

# **Multiplex Flow Cytometric Assays for Markers of Inflammation**

**Development and Application in Bovine Samples**

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## Abstract

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The aim of this thesis was to develop new techniques for quantification of bovine pro-inflammatory markers, with emphasis on cytokines and acute phase proteins, and to apply the techniques, using mastitis as disease model.

Singleplex, duplex and triplex xMAP assays were developed for the bovine cytokines tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and the bovine acute phase proteins serum amyloid A (SAA) and lipopolysaccharide binding protein (LBP). Detection limits, linear ranges and intra- and inter-assay variations differed between assays, but generally, lower detection limits and wider linear ranges were observed in singleplex assays than in duplex and triplex assays. The detection limits for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were satisfactory in the singleplex assays. Further development work is required before the multiplex formats can be used in assays where very low cytokine concentrations are of interest. In the assays for acute phase proteins the detection limits and linear ranges were satisfactory, but could probably be further improved.

All xMAP assays could be used for quantification of the analytes in milk and plasma samples from cows with experimentally induced *Escherichia coli* or *Staphylococcus aureus* mastitis. Simultaneous detection of IL-1 $\beta$ , SAA and LBP was performed in plasma. LBP is secreted solely during bacterial infections, and cytokines and acute phase proteins are secreted at different phases of the inflammation. Thus a time perspective and information whether the infection is of bacterial origin or not is provided by measuring IL-1 $\beta$ , SAA and LBP simultaneously.

In one of the studies, ten cows were grouped as high or low responders for TNF- $\alpha$ , IL-1 $\beta$  and IL-6, based on their cytokine response in an *ex vivo* whole blood stimulation assay (WBA) with lipopolysaccharide (LPS) and *E. coli*. After the WBA, the cows were intramammary inoculated with *E. coli*, and the WBA was evaluated for its usefulness as a predictive tool of the severity of *E. coli* mastitis. The pre-inoculation WBA with used stimulation doses and incubation times could not predict the severity of an *E. coli* induced mastitis.

**Keywords:** bovine, multiplex particle based flow cytometry, immunoassay, suspension array, biomarkers, pro-inflammatory cytokines, acute phase proteins, mastitis, whole blood stimulation assay, milk, blood

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*The most exciting phrase to hear in science,  
the one that heralds the most discoveries,  
is not "Eureka!" but "Hm... that's funny..."*

*~ Isaac Asimov*

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# Appendix

## Papers I-IV

This thesis is based on the following papers which will be referred to in the text by their Roman numerals:

- I.** Dernfalk, J., Persson Waller, K., Johannisson, A. 2004. Commercially available antibodies to human tumour necrosis factor- $\alpha$  tested for cross-reactivity with ovine and bovine tumour necrosis factor- $\alpha$  using flow cytometric assays. *Acta Veterinaria Scandinavica* 45, 99-107.
- II.** Dernfalk, J., Persson Waller, K., Johannisson, A. 2007. The xMAP™ technique can be used for detection of the inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in bovine samples. *Veterinary Immunology and Immunopathology* 118, 40-49.
- III.** Dernfalk, J., Persson Waller, K., Johannisson, A., Røntved, C.M. TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production *ex vivo* after *Escherichia coli* and LPS stimulation, and its associations with *Escherichia coli* mastitis and cytokine production in dairy cows. *In manuscript*.
- IV.** Dernfalk, J., Persson Waller, K., Johannisson, A. Simultaneous detection of interleukin-1 $\beta$ , serum amyloid A and lipopolysaccharide binding protein in bovine plasma using multiplex xMAP technology. *In manuscript*.

Papers **I** and **II** are reproduced with kind permission from the journals concerned.

## Abbreviations

The following abbreviations will be used in the text:

ANCOVA	Analysis of covariance
APC	Antigen presenting cell
APP	Acute phase protein
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming units
CV	Coefficient of variation
DMEM	Dulbecco's modified Eagle's medium
<i>E. coli</i>	<i>Escherichia coli</i>
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
GLM	General linear model
IL	Interleukin
LBP	LPS-binding protein
LOD	Limit of detection
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
PAMP	Pattern-associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
pi	Post inoculation
PGN	Peptidoglycan
rboIL-6	Recombinant bovine IL-6
rboSAA	Recombinant bovine SAA
rboTNF- $\alpha$	Recombinant bovine TNF- $\alpha$
rhuLBP	Recombinant human LBP
rhuTNF- $\alpha$	Recombinant human TNF- $\alpha$
rovIL-1 $\beta$	Recombinant ovine IL-1 $\beta$
rovTNF- $\alpha$	Recombinant ovine TNF- $\alpha$
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SAA	Serum Amyloid A
SCC	Somatic cell count
SD	Standard deviation
Sulfo-NHS	<i>N</i> -hydroxysulfosuccinimide
TLR	Toll-like receptor
TNF- $\alpha$	Tumour necrosis factor- $\alpha$
Tris	Tris-buffered saline
WBA	Whole blood stimulation assay



# Introduction

Bovine infectious diseases, especially mastitis, generate considerable animal welfare problems and economical losses in the dairy industry worldwide and further investigations on preventive measures, diagnostic methods and alternatives for treatment are therefore needed. Knowledge of the innate immune response in general and of the early host response to intramammary pathogen invasion is essential. By mapping different inflammatory markers, the progress of an infection can be studied in detail. The ability to characterize and quantify cytokines and acute phase proteins (APPs) is fundamental to understand the inflammatory responses to infectious diseases.

