defense in mammals in any type of invasion because of its capacity to recognize a broad spectrum of pathogens by using a repertoire of invariant receptors. Macrophages and polymorponuclear cells (PMNs) are capable of recognizing self from non-self via germ line-encoded receptors referred to as pattern-recognition receptors. These receptors are primarily identified in *Drosophila melanogaster* as the *Drosophila* Toll, one of the 12 maternal effect genes that function in a pathway required for dorso-ventral axis formation in fly embryos. They are also involved in the response to fungal infections (Janeway et al. 2002). Toll Like Receptors (TLRs) are transmembrane proteins characterized by an extracellular leucine-rich domain and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain. The identification of IL-1R receptors in humans with possible functional similarities to the Toll receptor demonstrated that through evolution these receptors were conserved for their ability to respond and initiate inflammatory and immune responses. TLRs are predominantly expressed in tissues involving immune function, such as spleen and peripheral blood leukocytes. Lines of evidence also indicate that cells exposed to the external environment such as lung, gastrointestinal tract and the mammary glands participate in immune responses (Zarember et al., 2002. Goldammer et al., 2004). Up to now, ten human and nine murine TLRs have been mapped to their appropriate chromosomal locations and have been characterized with their inducing ligands (Underhill and Ozinsky, 2002; Takeda et al., 2003). TLRs become activated when they recognize highly conserved structural motifs specifically expressed by microbial pathogens, so called pathogen-associated microbial patterns (PAMPs). These PAMPs include various bacterial cell wall components such as lipopolysaccharides (LPS), Lipoteichoic acids (LTA) and peptidoglycans (PGN), as well as flagellin, bacterial DNA and viral double-stranded RNA. Stimulation of the TLRs by their specific PAMPs initiates a signaling cascade that involves a number of proteins, such as MyD88 and IRAK in the activation of the NF-κB

transcription factor which upregulates the expression of pro-inflammatory cytokine mRNA such as IL-1, IL-6, IL-8, and TNF-α to subsequently excrete the cytokines in the recruitment of effector immune cells. Amongst the different TLRs, mouse models have specifically demonstrated that TLR 4 and TLR 2 recognize Gram negative and Gram-positive bacteria respectively (Hoshino et al., 1999, Takeuchi et al. 2000). *E. coli* and *S. aureus* pathogens are most prominent bacteria to cause infections in all types of tissues (Eckmann et al., 1993; Goldammer et al., 2004; Guillot et al., 2004; Harada et al., 2003; Uehara et al., 2002). Lipopolysaccaride (LPS), the structural component of Gram-negative bacteria (such as *E. coli*), is recognized by the TLR 4 whereas TLR 2 has been demonstrated to recognize bacterial lipoproteins, peptidoglycan (PGN), and lipoteichoic acids (LTA), which are mainly constituents of Gram-positive bacteria such as *Staphylococcus aureus* (Deininger et al. 2003; Takeda et al., 2003; Takeuchi and Akira, 2002). During an infection from either *E. coli* or *S. aureus* in the mammary gland it would be expected that the TLR 4 and 2 mRNA levels be upregulated and present in high numbers on the membrane in order to facilitate the recognition of pathogen and/or PAMPs. Previously in MAC-T cells, a non differentiated bovine mammary epithelial cell line, the expression of mRNA for cytokine IL-1 and chemokine IL-8 was identified after stimulation with LPS (Boudjellab et al., 2000) demonstrating involvement of mammary epithelial cells in pathogen recognition. In this study we used a primary bovine mammary duct epithelial cell line to monitor the presence of TLR 4 and 2 mRNA by quantifying their expression levels after stimulation with LPS, LTA and PGN. Bovine mammary glands were also challenged with either *E. coli* or *S. aureus* to monitor the TLR 4 and 2 mRNA expressions in milk somatic cells, mostly comprising of leukocytes during an infection.

METHODS AND MATERIALS

Animals. The experiment was conducted in parallel to our previous study (Bannerman et al., 2004), using the same animals. Briefly, sixteen clinically healthy Holstein cows in mid lactation (214 ± 8.67 DIM) were selected based on milk SCC (< 500,000 cells/ml) and the absence of bacteria from three daily, consecutive, aseptically collected milk samples. The use of animals for this study was approved by the Animal Care and Use Committee of Beltsville Agriculture Research Center.

Preparation of bacteria. The organisms used were serum-resistant *E. coli* strain P4 and *S. aureus* strain 305, which were originally isolated from clinical cases of bovine mastitis and have been shown to induce experimental mastitis successfully (Alluwaimi et al., 2003; Lee et al., 2003). Before challenge exposure, 10 ml of brain heart infusion broth (Becton-Dickinson Diagnostic Systems, Inc., Spark, MD) were inoculated with either strain and incubated for 6 h at 37°C. Thereafter, 1 ml of the inocula was transferred to aerating flasks containing 99 ml of tryptic soy broth (TSB, Difco, Detroit, MI) and incubated overnight at 37°C. After incubation, the flasks were placed in an ice water bath and mixed by swirling. One ml from each flask was serially diluted in PBS and 1 ml of the resulting dilution was mixed with 9 ml of pre-melted trypticase soy agar in petri dishes. The plates were allowed to solidify at room temperature and then transferred to a 37°C incubator overnight. The aerating flasks containing the stock inoculum were maintained at 4°C overnight. Once the concentration of the stock has been determined based on the prepared poured plates, the stock was diluted in PBS to a final concentration of 40 CFU/ml.

Intra-mammary challenge. One front or rear quarter of each cow was infused with 2 ml (40 CFU/ml) inoculum of either *E. coli* (n = 8) or *S. aureus* (n = 8) immediately

after the morning milking. The contralateral quarter of each challenged quarter was infused with 2 ml of sterile PBS. The actually inoculated number of bacteria, determined by pour-plating, was confirmed to be 72 and 74 CFU/quarter for *E. coli* and *S. aureus*, respectively. Milk sample collection and rectal temperature measurement were carried out at 0, 8, 16, 24, 32, 40, 48, and 72 h relative to the challenge.

Bacteriology and determination of SCC. Aseptically collected milk samples, with or without serial dilutions, were plated onto blood agar plates and the number of CFU was numerated after 16 h of incubation at 37°C. The confirmatory identification was performed by Maryland Department of Agriculture Animal Health Section (College Park, MD). A 2-ml aliquot of milk was heated for 15 min at 60°C and maintained at 40°C until counted by an automated cell counter (Fossomatic 90, Foss Electronic, Helleroed, Denmark) in duplicates.

Isolation of milk somatic cells. Fifty ml of aseptically collected milk samples were diluted with an equal volume of sterile PBS and centrifuged at 700 × g for 20 min at 20°C. After the fat layer and the supernatant were discarded, the cell pellet was washed twice and suspended in sterile PBS. A small portion of the cell suspension was properly diluted and cytospin-centrifuged for differential counting. The remaining cells were numerated and spun down for total RNA extraction.

RNA extraction and reverse transcription. Total RNA extraction was performed using TRIZOL (GIBCO/BRL, Gaithersburg, MD) according to the manufacture's instructions. The pellet of 10^6 milk somatic cells were lysed by 1 ml of TRIZOL reagent, and centrifuged after adding 0.2 ml chloroform (phase separation). The RNA, retained in the aqueous phase, was precipitated by mixing with equal volume of isopropanol and

washed with 75% ethanol twice. Afterward, an appropriate amount of DEPC-treated water was added to dissolve the RNA, and the concentration was determined by the optical density value at 260nm. The reverse transcription (RT) was conducted in a total volume of 20 μl containing 2 μg of total RNA, 0.5 μg of oligo (dT_{12-18}), 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 5× first strand buffer (Invitrogen Canada Inc. Burlington, Ontario) and 200 U Superscript II RNase H^- Reverse Transcriptase (Gibco/BRL). The mixture was heated at 70°C for 10 min, placed on ice for 2 min, and subsequently incubated at 42°C, 50 min, for the RT reaction. Thereafter, the temperature was raised to 70°C for 15 min to inactivate the reverse transcriptase. Synthesized cDNA was kept at -20°C until being used.

Cell Culture. Bovine mammary ductal epithelial cells (kindly provided by Dr. Guidry (Guidry and O'Brien, 2002; Smits et al., 1996)) were cultured at 37° C in 5% CO_2 humidified atmosphere. 2.1×10^6 ductal epithelial cells are grown on 100mm x 20mm plastic tissue culture plates in growth medium containing 45% Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), 45% RPMI-1640 medium (Gibco), 10% Fetal Bovine Serum (FBS; Hyclone, Logan, USA), 2% antibiotic-antimycotic solution.

Stimulation of Bovine Mammary Duct Epithelial cells with LPS, LTA, or PGN for specified time points. Bovine mammary duct epithelial cells were subsequently induced with LPS (Sigma) at a concentration of 1μg/ml, LTA(Medicorp) at a concentration of 2μg/ml, and PGN (Medicorp) at a concentration of 2μg/ml. RNA sample collections were carried out at 0h, 2h, 6h, 12h, 24h, 48h, and 72h relative to the induction with the use of TRIZOL (GIBCO/BRL, Gaithersburg, MD).

RNA extraction and reverse transcription. Total RNA extraction was performed using TRIZOL (GIBCO/BRL, Gaithersburg, MD) according to the manufacture's instructions. The monolayer of bovine mammary duct epithelial cells was lysed by the addition of 5 ml of TRIZOL reagent directly into the culture dish having an area of 157cm^2. After subsequent homogenization 0.2 ml of chloroform (phase separation) was added to 1 ml of homogenization the mixture and centrifuged at 12 000 x g for 15 min, 4°C. The RNA, retained in the aqueous phase, was precipitated by mixing with equal volume of isopropanol and washed with 75% ethanol twice. Afterward, an appropriate amount of DEPC-treated water was added to dissolve the RNA, and the concentration was determined by the optical density value at 260nm. The reverse transcription (RT) was conducted in a total volume of 20 μl containing 2 μg of total RNA, 0.5 μg of oligo (dT_{12-18}), 10 mM dithiothreitol (DTT), 0.5 mM of each dNTP, 5× first strand buffer (Invitrogen Canada Inc. Burlington, Ontario) and 200 U Superscript II RNase H$^-$ Reverse Transcriptase (Gibco/BRL). The mixture was heated at 70°C for 10 min, placed on ice for 2 min, and subsequently incubated at 42°C, 50 min, for the RT reaction. Thereafter, the temperature was raised to 70°C for 15 min to inactivate the reverse transcriptase. Synthesized cDNA was kept at -20°C until being used.

Real Time RT PCR quantification of mRNA for TLR 4 and TLR 2 expression. The Lightcycler real-time polymerase chain reaction (PCR) was carried out as described (Pfaffl, 2001) with modifications. Briefly, primers for specific bovine genes, as listed in Table 1, were synthesized (Invitrogen, Burlington, Ontario). The reaction condition for each individual gene was optimized using a QuantiTect SYBR Green PCR kit (Qiagen) in a LightCycler system (Roche) and applied to the following protocol. The cDNA was

analyzed in 20 µl PCR mixture containing a final concentration of 0.5 µM primer, 1 µl of cDNA, and 2× QuantiTect SYBR green PCR mastermix. The PCR master mix contains HotStartaq DNA polymerase, SYBR green PCR buffer, dNTP mix including dUTP, SYBR green I, ROX (passive reference dye) and $MgCl_2$ (3 mM for GM-CSF and TNF-α; 2.5 mM for the others). The PCR mixture was added into a cold PCR capillary (Roche), centrifuged, and placed in the LightCycler system. The LightCycler was programmed in 4 steps: 1) denaturation at 95°C for 15 min 2) amplification for 50 cycles of denaturation at 94°C for 15 sec, annealing at 60°C (TLR 4) for 30 sec / 55°C (TLR 2) for 30 sec, and extension at 72°C (depending on the product length, 5 sec per 100 bp) 3) melting curve by 95 °C for 5 sec, 65 °C for 15 sec, and 95 °C for 0 sec. 4) cooling at 40 °C.

Relative quantification. The expression of each gene was analyzed using the relative quantification method described by Pfaffl (2001). In brief, a slope was determined from the exponential phase, under the optimized real-time PCR amplification condition, of each target gene or the reference gene (bovine β-actin). The amplification efficiency (E) was calculated based on the slope, where $E = 10^{[-1/\text{slope}]}$. The fold was then calculated by dividing the target gene with that of the reference gene for that particular time point or concentration used.

Statistical analysis. Sample folds were analyzed by multiple comparison using the PROC MIXED function in the SAS software (SAS/STAT User Guide, 2000) at a level of significance $p < 0.05$. For the different concentrations used of LPS, LTA and PGN the folds were compared in triplicates to the 0 µg/ml value. Whereas for the different time points 2, 6, 12, 24, 48, and 72h the folds were compared in quatripletes to the 0h value. For the data from cows, 16 cow RNA samples were obtained for either *E. coli* (8) or *S. aureus* (8)

challenged quarters. However, only seven samples were used for the real-time PCR. Since there were big individual variabilitions, the results were represented on a cow basis, as a dot blot.