## Bovine infectious diseases

The term infectious diseases did not exist in early human communities (Last, 1998). Although fatal diseases, such as smallpox and plague, spread through the world and people died en masse, connections were not drawn between an individual's survival of a disease and resistance to the same disease during the next outbreak. It was not until the tenth century that Chinese physicians observed that a patient obtained immunity after survival of a smallpox infection and used that knowledge for a first vaccination attempt (Gross & Sepkowitz, 1998). They developed a method, variolation, where they dried material from smallpox scabs and induced a mild smallpox infection. The technique spread and it had reached the whole civilized world by the 1750s (Feery, 1976; Barquet & Domingo, 1997).

Inspired by the progress in human immunology, veterinarians in 1754 started to inoculate cows with mild forms of cattle plague successfully inducing resistance (Tizard, 2004). During the nineteenth century, epochal advances in immunological research in both animal and man were achieved by *e.g.* Edward Jenner, Louis Pasteur and Daniel Elmer Salmon constituting the fundamental knowledge about infectious diseases and the immunological response to them (Gross & Sepkowitz, 1998; Last, 1998).

Although research in the fields of immunology, microbiology, hygiene and nutrition has led to eradication of several dangerous diseases, infectious diseases are still a large problem (BSE Inquiry: The Report, 2000). Infectious pneumonia with bovine respiratory syncytial virus (BRSV) and parainfluenza type 3 virus (PI-3 virus) as important pathogens cause major fatalities in dairy calves worldwide (Bryson, 1985; Ames, 1997). Another important health problem in cattle is different diarrhoeal diseases, for example winter dysentery and diarrhoeas caused by bovine viral diarrhoea virus (BVDV) (Tråvén, 1993; Kalaycioglu, 2007).

In Swedish cattle, diseases that are common in other parts of Europe, *e.g.* brucellosis, *Mycobacterium bovis*, tuberculosis, foot-and-mouth disease and Johne's disease are presently not found (Herlin, Hultgren & Ekman, 2007). With continuously increasing travelling and more flexible export- and import laws for animals within Europe, the risks of transfer of unwanted disease pathogens to Sweden increase. Improved methods for early detection of such diseases are

therefore important so that programmes to prevent spreading of the pathogens can be effective.

### *Mastitis*

Most cases of bovine mastitis are of infectious origin, but trauma, physical injury and hyper-active lymphocytes have also been reported as reasons for mastitis (Tournant, 1995; Bradely, 2002). It is found in a clinical form, characterized by local and systemic signs of inflammation and infection, and in a sub-clinical form, where the milk quality and quantity are deteriorated but clinical symptoms are absent. Most clinical cases of mastitis are acute, while the sub-clinical form of the disease frequently becomes chronic (Barkema, Schukken & Zadoks, 2006). Depending on the degree of local and systemic signs during acute clinical forms of mastitis, the disease is ranked as mild, moderate or severe (Wenz *et al.*, 2001). Severe cases of acute clinical mastitis can be fatal if left untreated (Eberhart, 1984). Despite extensive research on preventive strategies, control programs and treatments of mastitis during the last decades (Eberhart, 1986; Ziv, 1992; du Preez, 2000), it is still considered the most important production disease in cattle of developed countries. That is because of the economic consequences for the farmers, due to unacceptable milk quality and low quantity, veterinary costs, and culling (Rajala-Schultz & Gröhn, 1999; Seegers, Fourichon & Beaudeau, 2003).

Henceforth, attention will be focused on different forms of infectious mastitis. Bovine infectious mastitis can be caused by innumerable pathogens. Since the late 1800s, *streptococci* have been associated with mastitis (Jones, 2005), and by the mid-1900s it was well-established among veterinarians that infectious mastitis could be caused by *e.g. staphylococci, streptococci, coliform bacteria* and *mycobacteria* (Norcross & Stark, 1970; Schultze, Stroud & Brasso, 1985). Since then the list has grown to include *mycoplasma, virus, yeast* and *algae*. However, bacterial infections are still the major cause (Bradley, 2002; Wellenberg, van der Poel & van Oirschot, 2002).

Contagious pathogenic bacteria live and multiply in infected mammary glands and can spread from one udder quarter to another within an udder, or from cow to cow. This group of bacteria includes the Gram-positive *Staphylococcus aureus* (*S. aureus*), *Streptococcus agalactiae* and several *mycoplasma* and *Arcanobacterium species* (Bradley, 2002; Kerro-Dego, van Dijk & Nederbragt, 2002). Another group of pathogenic bacteria is present in the animals' environment and they usually invade the mammary gland by teat contamination. This group of bacteria includes the Gram-negative *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae*, the Gram-positive *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus uberis*, and different *Bacillus species* (Kerro-Dego, van Dijk & Nederbragt, 2002; Persson Waller & Unnerstad, 2004). In Sweden, the yearly incidence of infectious mastitis is about 60%, with most cases being sub-clinical (Swedish Dairy Association, 2007). The most prevalent pathogens, together explaining about half of all reported mastitis cases, are *S. aureus* and *S. dysgalactiae*. Another important pathogen, responsible for the most severe cases of clinical mastitis is *E. coli*.

Udder infections can occur at any time during a cows' lactation cycle, but higher prevalence of infections with environmental pathogens such as *E. coli* are observed around drying-off and calving (Burvenich *et al.*, 2003; Rajala-Schultz, Hogan & Smith, 2005; Burvenich *et al.*, 2007). Also, the parity number seems to be associated with the susceptibility and severity of clinical mastitis (Gilbert *et al.*, 1993; Vangroenweghe, Lamote & Burvenich, 2005). The variation in susceptibility to udder pathogens is associated with the status of the cow and her immune system. Pregnancy, calving, induction of milk production and drying-off are periods of considerable challenge to the body. These periods involve tissue remodelling and changed nutritional demands which affect the functions of the immune system, making it less capable of fighting infections (Burvenich, *et al.*, 2003, 2007; de Schepper *et al.*, 2008).

### **Innate immunity and innate immune responses**

The primary task of the immune system is to protect the host from environmental microbes that threatens it. In general, the immune system is characterized by its capacity to recognize and discriminate between self and non-self (Tizard, 2004). The host is protected by two forms of immune defence mechanisms, the innate and the acquired immunity, that interact closely to eliminate foreign invaders and protect the host. In the innate immunity, non-specific mechanisms recognise and kill microorganisms, while responses of the acquired immunity are specific, leading to selective elimination of pathogens. In addition, repeated exposure to the same antigen amplifies the immune responses of the acquired immune system, *i.e.* the responses are adaptive. Innate immune mechanisms dominate the early stages of an infection while later stages are characterized by actions of the acquired immunity. In this thesis, attention is focused on the induction of the innate immune response.

The first obstacle microbial invaders have to conquer before they can settle in a host is the anatomical and physical barriers of the body (Tizard, 2004). The skin and the mucosal membranes of the respiratory and gastro-intestinal tract constitute the basic physical defence barriers. The individual tries to remove the intruder by coughing, sneezing, vomiting and diarrhoea, but given time a persistent invader often overcomes the physical hindrances. If microorganisms manage to enter the body, the first line of immunological defence, the innate immune system, becomes activated (Chinen & Shearer, 2007; Opitz *et al.*, 2007).

In the innate immune system, phagocytic cells (*e.g.* neutrophils, monocytes and macrophages) together with natural killer (NK) cells, act as constant surveyors of the body, scanning for invaders (Tracey, 2002). When an attack is encountered, a localized inflammatory response is initiated. Inflammation is a local defence mechanism aiming to remove invading agents or initiate a healing process of damaged tissue. That is performed by enhanced blood flow to the site of invasion, locally increasing the quantity of phagocytic cells that can attack and destroy the invaders. Increased blood flow to the inflammatory site also accumulates other antimicrobial factors such as complement components and antibodies at the site of inflammation (Goldsby *et al.*, 2003). Neutrophils and macrophages are the major

phagocytic cells of the body, whereas NK-cells recognise and lyse cells without major histocompatibility complex (MHC) class I molecules on their surface (Tizard, 2004). Actions of the acquired immune system are initiated by antigen presenting cells (APC) displaying parts of invading organisms on their cell-surface receptors. Three major cell types act as APC, macrophages, dendritic cells and B-lymphocytes. The antigen presentation activates the functional cells of the acquired immunity, the lymphocytes. B-lymphocytes produce antibodies against invading microorganisms, helper-T-cells regulate the immune response and cytotoxic T-cells kill virally infected cells and cells without MHC class I on their surface (Tizard, 2004).

The alterations observed during acute inflammatory responses are mediated through release of various inflammatory mediators, foremost cytokines (Koj, 1996; Ebersole & Cappelli, 2000; Tracey, 2002). Cytokines involved in the inflammatory response can roughly be divided into three subgroups: (i) pro-inflammatory cytokines, that initiate and enhance the cascade of events; (ii) interleukin (IL)-6-type cytokines, that induce the systemic actions; (iii) anti-inflammatory cytokines, that down-regulate the inflammatory response. Important mediators in the group of pro-inflammatory cytokines are tumour necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-8. Among the IL-6-type cytokines, IL-6 is a prominent actor, but leukaemia inhibitory factor, IL-11, oncostatin M and cardiotrophin-1 are also important members of this group. In the set of down-regulating cytokines IL-4, IL-10, IL-13 and transforming growth factor (TGF)- $\beta$  have prominent roles.