RESULTS

In Vitro Experiment

TLR 4 and 2 mRNA Expression Monitored under LPS Stimulation in Duct Epithelial Cells

Initially the potency of LPS was assessed for various concentrations ranging from 0-10μg/ml in the bovine mammary duct epithelial cells. This step was necessary to determine the concentration at which LPS was most potent by quantifying the mRNA level expressed for TLR 4 after 24h. LPS is very efficient in initiating a response even at very minute amounts. The mammary duct epithelial cells responded to the presence of LPS but without any significant difference amongst the different concentrations tested (0-10 μg/ml). Either LPS has been internalized by the cells after 24h period or the cells have become tolerant to LPS thus making the LPS stimulation insignificant. Since there were no significant differences in TLR 4 mRNA expression we chose to use a modest range of 1μg/ml concentration, because the highest peak fold was at this concentration, even though it was not significantly different from the 0 μg/ml concentration (Figure 1.A). In the time course study both TLR 4 and 2 mRNA were analyzed, since LPS has also been shown to be involved in TLR 2 recognition. Results show that TLR 4 mRNA expression responded to LPS and was upregulated at time 2, 6, 12, 24, 48, and 72h significantly ($p<0.05$) in comparison to the 0h control (Figure 1. B). TLR 2 was also significantly ($p<0.05$) upregulated in the presence of LPS at times 12, 24, and 48h in comparison to the 0 h control (Figure 1. C).

TLR 4 and 2 mRNA Expression Monitored under PGN Stimulation in Duct Epithelial Cells

Different concentrations of PGN were assessed to determine the concentration with the highest cell response, by monitoring the levels of TLR 2 and 4 mRNA. We quantified for both TLR 2 and 4 because of the results obtained from the LPS stimulation. PGN significantly upregulated ($p<0.05$) TLR 4 during the 24h induction at 1 and 2 μg/ml concentration but not for the TLR 2 mRNA (Figure 2. A and B). As shown in Figure 2. C, a slight upregulation in TLR 2 occurred after PGN induction for 72h. The late response

observed from the PGN stimulation might be because these duct epithelial cells are not expressing the appropriate additional toll like receptor involved in its recognition. When TLR 4 mRNA was quantified after PGN stimulation, an upregulation at 24, 48, and 72h significantly ($p<0.05$) was observed in comparison with that of the 0 h control (Figure 2. D).

TLR 4 and 2 mRNA Expression Monitored under LTA Stimulation in Duct Epithelial Cells

LTA did not affect both TLR 2 and 4 mRNA expression after 24 h stimulation when monitored over a range of concentrations 0-10 μg/ml (Figure 3. A and B). However, longer time of stimulation did affect the both TLR 2 and 4 expression at the concentration of 2 ug/ml. Specifically, TLR 2 mRNA was significantly ($p<0.05$) upregulated at 48 and 72h (Figure 3. C). In Figure 3. D, TLR-4 mRNA was significantly ($p<0.05$) upregulated at 24, 48 and 72 hours.

In Vivo Experiment

TLR 4 and 2 mRNA Expression in Bovine Milk Somatic Cells under *E. coli* Intramammary Infection

Milk samples were collected from 8 cows in the non infected and infected quarters at 0, 8, 16, 24, 32, 40, 48, 72, and 96h. Quantification of the TLR 2 and 4 mRNA was performed by Real –Time PCR from the RNA extraction of the leukocytes obtained in the milk samples. Five cows were analyzed for TLR 4 expression due to lack of sufficient samples from 3 other animals. Large individual variability prevented us to have a meaningful statistical analysis. However, all five cows showed increased TLR 4 expression, at one or more time points (Figure 4 A). All cows showed at least a five fold increase for TLR 4 but at different times. Cow 1 was responding at time 40h, cow 2 was responsive at time 48h and continued to increase with a down expression, cow 3 was responding at time 6h and again higher at time 40, cow 4 also showed a response at 40h, and finally cow 5 responded at 16h. Because the cows responded to *E. coli* at different time

points we could not determine with statistical significance at which exact time point the response was initiated.

During the preliminary study, samples from 2 cows were analyzed for TLR 2 expression. Unlike TLR 4, no significant increases were observed for all time points. Thus, it is concluded that TLR 2 expression were not induced by *E. coli* challenge (Figure 4 B).

TLR 4 and 2 mRNA Expression in Bovine Milk Somatic Cells under *S. aureus* Intramammary Infection

Milk samples were collected from 3 cows in the non infected and infected quarters at 0, 8, 16, 24, 32, 40, 48, 72, and 96h. Quantification of the TLR 2 and 4 mRNA was performed by Real –Time PCR from the RNA extraction of the leukocytes obtained in the milk samples. Again, large individual variation was obvious. Cow 3 responded to *S. aureus* challenge with increased TLR 2 expression (Figure 5 A.), while cow 2 responded to *S. aureus* with increased TLR 4 expression (Figure 5. B). At the same time, TLR 2 or TLR 4 expressions in two other cows remained unchanged.

DISCUSSION

Together the innate immune cells and the epithelial cells function in the recognition of pathogens in the mammary gland. After initial identification of cytokine mRNA upregulation in milk somatic cells for *E. coli* and *S. aureus* challenged quarters of cows (Bannerman et al., 2004), we were interested in trying to identify by which signaling mechanism these mRNA cytokines were being upregulated. Both *in vitro* and *in vivo* analyses were performed in order to investigate if the mammary duct epithelial cells respond in a manner similar to milk somatic cells. In the case of *E. coli*, LPS is the component responsible for initiating an inflammation. For *S. aureus* infections, LTA and PGN have been reported to cause inflammatory responses. Specific Toll like Receptors have been identified to be involved with immune function and to recognize specific ligands. The presence of these types of receptors is very much defined in innate immune cells whereas there receptor populations on other tissues have been less well characterized. It has been demonstrated that vascular endothelial, adipocytes, intestinal epithelial cells do expresses the specific TLR under stimulation (Akira et al., 2001), but less is known or mentioned about TLR expression in mammary epithelial cells which compose the inner lining of the mammary gland. In order to identify if indeed these receptors are present and involved with the recognition of *E. coli* and *S. aureus* bacterial components we monitored their mRNA expression in a primary bovine duct epithelial cell line at different time points. Previously another cell line of bovine mammary epithelial cells, the MAC-T cells have demonstrated their ability to respond to LPS by upregulating IL-1 and 8 mRNA and protein levels (Boudjellab et al., 2000). Our findings demonstrate that both the Toll like Receptors 4 and 2 were upregulated significantly ($p<0.05$) after *E. coli* LPS and *S. aurues* LTA and PGN stimulation with differences in their time of onset. There was a significant ($p<0.05$) upregulation early on after LPS stimulation, thus demonstrating the potency of this bacterial component which is quick and strong. There was some difference observed as shown in Figure 1 A and C at 24 h. There are many parameters which can affect this observation, for example, stage of the cell growth, external environment, pipetting errors, etc. The primary difference was that the cells were not grown in parallel, and the

environmental factors surrounding the experiment might have had an effect on cell response.

PGN increased TLR 4 mRNA expression after 24h stimulation but had no effect on TLR 2 mRNA expression until 72h. It has been reported that other TLRs (1 and 6) are necessary for PGN functions and must heterodimerize before any response is mounted (Ozinsky et al., 2000). It also has been shown that for PGN the recognition occurs in phagosomes of macrophages which engulf the component. These might explain the late response of the epithelial cells to PGN. Why TLR 4 mRNA increased after PGN stimulation remains to be determined. To our knowledge no reports demonstrate the ability of TLR 4 to recognize PGN even though the bacteria possess this structural subunit. One of the reasons for which TLR 4 might be responding is because TLR 2 is not and that the epithelial cells are trying to mount an inflammation in its presence. This interesting observation needs further study.

Bovine mammary duct epithelial cells mounted a much quicker response after LTA stimulation. TLR 2 is being upregulated significantly after 48 and 72 hours ($p<0.05$), while TLR 4 mRNA was significantly upregulated at the 24, 48 and 72h time points. LTA is a more potent stimulator than PGN when it comes to initiating an inflammation in epithelial cells because LTA activates both the TLR 2 and 4 receptors (Janeway et al., 2002). LTA has been shown to stimulate lung epithelial cells (Wang et al., 2003) and bind epithelial cells. If LTA is similar in structure to LPS (Deininger et al., 2003), which has been demonstrated to be internalized inside the cells (Hornef et al., 2002) in order to stimulate a response then perhaps LTA is also being internalized and activating TLR 4. It remains to be determined why the epithelial cells under LTA stimulation would express both TLR 4 mRNA and TLR 2 mRNA in order to have a better recognition of the PAMP. One plausible explanation is that the epithelial cells are producing cytokines which regulate the expression of TLRs, as reported in Takeda et al. (2003). The upregulation of TLR during stimulation via LPS, LTA and PGN PAMPs demonstrates that mammary epithelial cells are capable of participating in pathogen recognition in order to possibly mount a quicker inflammatory response. Since the mammary gland is open to infection and that the

pathogen has reached the duct epithelial cells should set off an alarm and signal for reinforcements to control the invasion.

We also noticed that TLR 4 mRNA is preferentially up regulated earlier than TLR 2 mRNA in spite of the bacterial component used. This may be a defence mechanism of the cell which is interlinked to immune function since TLR 4 is capable of recognizing a broad range of different PAMPs, so it gets expressed before TLR 2. The TLR 4 and 2 genes are situated on different chromosomes 8 and 17 respectively (White et al. 2003) and may be regulated under different modulators, which might be a reason for differences seen in their time of expression.

A total of 16 cows were used to study *E. coli* and *S. aureus* pathogen induced inflammations. Quarters challenged with *E. coli* or *S. aureus* demonstrated clinical symptoms in elevated rectal temperature after 16h and 32h with an increase of milk somatic cells, primarily neutrophils, after 16h and 24h respectively when compared to the quarters injected with saline (Bannerman et al., 2004). Even though a large individual variability prevents us from determining the time point where all cows were responding, we observe trends within the cows for each of the TLR monitored. In the *E. coli* challenge five cows showed increased TLR 4 expression, at least during one time point (Figure 4 A). At least five fold increase was seen but appeared at different times from the initial challenge time. This observation simply demonstrated that cows did respond to *E. coli* by TLR 4 upregulation but varied in their response to the pathogen. TLR 2 is not upregulated in the two cows we tested, presumably due to the fact TLR 4, not TLR 2, responds to the pathogen *E. coli.*

In the *S. aureus* challenged cows, TLR 2 and 4 mRNA expression in the milk somatic cells seems to be early for TLR 2 mRNA analysis from cow 3 at 8h but in the case of TLR 4 only cow 2 showed a response. In order to limit the variability a larger sample size is needed. This will have to be taken into consideration for future studies.

In summary, it is possible that epithelial cells in the bovine mammary gland do participate in pathogen recognition through pathogen recognition receptors known as the

Toll like Receptors. we can conclude that the epithelial cells are expressing TLR 4 and 2 during pathogen structural component stimulation. Due to the large variation, no consistent responses were observed in the milk somatic cells.

ACKNOWLEDGEMENTS

This study was sponsored by the Dairy Cattle Genetics Research and Development Council of Canadian Dairy Network, Valorisation Recherche Québéc and Natural Sciences and Engineering Research Council of Canada awarded to Dr. Xin Zhao.

REFERENCES

Alluwaimi A. M., Leutenegger C. M., Farver T. B., Rossitto P. V., Smith W. L., Cullor J. S. 2003. The cytokine markers in *Staphylococcus aureus* mastitis of bovine mammary gland. J. Vet. Med. 50:105-111.

Akira S., Takeda K., Kaisho T. 2001.Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2:675-680.

Bannerman D. D., Paape M. J., Lee J.-W., Zhao X., Hope J. C., Rainard P. 2004. Escherichia coli and Staphylococcus aureus elicit different innate immune responses following intramammary infection. Clin. Diagn. Lab. Immunol. 11:463-472.

Boudjellab, N., H.S. Chan-Tang and X. Zhao. 2000. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp. Physiol. Biochem. 127:191-199.

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

Bustin S A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocri. 25:169-193.

Dalpke A. H., Lehner M. D., Hartung T, Heeg K. 2005. Differential effects of CpG-DNA in Toll-like receptor-2/-4/-9 tolerance and cross-tolerance. Immunol. 116:203-212.

Deininger S., Stadelmaier A, von Aulock S, Morath S, Schmidt RR, Hartung T. 2003. Definition of structural prerequisites for lipoteichoic acid-inducible cytokine induction by synthetic derivatives. J Immunol. 170: 4134-4138

Eckmann L, Kagnoff MF, Fierer J. 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. Infect Immun. 61:4569-4574.

Goldammer T., Zerbe H., Molenaar A., Schuberth H. J., Brunner R. M., Kata S. R. , Seyfert H. M. 2004. Mastitis Increases Mammary mRNA Abundance of β-Defensin 5, Toll-Like-Receptor 2 (TLR 2) and TLR 4 but not TLR 9 in Cattle. Clin. Diag. Lab. Immuno. 11:174-185.

Guidry A and O'Brien C.A. 2002. Bovine mammary endothelial/epithelial cell culture model of the blood/milk barrier. Methods Mol Biol. 188:85-98.

Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M, Si-Tahar M. 2004.Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR 4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR 4. J Biol Chem. 279:2712-2718.

Harada K, Ohira S, Isse K, Ozaki S, Zen Y, Sato Y, Nakanuma Y. 2003. Lipopolysaccharide activates nuclear factor-kappaB through toll-like receptors and related molecules in cultured biliary epithelial cells. Lab Invest. 83:1657-1667.

Hornef M. W., Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. J Exp Med. 195:559-570.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. 1999. Cutting edge: Toll-like receptor 4 (TLR 4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR 4 as the Lps gene product.J Immunol. 162:3749-3752.

Janeway C. A. Jr., and Medzhitov R. 2002. Innate Immune Recognition. Annual Review Immunology. 20:197-216.

Jia H. P., Starner T, Ackermann M, Kirby P, Tack B. F., and McCray P. B. J. 2001. Abundant human beta-defensin-1 expression in milk and mammary gland epithelium. J. Pediatr. 138:109–112.

Kawai T., Takeuchi O, Fujita T, Inoue J, Muhlradt P. F., Sato S, Hoshino K, Akira S. 2001. Lipopolysaccaride Stimulates the MYD88-Independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a sunset of lipopolysaccharide-iducible genes. J. Immunol. 167:5887-5894.