A wide variety of cells produce cytokines when activated by different stimuli, but macrophages are the dominant producers of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Tizard, 2004). The main functions of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are to induce the acute phase response, *i.e.* starting inflammatory processes such as production of APPs in the liver, attracting neutrophils to the site of inflammation, and activating B-, T- and NK-cells (Moshage, 1997; Goldsby *et al.*, 2003; Petersen, Nielsen & Heegaard, 2004). The initiation of the acute phase response is described in Figure 1.

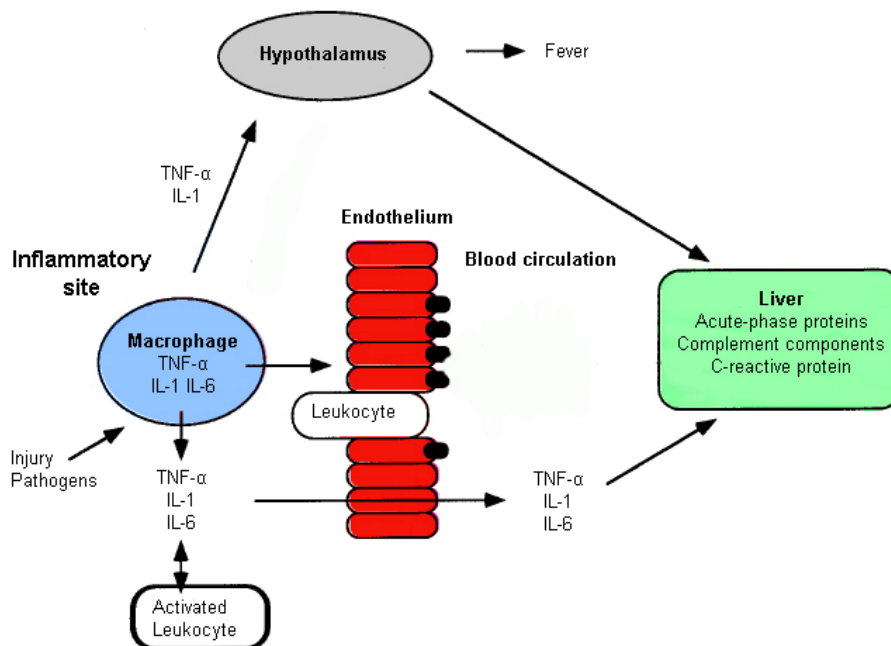


Figure 1. The cytokine-mediated activation of the acute phase response. When macrophages are exposed to stimulating agents they start to produce cytokines that act on neighbouring cells (monocytes, neutrophils, lymphocytes and endothelial cells). These cells become activated and produce more cytokines and express different membrane-bound receptors. Endothelial cells express selectins (black boxes) which attract circulating leukocytes and platelets from the bloodstream to the site of inflammation. Cytokines released into the bloodstream induce the hepatic acute phase response which involves increased synthesis of APPs. The cytokines also stimulate the central nervous system to e.g. induce fever (Modified from Jensen & Whitehead, 1998).

APPs are produced by hepatocytes, and their blood plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25% during inflammatory responses (Morley & Kushner, 1982). The positive APPs are produced within a few hours of injury and aid in eradication of pathogens and in wound healing by acting as components in clotting factors, as well as protease inhibitors and metal-binding proteins (Ramadori & Armbrust, 2001). Circulating IL-6 is believed to play the most important role in induction of APP production and the IL-6 synthesis is regulated by TNF- $\alpha$  and IL-1 $\beta$ . Thus, APP production could be considered a synergistic action of those cytokines (Heinrich, Castell & Andus, 1990; Akira, Taga & Kishimoto, 1993). The major functions of the pro-inflammatory cytokines and APPs in early innate immune responses are listed in Table 1.

The concentrations of the major APPs, C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin increase massively after cytokine stimulation of hepatocytes in many species (Gruys *et al.*, 2005; Ceciliani, Giordani & Spagnolo, 2002). The expression levels of the APPs differ, however, from species to species, proteins considered major APPs in one species might not be relevant in another

(Cecilian, Giordano & Spagnolo, 2002; Tizard, 2004). In cows, haptoglobin, SAA and  $\alpha_1$ -acid glycoprotein are major APPs, while CRP concentrations are not correlated to inflammatory responses in cows (Petersen, Nielsen & Heegaard, 2004).

Table 1. *Origin and some functions performed by a few important mediators during early innate immune responses to infections. Some of the functions described are performed synergistically by more than one cytokine. Information is compiled from Petersen, Nielsen & Heegaard (2004); Tizard (2004 and Horst Ibelgauf's COPE with cytokines (2008).*

Cytokine	Major source	Important activities in inflammation
Tumour necrosis factor- $\alpha$ (TNF- $\alpha$ )	Macrophages Monocytes Neutrophils Lymphocytes	Chemoattractant for neutrophils Inducing IL-1 $\beta$ and IL-6 Enhancing B- and T-cell proliferation
Interleukin-1 $\beta$ (IL-1 $\beta$ )	Monocytes	Chemoattractant for neutrophils Stimulating B-, T- and NK-cells Inducing IL-6, IL-8 and APP Inhibiting TNF- $\alpha$ , IL-1 $\beta$ , IL-6
Interleukin-6 (IL-6)	Monocytes Fibroblasts Endothelial cells	Inducing APP production Aiding in B-cell differentiation Stimulating B- and T-cells Inhibiting TNF- $\alpha$ , IL-1 $\beta$ , IL-6
Interleukin-8 (IL-8)	Monocytes Macrophages Fibroblasts	Activating neutrophilic oxidative burst Chemoattractant for all known migratory immune cells
Acute phase protein	Major source	Important activities in inflammation
C reactive protein (CRP)	Hepatocytes	Activating monocytes, macrophages and the complement system Inducing production of IL-1
Haptoglobin	Hepatocytes	Binding haemoglobin Inhibiting oxidative burst of neutrophils
Lipopolysaccharide binding protein (LBP)	Hepatocytes	Facilitating binding of PAMPs to TLRs
Serum Amyloid A (SAA)	Hepatocytes	Inhibiting fever, oxidative burst of neutrophils and platelet functions Chemoattractant for leukocytes

The innate immune system recognizes a great number of different pathogens by locating highly conserved motifs shared by several pathogens. These motifs are commonly referred to as pathogen-associated molecular patterns (PAMPs), and include *e.g.* mannose, double-stranded RNA, cytosine-guanine-rich DNA, and the bacterial cell wall constituents lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA) (Aderem & Ulevitch, 2000; Zeytun *et al.*, 2007). Cells of the immune system utilize evolutionary conserved pattern recognition

receptors, Toll-like receptors (TLRs), for recognition of PAMPs. At least ten functional TLRs have been identified in mammals, where each receptor has specificity for PAMPs in bacteria, viruses, fungi or parasites (Chen *et al.*, 2007). Interaction between PAMPs and TLRs on the immune cells induces production of cytokines and other immunological mediators that are important in elimination of pathogenic microorganisms.

A component of the Gram-negative bacterial cell wall is LPS, a component not found in Gram-positive bacteria, where instead PGN is the major component. PGN can also be found to a lesser extent in the walls of Gram-negative bacteria. (Trent *et al.*, 2006) (Figure 2). LPS is recognized by the host mainly through TLR-4, and PGN is detected by TLR-2 (Underhill, 2003). The TLRs are however not capable of inducing inflammatory responses on their own, but need help from other immune-recognition proteins. Both LPS and PGN recognition is associated with the soluble protein lipopolysaccharide binding protein (LBP), and the membrane bound proteins CD14 and MD-2 (Dziarski, R., Tapping, R.I. & Tobias 1998; Dziarski & Gupta, 2000; Beutler, 2000, Weber *et al.*, 2003). Although TLR-4 and TLR-2 elicit different intracellular signalling cascades and therefore different immune responses, the production of pro-inflammatory cytokines is induced by both TLRs (Werling & Jungi, 2003; Bannerman *et al.*, 2004).

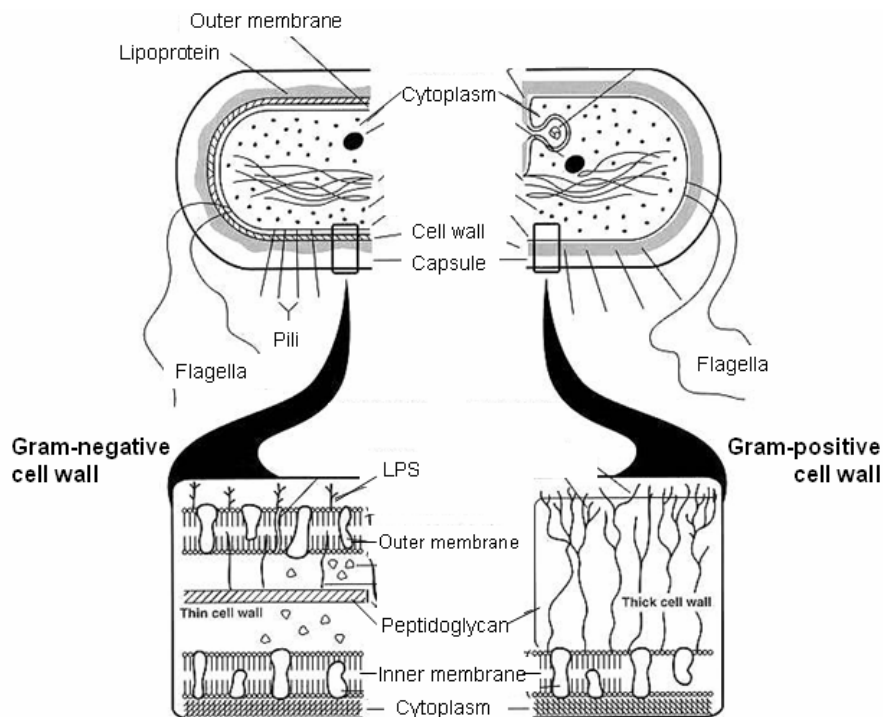


Figure 2. Construction of the cell walls of Gram-negative (left) and Gram-positive (right) bacteria. The peptidoglycan layer is thicker in walls of Gram-positive bacteria than in walls of Gram-negative bacteria, where instead lipopolysaccharide (LPS) is found in the outer membrane (Modified from [www.gsbs.utmb.edu/microbook](http://www.gsbs.utmb.edu/microbook), Jan-11, 2008).