Lee J.-W., Paape M.J., Elsasser T.H., Zhao X. 2003. Recombinant soluble CD14 reduces severity of intramammary infection by *Escherichia coli*. Infect. Immun. 71: 4034-4039.

Medzhitov R., Preston-Hurlburt P, Janeway C. A. Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 388:394-397

Nagai Y., Akashi S., Nagafuku M., Ogata M., Iwakura Y., Akira S., Kitamura T., Kosugi A., Kimoto M., Miyake K. 2002. Essential role of MD-2 in LPS responsiveness and TLR 4 distribution. Nat Immunol. 3:667-672.

Pfaffl M.W. 2001. A new mathematic model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:2002-2007.

SAS/STAT User Guide, Version 8, SAS Institue Inc., Cary, NC, 2000.

Smits E, Cifrian E, Guidry A. J., Rainard P, Burvenich C, Paape M. J. 1996. Cell culture system for studying bovine neutrophil diapedesis. J Dairy Sci. 79:1353-1360.

Takeda K., Kaisho T., Akira S. 2003. Toll-Like Receptors. Ann. Rev.Immunol. 21:335-376.

Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S. 1999. Differential Roles of TLR 2 and TLR 4 in Recognition of Gram–Negative and Gram-Positive Bacterial Cell Wall components. Immunity. 11:443-451.

Takeuchi O, Hoshino K, Akira S. 2000. Cutting Edge: TLR 2-Defficient and MyD88-Deficient Mice Are Highly Suceptible to Staphylococcus aureus Infection. J. Immuno. 165:5392-5396.

Takeuchi O. and Akira S. 2001. Toll-like receptors; their physiological role and signal transduction system. Rev.Inter.Immunopharm. 1:625-635.

Takeuchi O. and Akira S. 2002. Genetic approaches to the study of Toll-like receptor function. Micro. Infec. 9:887-895.

Uehara A, Sugawara S, Takada H. 2002. Priming of human oral epithelial cells by interferon-gamma to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. J Med Microbiol. 51:626-634.

Underhill DM. and A. Ozinsky, 2002. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol. 14:103-110

Wang X., Zhang Z., Louboutin J. P., Moser C., Weiner D. J., Wilson J. M. 2003. Airway epithelia regulate expression of human beta-defensin 2 through Toll-like receptor 2. FASEB J.17:1727-1729.

White S. N., Kata S. R., Womack J. E. 2003. Comparitive fine maps of bovine toll-like receptor 4 and toll-like receptor 2 regions. Mamm Genome. 2:149-155

Zarember K. A. and Godowski P. J. 2002. Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNA in Leukocytes in Response to Microbes, Their Products, and Cytokines. J. of Immunol. 168:554-561.

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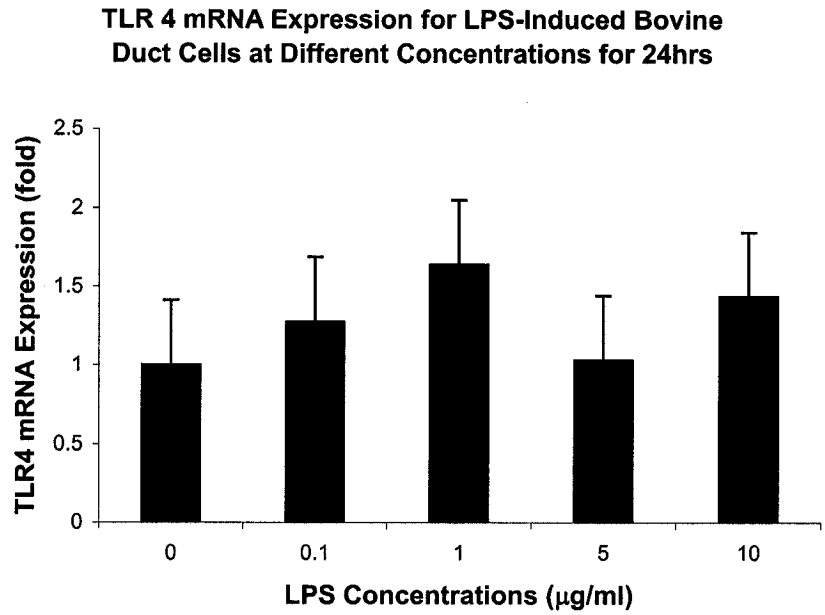

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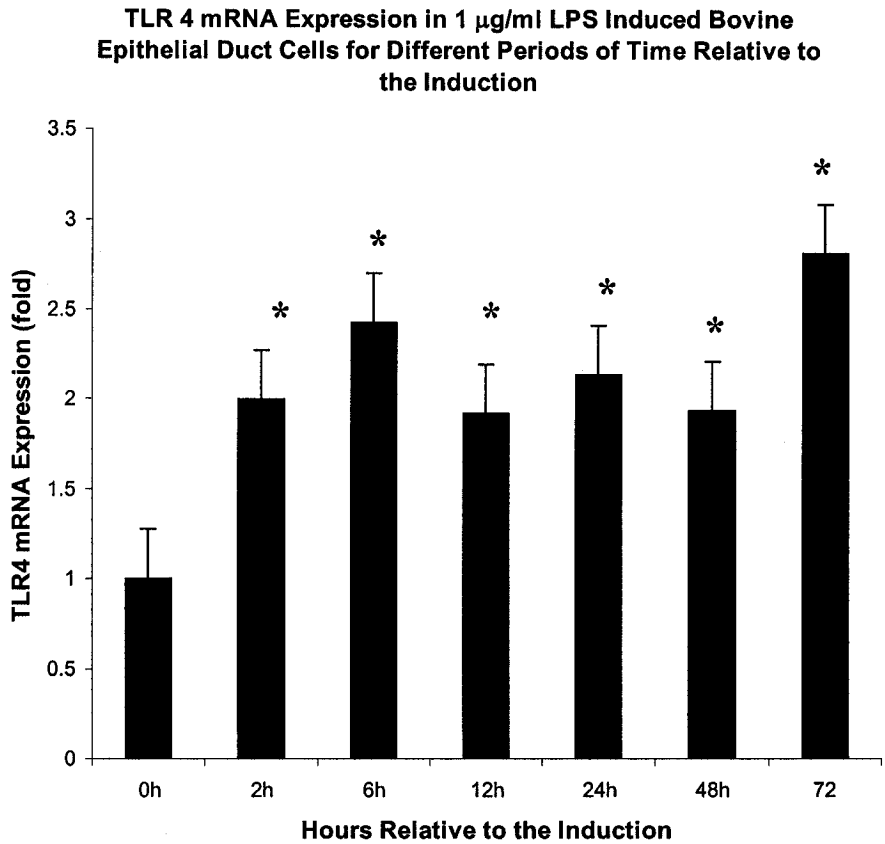
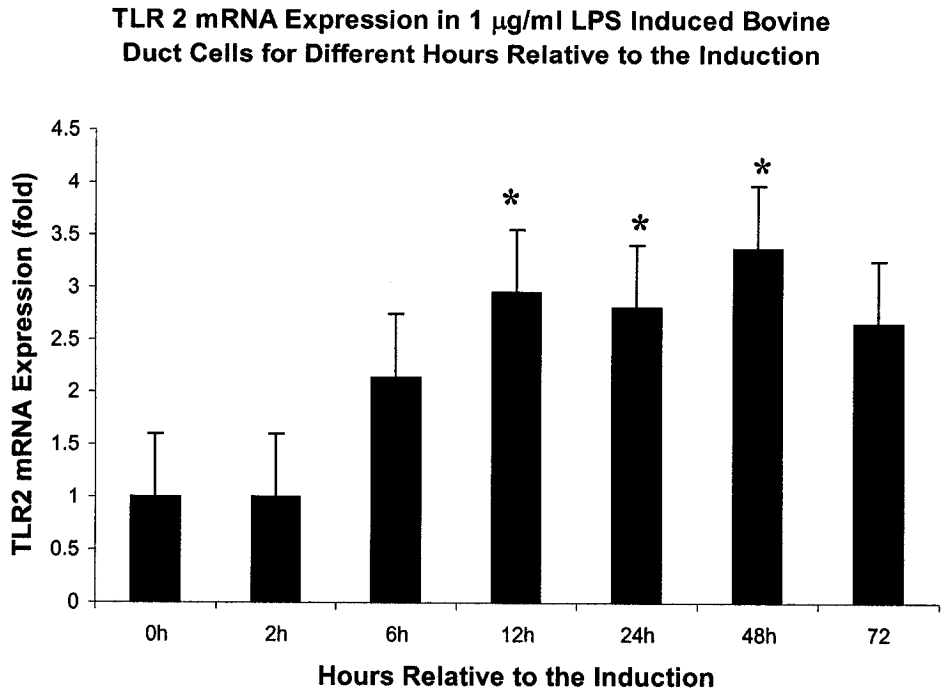


* $P < 0.05$ when compared to the 0h

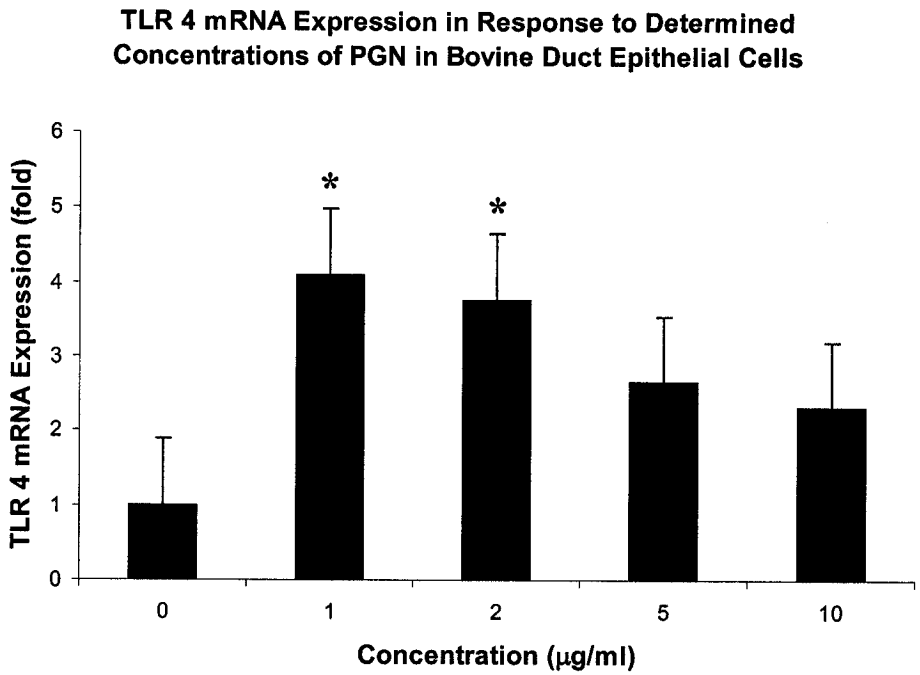
C



* $P < 0.05$ when compared to the 0h

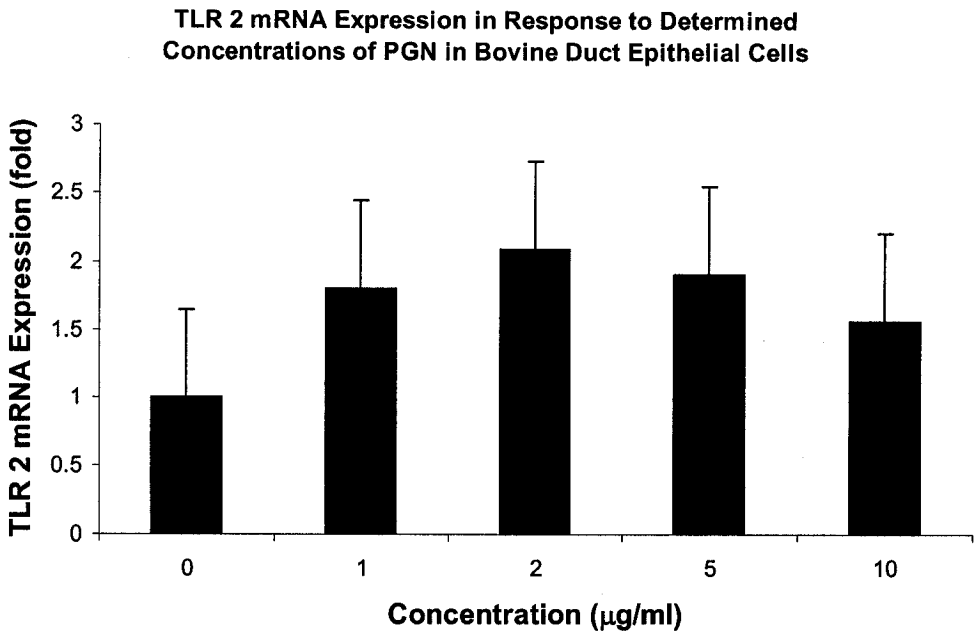
Figure 1. A. TLR 4 mRNA expression in response to determined concentrations of LPS in bovine duct epithelial cells. **B.** TLR 4 mRNA expression in response to 1μg/ml LPS in bovine duct epithelial cells for determined periods relative to the induction. **C.** TLR 2 mRNA expression in response to 1μg/ml LPS in bovine duct epithelial cells for determined periods relative to the induction.