## **Innate immune responses to bacterial mastitis**

During clinical mastitis the cardinal symptoms of inflammation *i.e.* redness, heat and swelling can be observed locally in the udder. The animals often show signs of pain, loss of appetite, fever, increased heart- and respiration rates, diarrhoea, slow reticulo-rumen motility and weakened skin turgor can be present (Lohuis *et al.*, 1988; Vangroenweghe *et al.*, 2005). Other important clinical manifestations are considerably reduced milk yield and changed milk composition (Shuster *et al.*, 1991; Rajala-Schultz *et al.*, 1999). The clinical symptoms during mastitis have mainly been explained by the immune mediated shock and the extensive inflammatory process, and not so much by the invading pathogen (Dosogne *et al.*, 2002; Hoeben *et al.*, 2000; Burvenich *et al.*, 2007).

Ehrlich (1892) was first to report presence of antibodies in milk. He suggested that the mammary gland functioned as a reservoir for antibodies, but had no capacity to produce them. Today it is well established that the mammary gland has substantial immunological capacity, which is of cardinal importance in the defence against pathogenic udder invasion. The basic defence mechanisms include anatomical, cellular and soluble factors that act in coordination and are crucial for mammary gland resistance to bacterial invasion (reviewed by Oviedo-Boyso *et al.*, 2007). During lactating periods, the flushing of milk physically hinders infection and in addition, the milk contains antimicrobial substances such as complement components, lactoferrin, lactoperoxidase and thiocyanate ions, together with different immune cells (Tizard, 2004).

Macrophages, lymphocytes, neutrophils and epithelial cells are the cell types found in healthy milk. The macrophages are the dominant celltype, guarding the gland against intruders (Paape *et al.*, 2000 & 2003). In addition, the antibody subsets IgA and IgG<sub>1</sub> are present in milk. IgA is synthesized locally in the udder and IgG<sub>1</sub> is actively transferred there from serum (Tizard, 2004). The concentrations of IgA and IgG<sub>1</sub> in milk are, due to the continuous milk flow, unfortunately not high enough to exert adequate protective influence.

When bacteria colonize the udder and start proliferating, PAMPs, *e.g.* LPS, PGN and LTA, are released from dead bacteria. These PAMPs are caught on TLRs on macrophages in the mammary gland. The macrophages internally process their PAMPs, become activated and start to launch the acute mammary inflammatory response as well as activating the adapted immunity. The macrophages perform this by secreting *e.g.* complement factors, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and arachidonic acid metabolites such as leukotrienes, prostaglandins and platelet activating factor (Tizard, 2004). These mediators guide leukocytes, mainly neutrophils, from blood to the affected mammary gland (Rainard & Riollet, 2006). In healthy mammary glands, the milk usually contains < 100000 cells/ml, a number that within hours of bacterial infection of the udder can increase to > 1million cells/ml (Harmon, 1994).

Neutrophils and macrophages in the udder ingest and kill pathogens by different means *e.g.* by production of reactive oxygen intermediates, reactive nitrogen intermediates, hydrolytic enzymes and defensins that all are toxic for devoured pathogens (Paape *et al.*, 1979; Tizard, 2004). Neutrophils are the major effector



cells in bovine innate and adaptive immunity and have been extensively studied (reviews by Paape *et al.*, 2000, 2003; Rainard & Riollet, 2006, Lun *et al.*, 2007). In order to be effective during bovine mastitis, neutrophils have to migrate rapidly to the site of infection and start to phagocyte and kill bacteria immediately (Paape *et al.*, 2003). Neutrophils can only ingest and kill a limited number of pathogens before they undergo apoptosis, while macrophages can ingest and kill numerous particles during their longer life-span (Paape *et al.*, 2000).

The release of pro-inflammatory cytokines during bacterial mammary gland infections also affects the acquired immune system by activating B- and T-lymphocytes. Also, as mentioned before TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are responsible for the systemic symptoms such as fever, lethargy, malaise, loss of appetite and weight loss seen during some forms of mastitis, in addition with the induction of APP production (Tizard, 2004). (Figure 3).

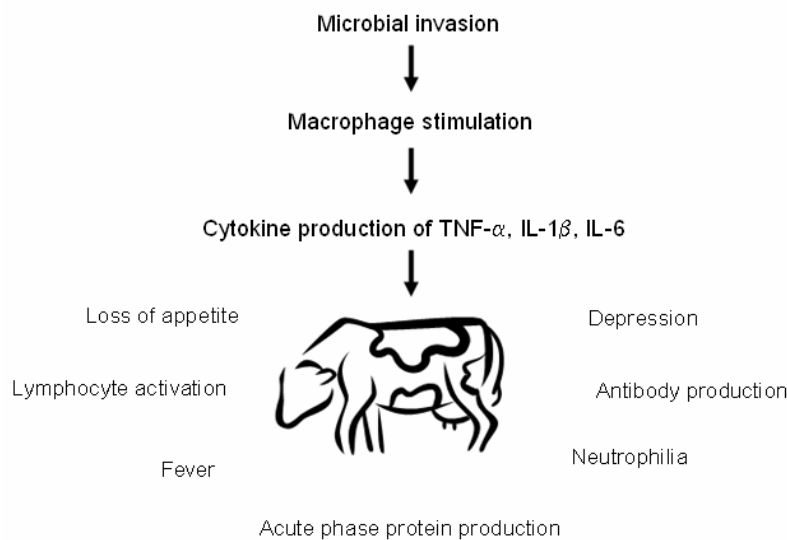


Figure 3. A brief overview of the major mechanisms induced by TNF- $\alpha$ , IL-1 $\beta$  and IL-6 during the host response to microbial mammary gland infection.

## Methods to study innate immune responses

Different methods to describe, quantify and grade immune responses have been developed (Masseyeff, 1991; Ferré, 1994; Eckersall *et al.*, 1999; Sachdeva & Asthana, 2007). The first mediator in the group later called cytokines was described in the 1950s (Isaacs & Lindenmann, 1957). Since then, molecular characterization of many cytokines has been performed and their functions have been described (Ryan & Majno, 1977; Sikora, 1980; Dinarello, 2000). The cytokine family is continuously expanding and different cytokines have become useful tools for diagnosis and treatment of bovine immunological diseases (Lofthouse *et al.*, 1996; Moore, 1996; Wood & Jones, 2001). Also, APPs frequently serve as markers of inflammation and infection in various species and diseases (reviewed by *e.g.* Petersen *et al.*, 2004). In cattle, quantification of APPs

in blood plasma, especially SAA and haptoglobin, have been shown to discriminate between acute and chronic inflammatory conditions (Horadagoda *et al.*, 1999; Eckersall *et al.*, 2001).

### *Quantification of cytokines and APPs*

Under normal circumstances cytokines are undetectable or found in very low levels in body fluids and tissues (Sachdeva & Asthana, 2007), but high levels can be found early in the inflammatory process. Since different cytokines can be related to specific groups of pathogens it is valuable to quantify them as early as possible. A wide range of assays for detection of cytokines in different biological samples are available. Depending on the type of information required, different ways to detect the cytokines are used, some methods assess the biological activity, others the mRNA expression, and yet others the secretion of a cytokine or its soluble receptor.

Cytokines and their soluble receptors are usually analysed in body fluids and cell-supernatants with Enzyme-Linked Immunosorbent Assay (ELISA), Radioimmunoassays (RIA), chemiluminescence or bioassays. When the cytokine production from specific cell populations or individual cells are of interest, methods such as multi-parametric flow cytometry, different mRNA-based assays (reverse-transcriptase linked polymerase chain reaction (RT-PCR), Northern blotting, *In situ* hybridisation (ISH), RNA-protection assays), intra-cytoplasmic cytokine staining, and Enzyme-Linked Immunospot (ELISPOT) can be used. To detect presence of cytokines in tissues, immunostaining and different mRNA based assays usually are performed. Each assay has its advantages and limitations. Standard ELISA is for instance not suited for high throughput analyses since it only measures one analyte at the time, and RT-PCR, which can be performed in multiplex style, measure gene expression and not the proteins.

Coming technologies for cytokine quantification seem to be in multiplex formats, *i.e.* several cytokines are analysed simultaneously in the same sample. Examples of multiplex technologies are multiplex ELISA, DNA- and protein micro-arrays and microsphere-based multiplex flow cytometric assays (Grøndahl-Hansen, *et al.*, 2003; Wilson *et al.*, 2005; Andersson *et al.*, 2007). Simultaneous assessment of several cytokines in a biological sample is probably more valuable than measuring the absolute concentrations of a single component, since they act together (Ebersole & Cappelli, 2000; Sachdeva & Asthana, 2007).