A

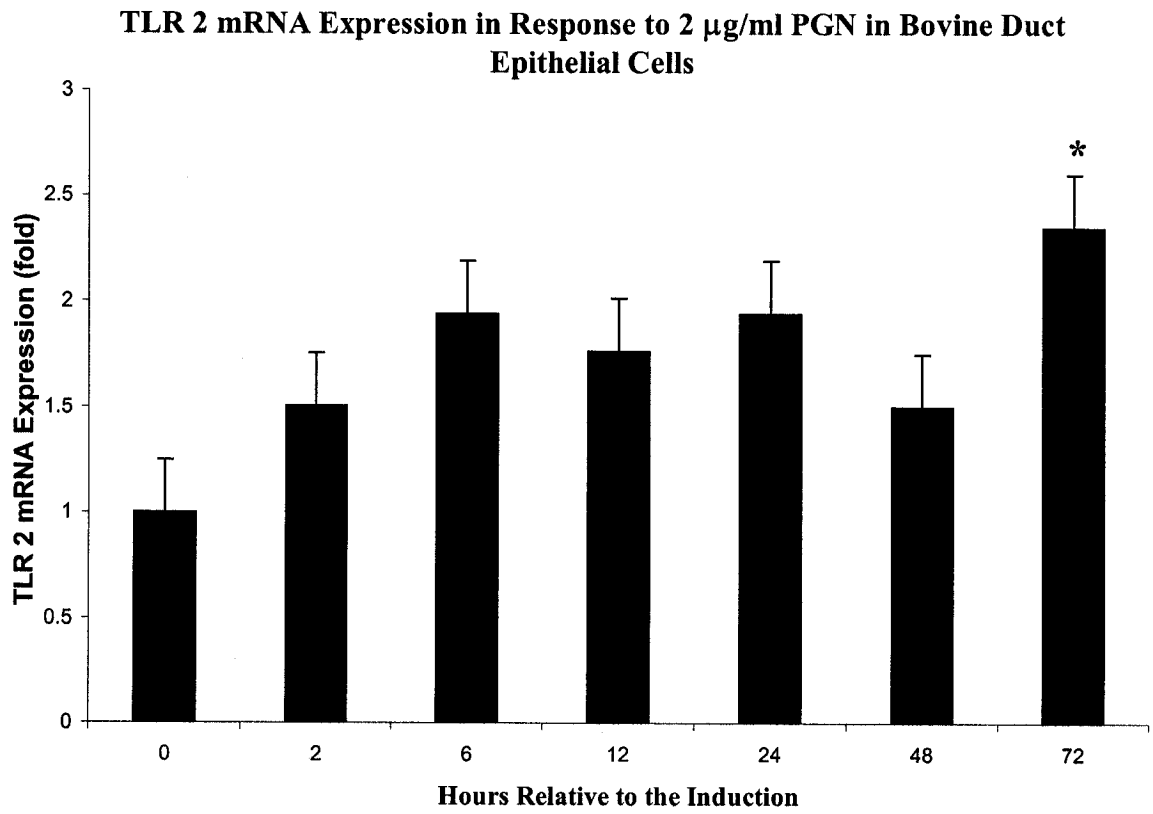


* $P < 0.05$ when compared to the 0 μg/ml concentration

B

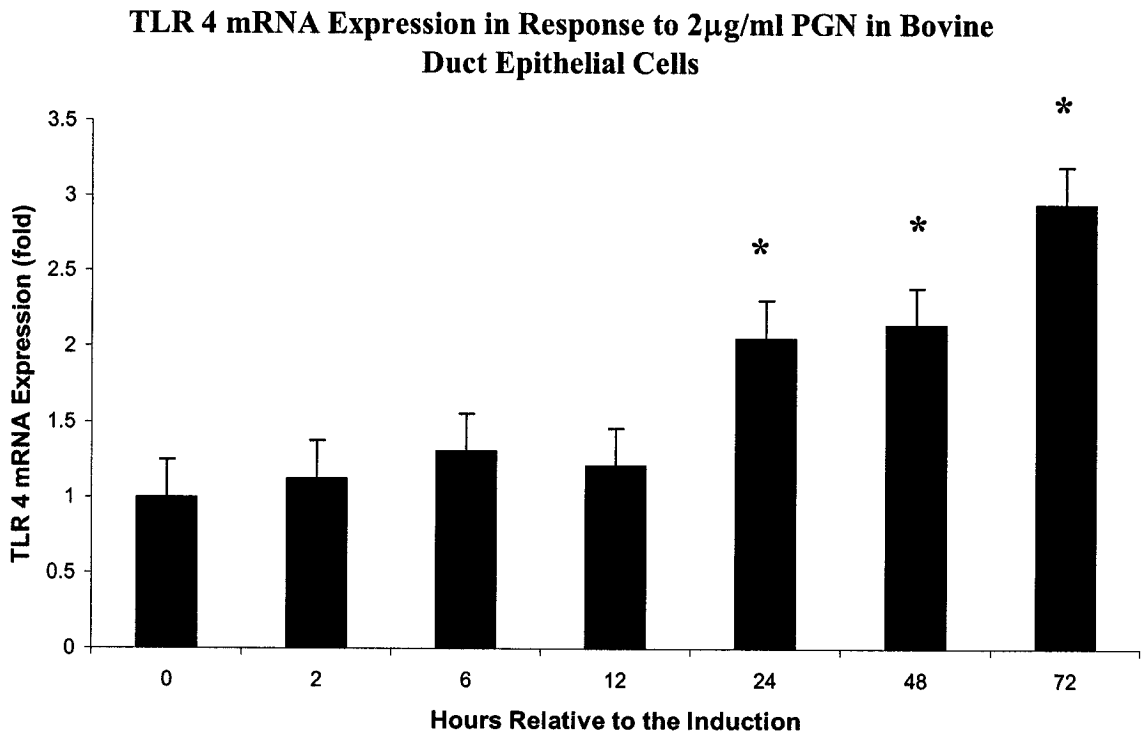


C



* $p < 0.05$ when compared to 0hr

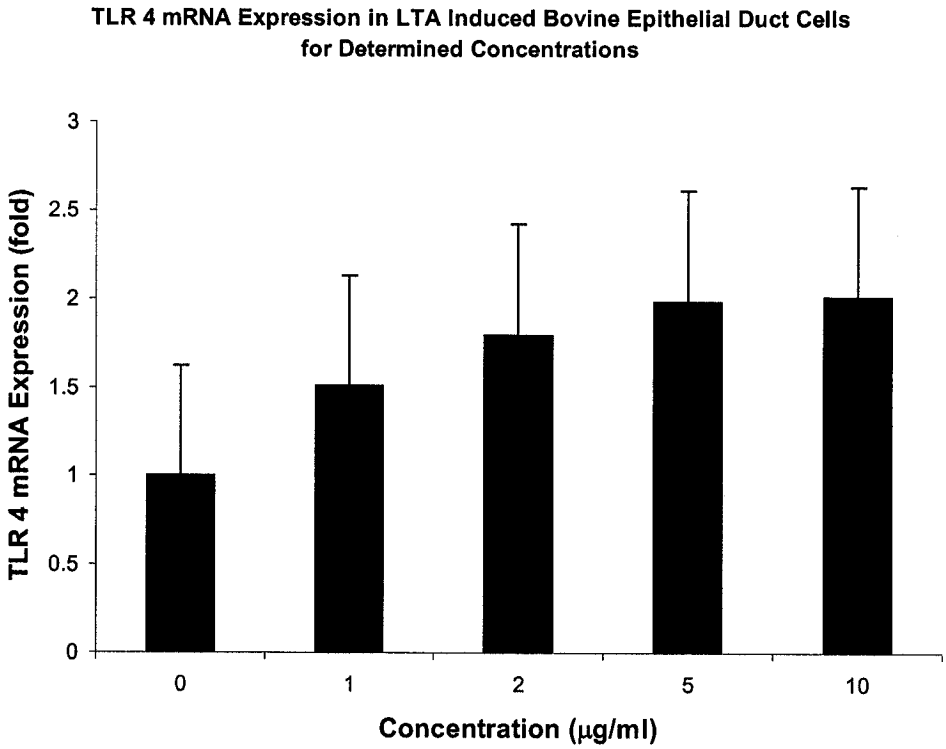
D



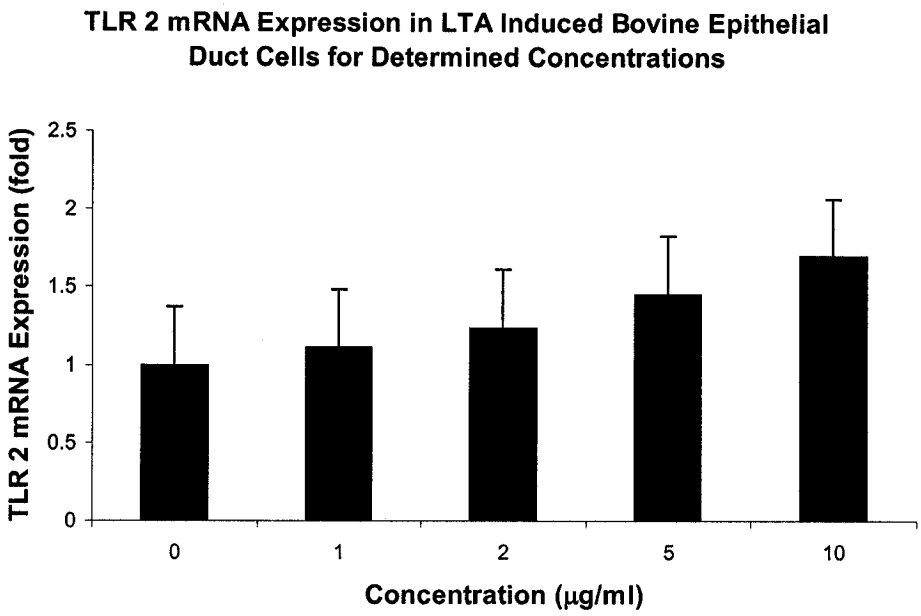
* $P < 0.05$ when compared to 0hr

Figure 2. A. TLR 4 mRNA expression in response to determined concentrations of PGN in bovine duct epithelial cells **B.** TLR 2 mRNA expression in response to determined concentrations of PGN in bovine duct epithelial cells **C.** TLR 2 mRNA expression in response to 2μg/ml PGN in bovine duct epithelial cells for determined periods relative to the induction. **D.** TLR 4 mRNA expression in response to 2μg/ml PGN in bovine duct epithelial cells for determined periods relative to the induction.

A


TLR 4 mRNA Expression in LTA Induced Bovine Epithelial Duct Cells for Determined Concentrations
TLR 4 mRNA Expression (fold)
3
2.5
2
1.5
1
0.5
0
0
1
2
5
10
Concentration (μg/ml)

B


TLR 2 mRNA Expression in LTA Induced Bovine Epithelial Duct Cells for Determined Concentrations
TLR 2 mRNA Expression (fold)
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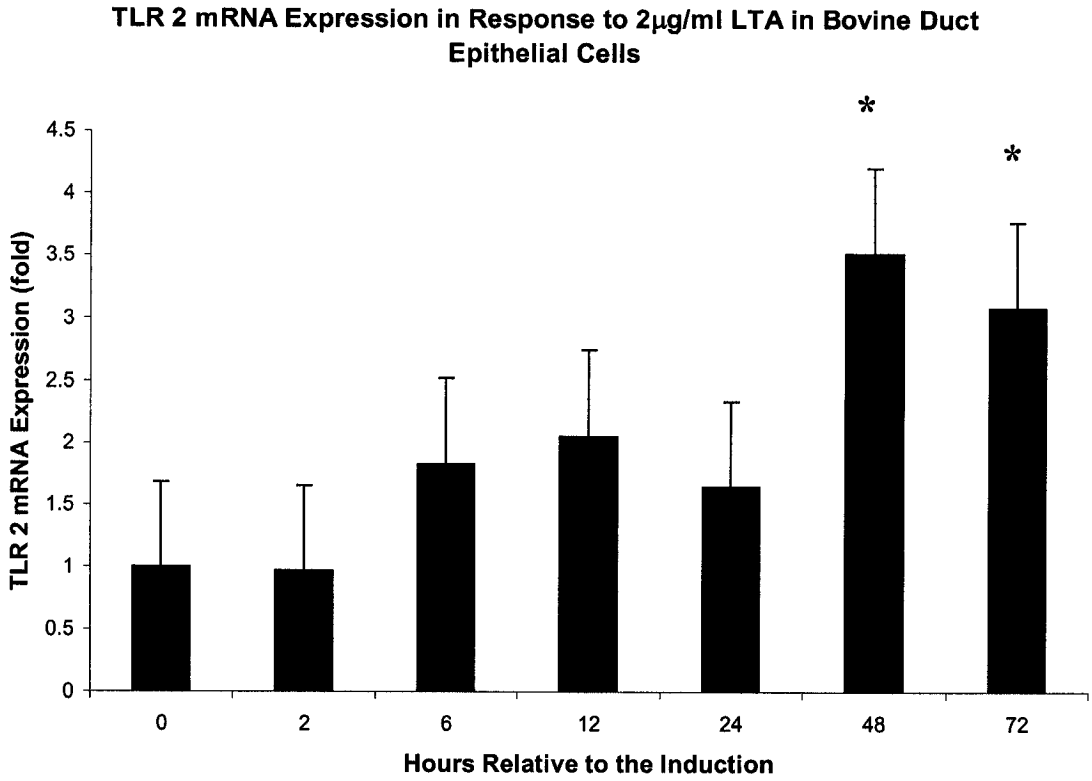
2
1.5
1
0.5
0
0
1
2
5
10
Concentration (μg/ml)

C



* $P < 0.05$ when compared to 0hr

D

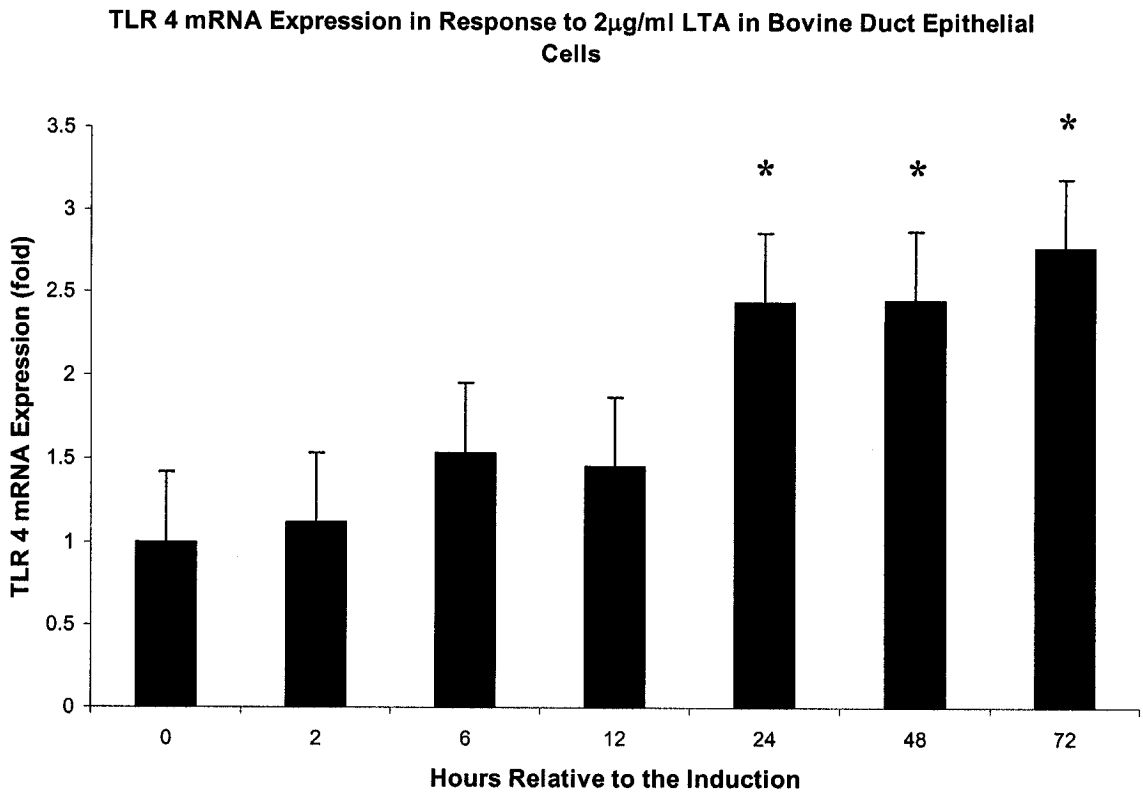
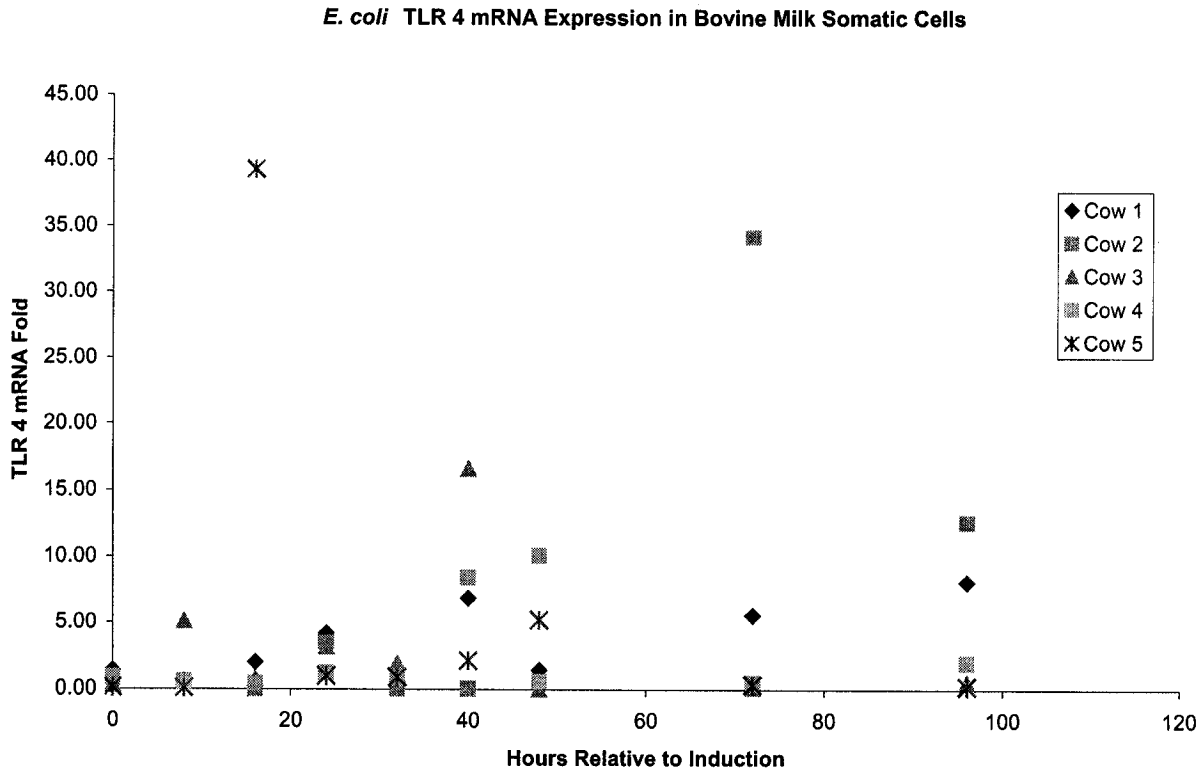


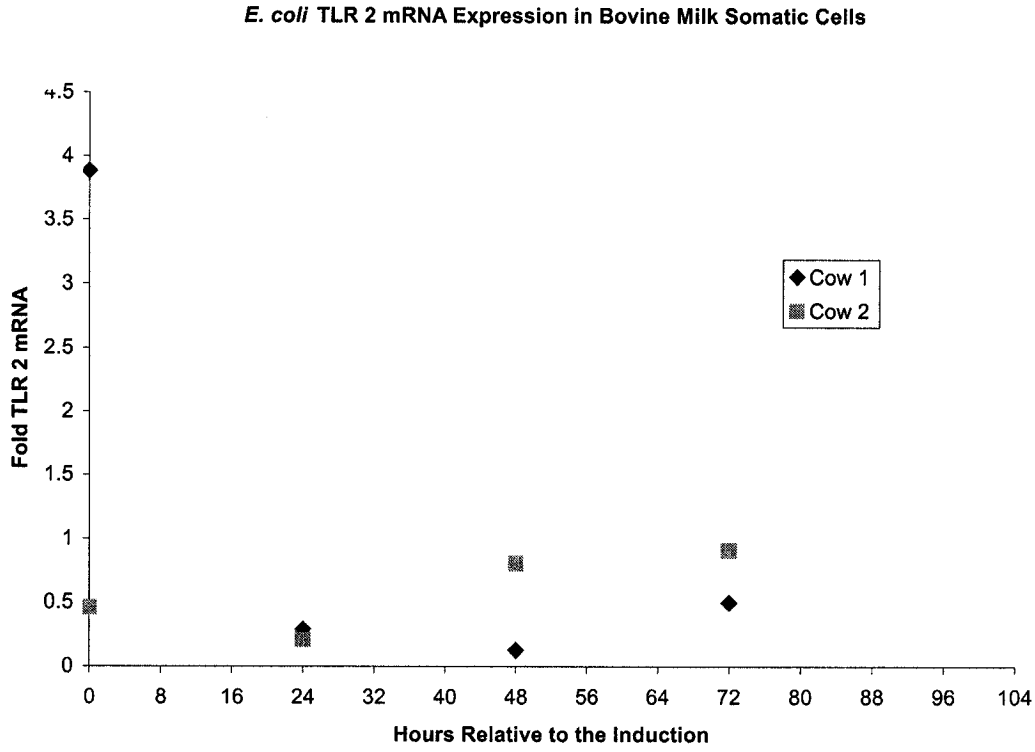
Figure 3. A. TLR 4 mRNA expression in response to determined concentrations of LTA in bovine duct epithelial cells **B.** TLR 2 mRNA expression in response to determined concentrations of LTA in bovine duct epithelial cells **C.** TLR 2 mRNA expression in response to 2μg/ml LTA in bovine duct epithelial cells for determined periods relative to the induction. **D.** TLR 4 mRNA expression in response to 2μg/ml LTA in bovine duct epithelial cells for determined periods relative to the induction.

A



Cow 1 = Induced Quarter over Control Quarter
Cow 2 = Induced Quarter over Control Quarter
Cow 3 = Induced Quarter over Control Quarter
Cow 4 = Induced Quarter over Control Quarter
Cow 5 = Induced Quarter over Control Quarter
Cow 6 = Induced Quarter over Control Quarter

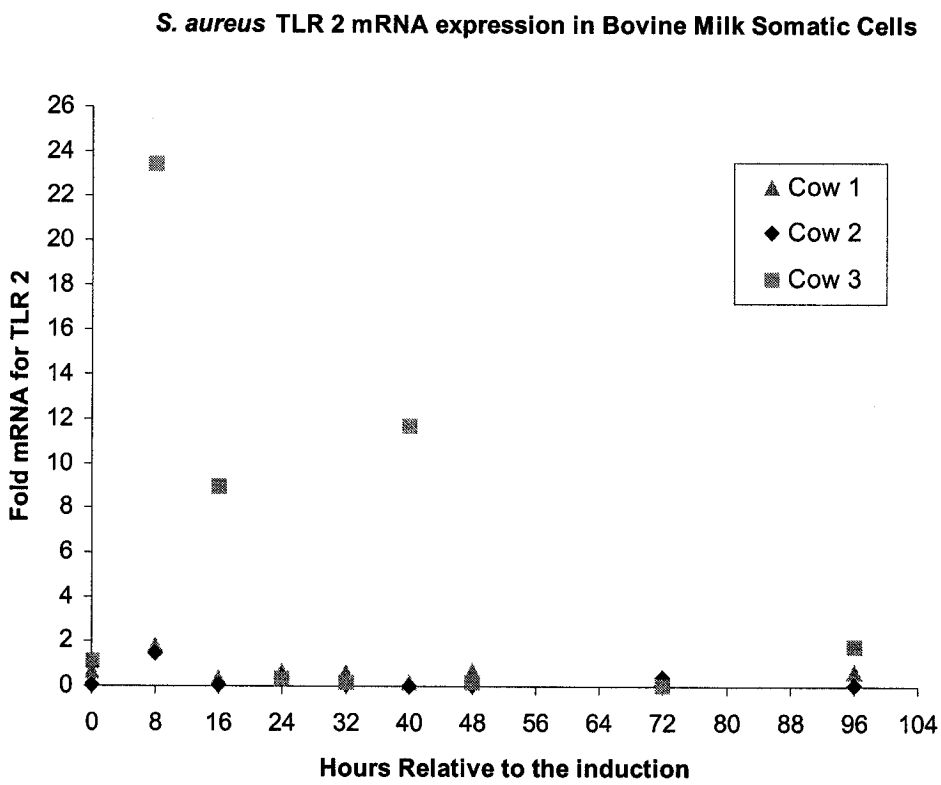
B



Cow 1 = Induced Quarter over Control Quarter
Cow 2 = Induced Quarter over Control Quarter

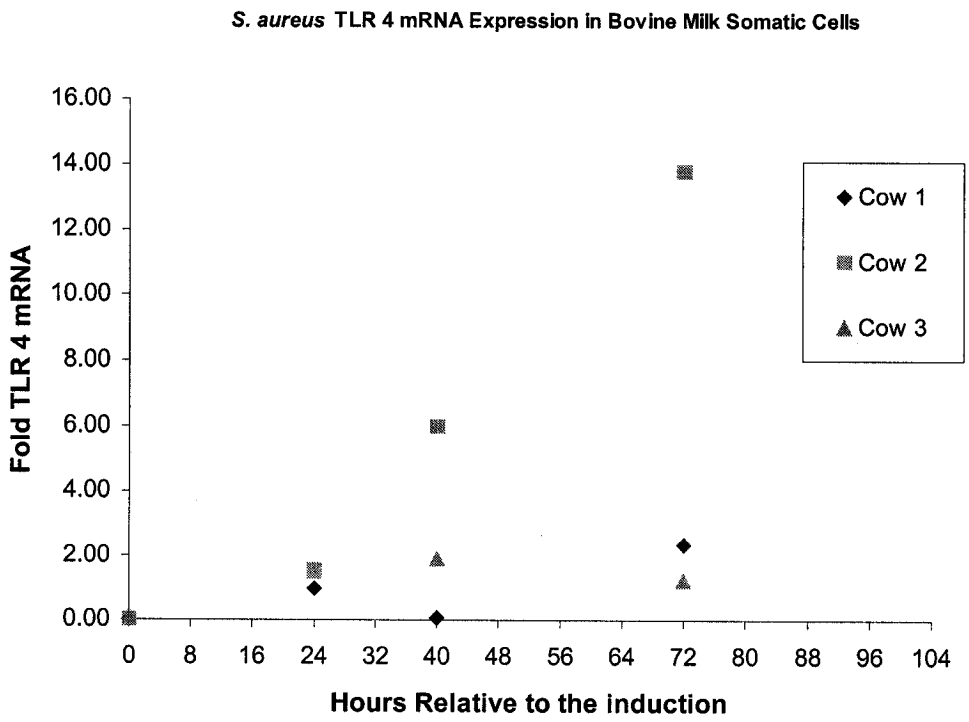
Figure 4. A. TLR 4 mRNA expression in cow somatic cells from induced quarter versus control quarter for *E. coli* **B.** TLR 2 mRNA Expression in cow somatic cells from induced quarter versus control quarter for *E. coli.*

A



Cow 1 = Induced Quarter over Control Quarter
Cow 2 = Induced Quarter over Control Quarter
Cow 3 = Induced Quarter over Control Quarter

B



Cow 1 = Induced Quarter over Control Quarter
Cow 2 = Induced Quarter over Control Quarter
Cow 3 = Induced Quarter over Control Quarter

Figure 5. A. TLR 2 mRNA expression in cow somatic cells from induced quarter versus control quarter for *S. aureus* **B.** TLR 4 mRNA expression in cow somatic cells from induced quarter versus control quarter for *S. aureus*.

Gene	Primer Name	Sequence (5'-3')	Length	Accession
TLR 4	TLR 4.f20 TLR 4.r400	GGCTGCGGCTCTGATCCCAG CGGCCACCAGCTTCTGTAAAC	380	NM_174198
TLR 2	TLR 2.f2228 TLR 2.r2369	GACTTCATTCCTGGCAAGTG AGAGACGGAAATGGGAGAAG	141	AF368419
β-actin	β-actin.f38 β-actin.r428	CCTTTTACAACGAGCTGCGTGTG ACGTAGCAGAGCTTCTCCTTGATG	391	AH00130

Table 1. Sequences of primers for bovine TLRs and β-actin in real time PCR.

CONNECTING STATEMENT I

In Chapter II, the identification of Toll like Receptors in the bovine mammary duct epithelial cells defines the route by which these cells are involved in initiating a response due to pathogens. The identification of other genes involved in either *E. coli* or *S. aureus* induced infections, is crucial to understanding host-pathogen interactions. By determining the genes responsible in the infection one can then use this acquired information to design a technique to differentiate the mastitis resistant from the mastitis prone cows by use of gene specific targets.

CHAPTER III. IDENTIFICATION AND CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED GENES BY BOVINE MAMMARY CELLS IN RESPONSE TO CELL WALL COMPONANTS OF *ESCHERICHIA COLI* AND *STAPHYLOCOCCUS AUREUS* BY DIFFERENTIAL DISPLAY RT-PCR

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ABSTRACT

Mastitis is most often caused by pathogens such as *E. coli* and *S. aureus* and results in enormous economic losses for the dairy industry. There is a great need to discover new routes to treat the infection without the use of antibiotics because of the problems associated with their administration. By using a molecular approach to identify the response observed in the presence of an invasion it will allow us to monitor which genes get turned on or off as a consequence. With the identification and characterization of specific genes expressed within a particular invasion will help to design methods targeted towards those genes. Until now the identification of genes in the mammary gland of the bovine is very limited to certain cytokines which are involved with the initiation of the inflammatory response. DD-PCR was used to identify gene differences between the control non-induced and induced bovine duct epithelial cells for the immunogenic components of *E. coli* LPS and *S. aureus* LTA, and PGN. Fragments were obtained and sequenced with limited homology for the *E. coli* samples due to the size in base pairs obtained, where as for *S. aureus* LTA the identification of a chemokine CXC-6/GCP-2 was identified, confirmed ($p<0.001$) and characterized to be involved in neutrophil migration in conjunction with IL-8 who also demonstrated to be significantly upregulated in the induced samples 15.49 ± 2.98 folds by LTA, but not PGN, stimulation compared to the control ($p<0.01$). In the case of *E. coli* inflammatory response in epithelial cells numerous fragments demonstrate to be different in their expression level either up or down, but the small fragment sizes limit our ability to properly identify their homologues. Two of these fragments identified genes involved in ATP production and α-casien down regulation but these results need to be further confirmed. These results demonstrate that it is possible to identify genes for infections but their subtle differences in their change make it a challenge in identifying them.

INTRODUCTION

Mastitis is characterized as an inflammatory response of the mammary gland due to the invasion of bacteria through the teats. The response of host innate immunity to infectious pathogens plays a pivotal role in the infection. The mammary gland of a cow is a good model to study inflammatory response induced by foreign intruders. Infections are very complex in terms of their gene expression profiles. Identification and study of these genes are crucial in understanding how the host cells respond to pathogens. Cows have become more and more susceptible to mastitis because of the increased demands in milk production on their immune systems. It has been shown that cows have different levels of resistance to mastitis-causing pathogens. The profile of gene expression in response to infection might be responsible, at least in part, for the natural resistance of an animal to mastitis. Finding genes that are differentially expressed during mastitis might be helpful to understand cellular defensive mechanisms to infectious pathogens.

During the course of infection, the expression of a gene can be altered, either up- or down-regulated in cells at site of infections, in order to mount a proper defensive mechanism to combat invading pathogens. Some genes are silent under normal conditions and are only turned on specifically to certain stimuli. To identify these differentially regulated genes, many approaches have been applied, including microarray, suppressive subtractive hybridization (SSH), serial analysis of gene expression (SAGE), and differential display (DD)-PCR. All of these techniques are capable of identifying differential gene expression but differ from one another when it comes to accuracy rapidity, ability to identify novel genes, and in their ability to differentiate genes within the same cell type. In this study the differential Display PCR technique designed by GenHunter was used because of its practical method for identifying possible genes under two differing conditions (non induced/ induced) as well as its accuracy and rapidity. The technique is based on using G, A and C anchor primers to separate the mRNA population into three sub groups where arbitrary primers are designed to randomly pick up genes within a particular mRNA/cDNA population. With the use of this technique it is possible to study different types of conditions for various types of cells. This technique is also the most preferred technique by scientists compared to all the other techniques available to study gene expression profiles.

Leukocytes, especially macrophages, are very sensitive to bacteria, or their components. A number of bacterial cell wall components, including lipoteichoic acid (LTA) and peptidoglycan (PGN) from Gram-positive bacteria, as well as lipopolysaccharide (LPS) from Gram-negative bacteria, have been demonstrated to elicit different profiles of gene expression in human and mouse macrophages (Jin et al., 1998; Boldrick et al., 2002; Nau et al., 2002). Accumulated lines of evidence indicate that epithelial cells are also involved in the recognition of infectious microorganmisms. A previous study showed that cultured bovine mammary epithelial cells (MAC-T) increased the expression of IL-1α mRNA in response to the stimulation of LPS in a dose-dependent manner (Boudjellab et al., 2000). However, a more comprehensive investigation upon the profile of gene expression in bovine mammary epithelial cells in response to bacterial components has never been implemented. Studying genes that are differentially expressed in mammary cells during infections induced by various bacterial cell wall components can help to decipher the genes which are involved in inflammatory responses. Identification of such genes is fundamental to designing better control strategies to prevent mastitis.

MATERIALS AND METHODS

Cell Culture. Bovine mammary ductal epithelial cells (kindly provided by Dr. Guidry, USDA (Guidry and O'Brien, 2002)) were cultured at 37° C in 5% CO_2 humidified atmosphere in growth medium containing 45% Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), 45% RPMI-1640 medium (Gibco), 10% Fetal Bovine Serum (FBS; Hyclone, Logan, USA), and 2% antibiotic-antimycotic solution.

Stimulation of Bovine Mammary Ductal Epithelial cells with bacterial cell wall components. Bovine Mammary Duct Epithelial cells, when reached 90% confluence, were incubated in stimulating medium (DMEM/RPMI-1640 supplemented with 1% inactivated FBS) containing lipopolysaccaride (LPS, 10 μg/ml)) (sigma), lipoteichoic acid (LTA, 2μg/ml) (Medicorp), or Peptidoglycan (PGN, 2 μg/ml) (Medicorp) for 24 h. Cells cultured in stimulating medium without any stimulus were used as the control.

RNA Extraction. At the end of incubation, total RNA extraction was performed by using TRIZOL (GIBCO/BRL, Gaithersburg, MD) according to the manufacture's instructions. The monolayer of bovine mammary duct epithelial cells was lysed by adding 5 ml of TRIZOL reagent directly into the culture dish with an area of 157cm^2. For each ml of the mixture, 0.2 ml of chloroform (phase separation) was added followed by centrifugation at 12 000 x g for 15 min, 4°C. The RNA, retained in the aqueous phase, was precipitated by mixing with an equal volume of isopropanol and washed with 75% ethanol twice. Afterward, an appropriate amount of DEPC-treated water was added to dissolve the RNA,

and the concentration was determined by the optical density value at 260nm. RNA was stored at –80°C until use.

Infection Identification/Confirmation. Cells subjected to LPS induction versus the controls were monitored for IL-1 α presence or not by RT PCR. For cells induced with LTA and PGN have been demonstrated to express IL-8 (Kumar et al. 2004; Uehara A. et al.2002) and the cells were monitored for the expression of this gene in both the induced and controls by RT PCR.

Differential Display Reverse Transcription-Polymerase Chain Reaction (DDRT-PCR). DDRT-PCR was conducted, according to the general protocol provided by Liang and Pardee (1992), with the kits purchased from Genhunter Corporation Inc. The RNAimage kits composed of oligo-anchor primers and arbitrary primers were used to identify any differentially expressed unknown genes in bovine mammary ductal epithelial cells before and after treatment with LPS (10mg/ml), LTA (2mg/ml) and PGN (2mg/ml) after a 24-hour induction.

The reverse transcription (RT) reaction was conducted using the RNaimage Kit (GenHunter Corporation) in a total volume of 21 μl containing 0.2 μg of total RNA, 0.2 μM of one-based-anchored oligo (AAGC-T_{11}M, M = C, A, T), 19 μM of dNTP, 5× first strand buffer (GenHunter, RNaimage Kit), 2 μg RNase Out (Invitrogen Canada Inc. Burlington, Ontario) and 100 U MMLV Reverse Transcriptase (GenHunter, RNaimage Kit). The mixture was heated at 65°C for 5 min, incubated at 37°C for 60 min, for the RT reaction. Thereafter, the temperature was raised to 70°C for 5 min to inactivate the reverse transcriptase. Synthesized cDNA was kept at -20°C until being used.

The Polymerase Chain Reaction was conducted using the RNaimage Kit (GenHunter Corporation) in a total volume of 20 μl containing 2.0 μl of cDNA from the previous RT reaction, 2 μM of dNTPs, 0.2 μM of one-based-anchored oligo (AAGC-T_{11}M, M = C, A, T), 0.2 μM of arbitrary primer (see appendix for all primer sequences used), 10X PCR Buffer (GenHunter, RNaimage Kit), 0.05 U Taq DNA Polymerase (Qiagen), 20 Ci/mmole of α-[^{33}P]dATP (Amersham). The mixture was heated at 94°C for 30 seconds, 40°C for 2 min, 72°C for 30 seconds for 40 cycles for the PCR reaction. Thereafter, the temperature was raised to 72°C for 5 min. The PCR products were then electrophoresed in a 6% denaturing polyacrylamide gel, transferred to 3M paper and dried at 80C for 1 hour. The identification of differentially expressed genes was obtained by Kodak MS film radiography overnight. Bands of interest were then reamplified in a total volume of 40 μl containing 20 μM of dNTPs, 0.2 μM of each of the appropriate primer combination, 4.0 μl of cDNA band mix from gel extraction, 10 X PCR Buffer (GenHunter, RNaimage Kit), 0.05 U Taq DNA Polymerase (Qiagen). The reamplification products were then electrophoresed in a 1.5 % agarose gel to confirm their presence and appropriate molecular weights.

Cloning and Sequencing. The pGEM-T easy vector system II (Promega) was used to ligate the reamplified cDNA fragments. After the ligation reaction the ligates were then subjected to a transformation reaction with Promega JM109 High Efficient Competent Cells and plated on LB ampicillin plates after the addition of 10 μl of X- gal (50mg/ml) and 50 μl of IPTG (0.1M) in the cell mixture. Plates were left to grow overnight at 37 C for blue and white colony selection. Negative and positive controls were also set up to guide

the experiment. The white colonies were selected and streaked onto new LB ampicillin plates for purification purposes and incubated overnight at 37 C. Single colonies were then inoculated in LB and ampicillin medium overnight at 37 C with aeration/shaking. Plasmid extraction (BIO-RAD; Quantum Prep Plasmid Miniprep) was conducted on the inoculants for verification by EcoRI (Amersham GE Healthcare BioScience) restriction enzyme digestion of the fragments from the vector before sent for sequencing at the McGill Genome Center (McGill University and Genome Quebec Innovation Center; http://www.genomequebec.mcgill.ca/centre.php).

The sequences were then subjected to BLAST analysis at the NCBI database. Real-Time PCR was used to confirm fragments having the greatest differential potential expression.

Real Time RT PCR quantification of mRNA for differentially expressed genes. The Lightcycler real-time polymerase chain reaction (PCR) was carried out as described by Pfaffl 2001, with modifications. Briefly, primers for specific bovine genes, as listed in Table 1, were synthesized (Invitrogen, Burlington, Ontario). The reaction condition for each individual gene was optimized using a QuantiTect SYBR Green PCR kit (Qiagen) in a LightCycler system (Roche) and applied to the following protocol. The cDNA was analyzed in 20 μl PCR mixture containing a final concentration of 0.5 μM primer, 1 μl of cDNA, and 2× QuantiTect SYBR green PCR mastermix. The PCR master mix contains HotStartaq DNA polymerase, SYBR green PCR buffer, dNTP mix including dUTP, SYBR green I, ROX (passive reference dye) and $MgCl_2$ (3 mM for GM-CSF and TNF-α; 2.5 mM for the others). The PCR mixture was added into a cold PCR capillary (Roche), centrifuged,

and placed in the LightCycler system. The LightCycler was programmed in 4 steps: 1) denaturation at 95°C for 15 min 2) amplification for 50 cycles of denaturation at 94°C for 15 sec, annealing at appropriate temperature (T_m) (see table 1) for 30 sec, and extension at 72°C (depending on the product length, 5 sec per 100 bp) 3) melting curve by 95 °C for 5 sec, 65 °C for 15 sec, and 95 °C for 0 sec. 4) cooling at 40 °C. The experiment was repeated 4 times for bovine GCP-2 gene.

Relative Quantification. The expression of each gene was analyzed using the relative quantification method described by Pfaffl (2001). In brief, a slope was determined from the exponential phase of set dilutions (1μg, 0.5μg, 0.1μg, 0.05μg, 0.01μg, 0.05μg) where the log of these dilution concentrations in relation to the specific fluorescence obtained from the cross point values under the optimized real-time PCR amplification condition, of each target gene or the reference gene (bovine β-actin). The amplification efficiency (E) was calculated based on the slope, where $E = 10^{[-1/\text{slope}]}$.

Statistical Analysis. Four sample folds for LTA mRNA expression were analyzed by multiple comparison, using the PROC MIXED function in the SAS software (SAS/STAT user's Guide, 2000) at a level of significance $p < 0.01$.

RESULTS AND DISCUSSION

A primary cell line of bovine mammary duct epithelial cell was used in this study as a model system. To confirm that this cell line responds to the bacterial components and to determine its suitability as a model, *E. coli* LPS and *S. aureus* LTA and PGN were used to demonstrate upregulation of already known immune regulated genes (Boudjellab et al., 2000; Kumar et al., 2004; Uehara et al., 2002). IL-1α previously identified in the MAC-T cell by Boudjellab et al. (1998 and 2000) and IL-8 were used to determine the effect the bacterial components on the duct epithelial cells (results not shown). Since positive results were observed, we decided to continue the identification of other genes possibly implicated in the presence of LPS, LTA or PGN.

Stimulation with LTA or PGN

By using the technique of differential display (DD), 28 differentially expressed genes were found in cultured bovine mammary duct epithelial cells in response to cell wall components of *Staphylococcus aureus*. After sequencing, an inflammation-related gene significantly upregulated by lipoteichoic acid (LTA), but not peptidoglycan (PGN), was confirmed to be bovine chemokine (C-X-C motif) ligand 6 (CXCL6)/granulocyte chemotactic protein-2 (GCP-2), a chemokine attracting neutrophils and inducing the release of gelatinase (MMP-9) (Figure 1). Expression of bovine GCP-2 gene after LTA stimulation was further confirmed by real-time PCR in quadruplet batches of samples. As we can see from Figure 2, GCP-2 /CXCL6, is significantly ($p < 0.01$) upregulated to 17.39 ± 1.39 folds by LTA, and not PGN. IL-8 and GCP-2 function by binding the same receptor when signaling for neutrophil recruitment (Gijsbers et al., 2005) therefore we then tested the IL-8 upregulation as well. Again, IL-8 gene was significantly ($p < 0.01$) increased 15.49 ± 2.98 folds by LTA, but not PGN, stimulation (Figure 3). The expression of a novel chemoattractant, GCP-2, as well as IL-8, is upregulated in response to LTA. Production of both chemoattractants might be associated with the recruitment of neutrophils, which facilitates clearance of invading bacteria. It seems that PGN does not activate the mammary duct epithelial cells even though it has been shown in other research to be a

potent inducer (Uehara et al., 2002). The recognition of PGN needs other TLRs such as TLR 1 and 6, to herterodimerize and be capable of recognition (Ozinsky et al., 2000). Our preliminary results show that bovine mammary epithelial cells are involved in the recognition of innate immunity toward cell wall components of *S. aureus*.

Stimulation with LPS

For the *E. coli* LPS induced duct epithelial cell samples run a denaturing gel, 6 bands which showed a change in expression were chosen to be sequenced. After sequencing the fragments were subjected to a BLASTN search for the closest homology of the query with all the genes available at the NCBI gene Bank. Each fragment identified to a gene but upon confirmation of the gene in the different batches of samples, they were not significantly found to be up regulated as seen on the denaturing gel film (Figures 4-9). Table 3 identifies which genes we found and tried to confirm by real time PCR. Because the fragments obtained are short ranging from 80 -250 bp, it is difficult to specifically identify the appropriate gene. The 2G3 gene demonstrated a 3 fold up regulation within its own sample batch subjected to DD-PCR, but was not observed in other sample batches (results not shown). One reason for this can be due to false positive identification of fragments which seem to be upregulated in one sample set because of extrinsic factors and are not observed in other batches of stimulated cells. An alternative needs to be designed to identify if these genes are indeed different in expression.

The Differential Display technique proves to be a useful tool in identifying subtle differences in gene expression as well as the potential to discover novel genes in mammary duct epithelial cells under invasion. In order to increase the probability in identifying novel genes, the use of more arbitrary primers is to key to increasing the chances.

ACKNOWLEDGEMENTS

This study was sponsored by the Dairy Cattle Genetics Research and Development Council of Canadian Dairy Network, Valorisation Recherche Québéc and Natural Sciences and Engineering Research Council of Canada awarded to Dr. Xin Zhao.

REFERENCES

Boldrick JC, Alizadeh AA, Diehn M, Dudoit S, Liu CL, Belcher CE, Botstein D, Staudt LM, Brown PO, Relman DA. 2002. Stereotyped and specific gene expression programs in human innate immune responses to bacteria. Proc Natl Acad Sci U S A.99:972-977.

Boudjellab, N., H.S. Chan-Tang, X. Li and X. Zhao. 1998. Bovine mammary epithelial cells secrete interleukin-8 in response to lipopolysaccharide stimulation. Am. J. Vet. Res. 59:1563-1567.

Boudjellab, N., H.S. Chan-Tang and X. Zhao. 2000. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp. Physiol. Biochem. 127:191-199.

Bustin S A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. of Mol. Endocri. 25:169-193.

Gijsbers K, Gouwy M, Struyf S, Wuyts A, Proost P, Opdenakker G, Penninckx F, Ectors N, Geboes K, Van Damme J. 2005. GCP-2/CXCL6 synergizes with other endothelial cell-derived chemokines in neutrophil mobilization and is associated with angiogenesis in gastrointestinal tumors. Exp Cell Res. 303:331-342.

Guidry A and O'Brien C.A 2002. Bovine mammary endothelial/epithelial cell culture model of the blood/milk barrier. Methods Mol Biol. 188:85-98.

Jin F, Nathan C, Ding A. 1998. Identification of genes involved in innate responsiveness to bacterial products by differential display. Methods. 16:396-406.

Kumar A., Zhang J. Yu F. S. 2004. Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: role of peptidoglycan in stimulating proinflammatory cytokine secretion. Invest Ophthalmol Vis Sci. 10:3513-3522

Liang P., Pardee A. B. 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Sci. 257:967-971.

Nau GJ, Richmond JF, Schlesinger A, Jennings EG, Lander ES, Young RA. 2002. Human macrophage activation programs induced by bacterial pathogens. Proc Natl Acad Sci U S A. 99:1503-1508.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29:2002-2007.

SAS/STAT User Guide, Version 8, SAS Institue Inc., Cary, NC, 2000.

Uehara A, Sugawara S, Takada H. 2002. Priming of human oral epithelial cells by interferon-gamma to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. J Med Microbiol. 51:626-634.

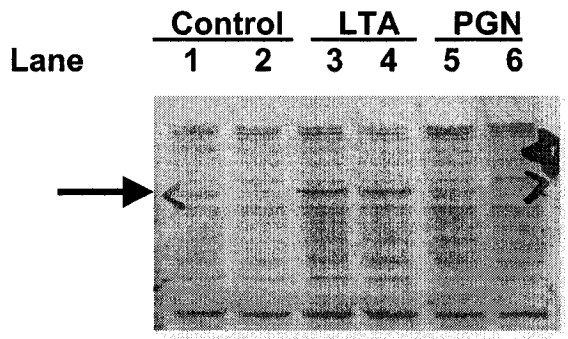

Figure 1. GCP-2/CXCL-6. *S. aureus* LTA and PGN induced bovine primary duct epithelial cells demonstrating up regulation in the LTA (2μg/ml) and not in the PGN (2μg/ml) versus the control sample.

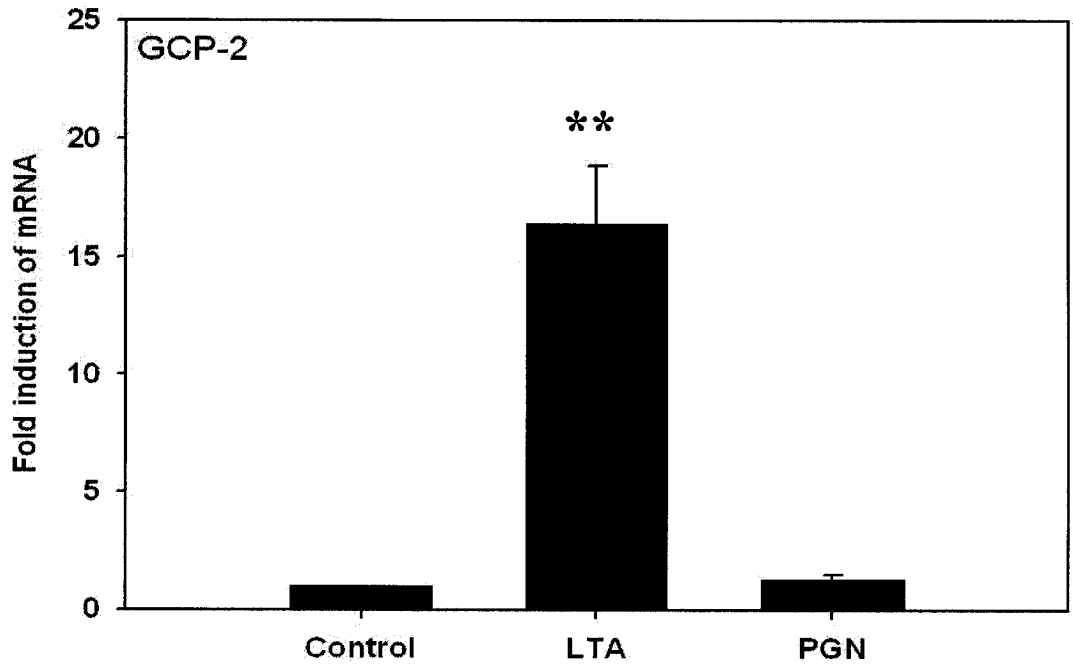

Figure 2. Fold Induction Increase of GCP-2 gene in *S. aureus* LTA and PGN **($P < 0.01$)

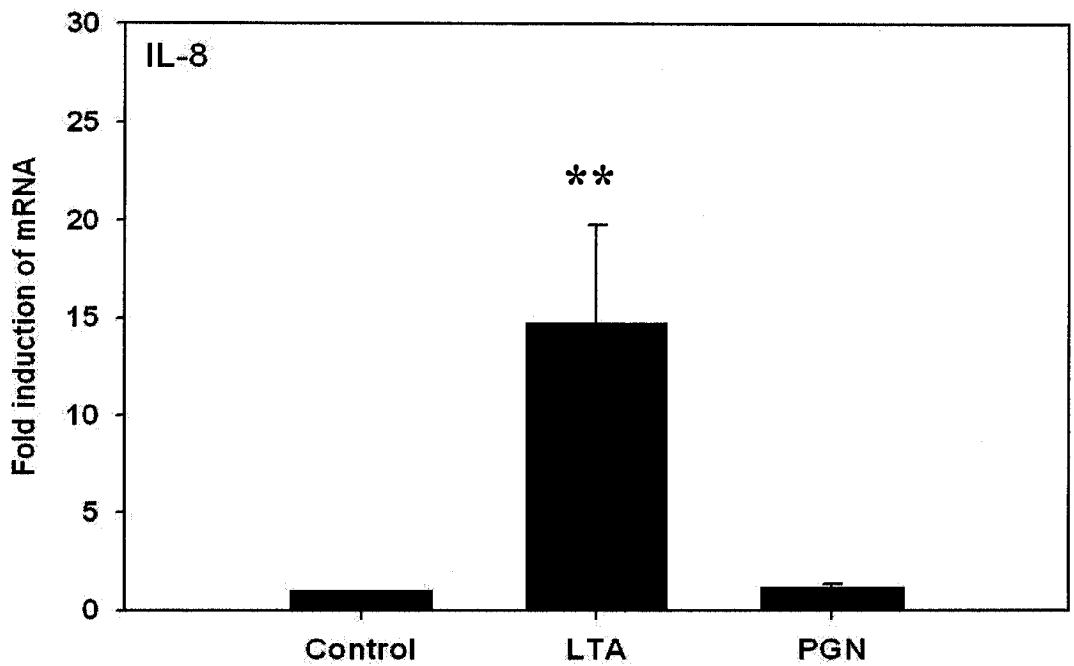

Figure 3. Fold Induction Increase of IL-8 gene in *S. aureus* LTA and PGN ** ($P < 0.01$)

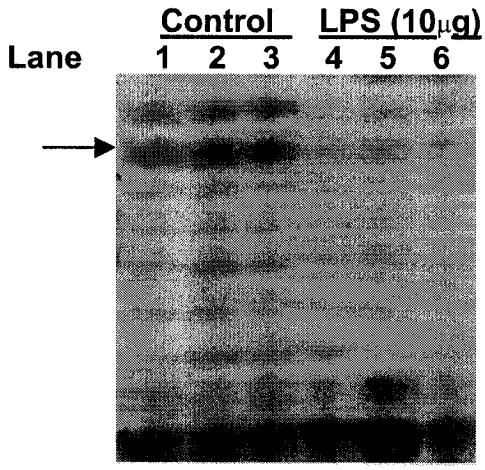

Figure 4- 3G29. *E. coli* LPS induced bovine primary duct epithelial cells demonstrating up regulation in the control versus the LPS (10μg) sample.

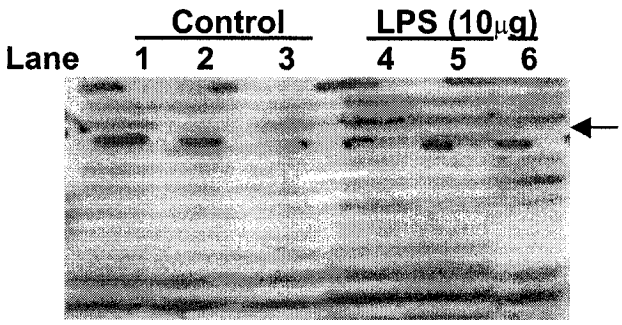

Figure 5- 4C25. *E. coli* LPS induced bovine primary duct epithelial cells demonstrating up regulation in LPS (10μg) versus the control sample.

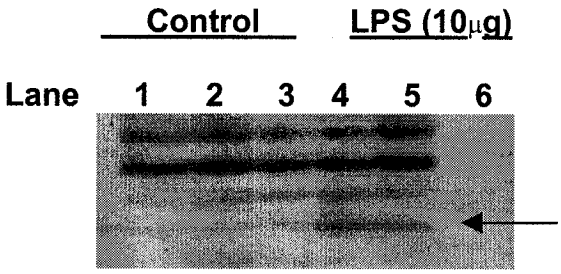

Figure 6- 4C27. *E. coli* LPS induced bovine primary duct epithelial cells demonstrating up regulation in LPS (10μg) versus the control sample.

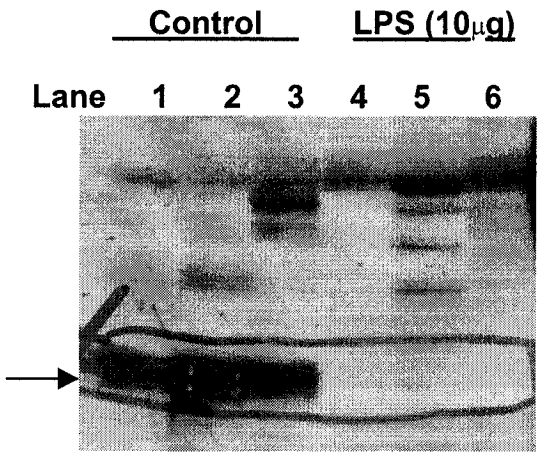

Figure 7- 2C28. *E. coli* LPS induced bovine primary duct epithelial cells demonstrating up regulation in the control versus the LPS (10μg) sample.

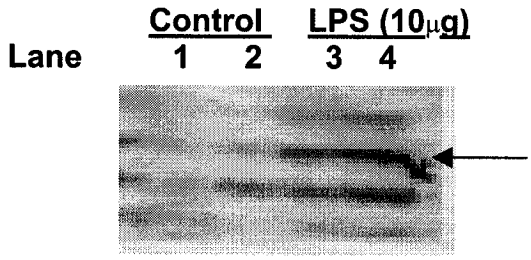

Figure 8- 4G3. *E. coli* LPS induced bovine primary duct epithelial cells demonstrating up regulation in LPS (10μg) versus the control sample

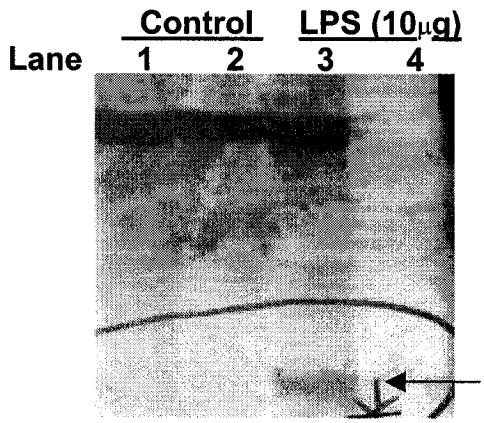

Figure 9- 2G3. *E. coli* LPS induced bovine primary duct epithelial cells demonstrating up regulation in LPS (10μg) versus the control sample.

Gene	Primer Name	Sequence(5'-3')	Length	Accession Number
2G3	2G3. f20 2G3. r400	GCTTTGGTCAGGAGGGCTAG GGTCCACCTTCTTAATTGTCTG	254	AY308069
3G29	3G29. f2228 3G29. r2369	CTCATCCTTACCTGTGTCTTGTG CATTGACCTTCTCCTTTCCAA	150	NM_175814
2C28	2C28. r 4C25. f	GGGAACCACAGCCAAAGAA CCCAAACACAGGGACTTCAC	236	AF013214
4C25	4C25. f 4C25. r	ACCTTCCCACTTGCTGAAAAG CGACTCCTCCGACTCCTCC	152	AF013214
GCP-2 (CXCL6)	GCP-2. f.218 GCP-2. r.479	GACTGGCAAAACCCTACTTA ACTTCTGTTCTTCGTTCCCG	262	NM_174300
IL-8	IL-8. f251 IL-8. r355	CACTGTGAAAATTCAGAAATCATTGTTA CTTCACAAATACCTGCACAACCTTC	105	NM_173925
β-actin	β-actin.f38 β-actin.r428	CCTTTTACAACGAGCTGCGTGTG ACGTAGCAGAGCTTCTCCTTGATG	391	AH00130

Table 1. Sequences of primers for bovine differentially expressed genes and β-actin, the housekeeping gene

GENE NUMBER	GENE MATCH IN BLAST SEARCH
4C25 AF013214	BOS Taurus acidic ribosomal phosphoprotein PO mRNA
2C28 AF013214	BOS Taurus succinate dehydrogenase complex subunit C, integral membrane protein
3G29 NM_175814	BOS Taurus gene for alpha S1-casein
4G3	Not known –maybe novel
2G3 AY308069	BOS Taurus isolate FL405 mitochondrion, partial genome
GCP-2 NM_174300 (CXCL-6)	CXCL6/granulocyte chemotactic protein-2 (GCP-2)
4C27 CB531811	754679 MARC 6BOV Bos taurus cDNA 3', mRNA sequence

Table 2. Gene Fragment Match in the BLAST Search. β-actin, the housekeeping gene was used as a standard for normalization. (See attached appendex for their sequence).

CHAPTER IV. CONCLUSIONS AND GENERAL DISCUSSION

Bovine mastitis most often results from either *E. coli* or *S. aureus* introduction into the teats. Each of these pathogens induces infections which are specific in their pathogenetic pathways. *S. aureus* develop more subclinical and chronic cases whereas *E. coli* infections most often result in clinical cases which can become fatal in the very most severe of cases. When it comes to studying the pathogenic pathways of each pathogen at a molecular level the knowledge is limited. Neutrophils are well defined to play a crucial role in recognizing the presence of pathogen but the limited knowledge concerning the role of the epithelial cells in the evolvement of the infection at the molecular level is gaining more interest. Genes get expressed as a consequence of the environment they reside in and any changes will affect what genes get turned on. In this study bovine epithelial cells of the duct lining of the mammary gland were kindly provided by Dr. Guidry, USDA (Guidry and O'Biran, 2000) and used as a primary cell line to identify the gene expression profiles expressed when under *E. coli* LPS, or *S. aureus* LTA or PGN induced mastitis. LPS is a well characterized immunogenetic component of *E. coli* cell wall to be involved with initiating the inflammatory response. On the other hand, S. aureus LTA and PGN, also structural components of *S. aureus* were not so extensively studied but have been demonstrated to induce inflammation (Goldammer et al., 2004; Yao et al., 1995)

The recognition of these bacterial structural components via TLR is well understood when leukocytes are concerned but when it comes to epithelial cells, there is a need to properly identify their presence as a possible pathway of their involvement during infection. We performed a TLR 4 and 2 study in the bovine duct epithelial cell line in *E. coli* LPS, or *S. aureus* LTA or PGN inductions to identified if these TLR mRNAs were being up regulated as a consequence of their presence. Indeed, we identified a direct effect for *E. coli* LPS for TLR 4 mRNA upregulation with some sign in TLR 2 up regulation which can be due to the close similarities LPS has with that of LTA. As it turns out during the *S. aureus* LTA and PGN inductions the TLR 2 and 4 responses were not as pronounced but did show a significant up regulation for each of these receptors regardless of their concentration. The upregulation of the TLRs during the induction demonstrates the importance for these genes to be regulated as they are involved with immune function.

This response monitored in these cells invokes the possibility of this pathway to be the reason for identifying a possibility of the epithelial cells to play a function in recognizing pathogens. Even though *E. coli in vivo* is soluble in the milk it still releases LPS which eventually binds with other structures to be then recognized by the cells which posses the proper equipment. The mechanism for LPS recognition in expressing IL-1 and 8 (Boudjellab et al., 1998; Boudjellab et al., 2000) is most possibly regulated through the TLRs in the mammary duct epithelial cells. Now that the presence of TLRs had been established as an indicative for a possible role of epithelial cells during mastitis, the need arise to identify other possibly implicated genes concerning the infection. During the *S. aureus* induction a total of 28 fragments demonstrated a difference in their expression level when analyzed on a denaturing gel, but one of those was confirmed to be GCP-2 also known as CXCL6, a chemokine involved with local recruitment of neutrophils. It has been previously identified in literature that IL-8, a potent chemoattractant, (Pugin et al., 1993; Lee and Zhao, 2000) is also systematically expressed and was also confirmed to be upregulated during the *S. aureus* LTA induction. Unfortunately for the *E. coli* induce epithelial cells a total of 6 possible fragments which demonstrated some potential remains to be confirmed. The fragments identify a gene in gene bank but numerous attempts have been set out to confirm the genes upreguation or down regulation with no success. An alternative method might be necessary to determine if indeed they are potential differentially expressed genes.

LPS needs to form a complex with MD-2 and CD14 to bind the Toll-Like receptor 4 on the surface of immune involved mammary cells. The binding of this complex to TLR 4 activates a signaling cascade through out the cell, for a certain transcription activator, NF-κB, to transcribe specific genes. Since multiple genes could be involved in mastitis resistance and none of them are truly identified, we propose to use powerful molecular biology methods (suppressive subtraction hybridization (SSH), cDNA micro-array, serial analysis of gene expression (SAGE) and Differential Display-PCR (DD-PCR)) to identify unknown genes which are involved in pathogenesis of bovine mastitis. The proven strength of these approaches in human medicine facilitates studying the expression of hundreds or even thousands of genes without biasing conclusions drawn from few genes known to be involved in a particular disease. Once the genes involved in the resistance of mastitis are

identified, we will continue our research into a large population for potential use of those identified genes as a basis for genetic selection of mastitis resistance. This will be beneficial to the dairy industry as well as animal welfare.

REFERENCES

Boudjellab, N., H.S. Chan-Tang, X. Li and X. Zhao. 1998. Bovine mammary epithelial cells secrete interleukin-8 in response to lipopolysaccharide stimulation. Am. J. Vet. Res. 59:1563-1567.

Boudjellab, N., H.S. Chan-Tang and X. Zhao. 2000. Bovine interleukin-1 expression by cultured mammary epithelial cells (MAC-T) and its involvement in the release of MAC-T derived interleukin-8. Comp. Physiol. Biochem. 127:191-199.

Goldammer T., Zerbe H., Molenaar A., Schuberth H. J., Brunner R. M., Kata S. R. , Seyfert H. M. 2004. Mastitis Increases Mammary mRNA Abundance of β-Defensin 5, Toll-Like-Receptor 2 (TLR 2) and TLR 4 but not TLR 9 in Cattle. Clin. Diag. Lab. Immuno. 11:174-185.

Guidry A and O'Brien C.A. 2002. Bovine mammary endothelial/epithelial cell culture model of the blood/milk barrier. Methods Mol Biol. 188:85-98.

Lee J.-W. and Zhao X. 2000. Recombinant human interleukin-8, but not human inteleukin1b, induces bovine neutrophil migration in an in vitro co-culture system. Cell Biol. Intl.24:889-895.

Pugin J., Shurer-Maly C., Leturcq D., Moriarty A., Ulevitch R.J., Tobias P.S. 1993. Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluable CD14. Proc.Natl Acad. Sci.USA 90:2744-2748.

Yao L, Bengualid V, Lowy FD, Gibbons JJ, Hatcher VB, Berman JW. 1995. Internalization of Staphylococcus aureus by endothelial cells induces cytokine gene expression. Infect. Immun.63:1835-1839.

Zarember K. A. and Godowski P. J. 2002. Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNA in Leukocytes in Response to Microbes, Their Products, and Cytokines. J. of Immunol. 168:554-561.

APPENDEX

4C25 - 126 bp

AAGCTTTCCTGGAGGATATGGGATTCGGTCTTTTTGACTAATCACTAAAAAGCAGCCAACTAGG
CCAGCATTATTTGTGAAATGAGAAAATAAAGGCTTACTTCTCTTACGAAAAAAAAAAAGCTT

4C27 – 101 bp

AAGCTTCTGCTGGGCCATAACAACTCATTTTCTCAGTGTGTCTTTTGCTAAATACACCCTTTTTAT
ATAAATAAAATATAATTTGGAAAAAAAAAAAGCTT

2C28 –185 bp

AAGCTTACGATGCAAGAAGAAAATCCAGAAAATTATTGTTGAATCTGTTACGGGTTATTTCTTTT
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3G29 – 81 bp

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4G3-81bp

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2G3

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GCP-2 CXCL6 gene sequence from gel 585bp

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