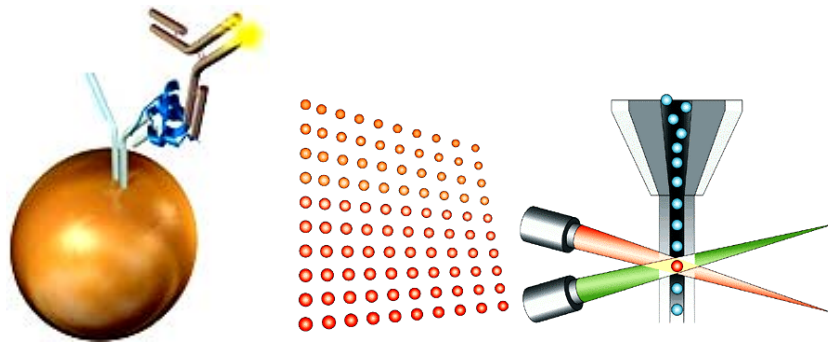
Unlike the cytokines, APPs can often be found in low levels in body fluids from healthy individuals of different species (Bannerman *et al.*, 2003; Ledeu & Rifai, 2003). The feature that makes them interesting as markers of inflammation and infection is their rapid elevation in blood plasma when the immune system is activated (Blackburn, 1994; Malle & DeBeer, 1996; Jensen & Whitehead, 1998; Heegard, *et al.*, 2000). In cows with mastitis, hundredfold higher SAA plasma concentrations have been recorded compared to concentrations in healthy cows (Eckersall *et al.*, 2001). Most frequently used assays for detection of bovine APPs are ELISA, mRNA based assays, bioassays and biosensor assays (Eckersall *et al.*, 1999, Åkerstedt *et al.*, 2006).

### *Particle based flow cytometry*

Flow cytometry is a powerful and fast technique where fluorescent and light scattering properties of cells or particles are analyzed (Shapiro, 2003). Particles are lead through an illuminating beam of coherent light, usually originating from a laser. The scattered laser light and emitted fluorescence is translated to information about the cell. Flow cytometry can be used to analyse intracellular and surface properties of cells and particles, distinction between live and dead cells can be made and physical sorting of cells with interesting properties can be performed.

In 1975 Knapp, *et al.* introduced antigen-coated microspheres with fluorescent labels as solid phase for capture and quantification of antigen-antibody reactions. That technique has been continuously improved and new applications have been found (Phillips *et al.*, 1980; Wanda & Smith, 1982; MacCrindle, Schwenzer & Jolley 1985; Frengen *et al.*, 1994). For instance, in 1982, Lisi *et al.* described a fluorescence immunoassay where diametrically different microspheres were used, enabling detection of different analytes in the same sample. In 1997 Fulton *et al.* presented the LabMAP technology (Luminex Corporation, Austin, TX, USA), presently called the xMAP technology. The new technique combines microsphere immunoassays, flow cytometry, rapid digital signal processing and multiplexing. Multiple analytes can individually and quantitatively be detected simultaneously in small volumes of samples with reliable statistics, as data are collected from at least 100 microspheres for each analyte.

Like in conventional immunoassays, the xMAP technique utilizes a solid and a soluble phase, where polystyrene microspheres function as the solid phase. Capture antibodies, often monoclonal, are covalently coupled to the microspheres, and the amount of bound analyte is determined with the use of fluorophore-coupled detection antibodies. In order to translate fluorescence intensities to quantitative data, the microspheres are analysed in a dedicated flow cytometer. To allow multiplexing, different microsphere subsets are internally dyed with a mix of far red and infrared fluorophores, giving them unique identities (Figure 4). By coupling different monoclonal antibodies to different microsphere subsets, multiplexing is possible. The dedicated flow cytometer used for xMAP assays is equipped with two lasers, one for classification of microspheres and one for quantification of the amount of analyte in the sample. For classification, a red laser exciting the red and infrared dyes inside the microspheres is used, and for quantification, a green laser exciting the molecules attached to the reporter antibody, resulting in orange fluorescence, is used.



*Figure 4.* Basic principles of the xMAP technique. To the left, an example of a sandwich immunoassay performed on a microsphere is shown. Capture antibodies are covalently coupled to microspheres, the analyte of interest binds to the capture antibody and is detected by a fluorophore-coupled reporter antibody. Note the proportionally different scales of the microsphere and the antibodies bound to it, the antibodies should be considerably smaller. In the middle, the 100 unique subsets of internally dyed microspheres are illustrated and to the right the two lasers in the flow cytometer, one for classification of the microsphere and one for quantification of analyte are depicted (*With permission from Breackmans et al., 2003*).

The microsphere subsets can either be purchased antibody-conjugated or conjugation-ready *i.e.* covered with avidin, carboxyl groups or oligonucleotide adapters. When microspheres with carboxyl groups are utilized, covalent coupling is usually performed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS). EDC reacts with the carboxyl groups on the microspheres, forming amine-reactive intermediates which sulfo-NHS stabilizes by converting them to amine-reactive sulfo-NHS esters, thus increasing the efficiency of the coupling reaction (Staros, Wright & Swingle, 1986).

Other multiplex particle based flow cytometric assays are available on the market. BD Biosciences (San Jose, CA, USA) has for instance developed the cytometric bead array (CBA), where microspheres with different fluorescence intensities from a single fluorophore are used for capture and quantification of soluble particles (Morgan *et al.*, 2004). Like the xMAP technique, an immunological sandwich assay is performed in the CBA. The main difference between the techniques, apart from the different internal colouring system, is that a dedicated flow cytometer is not necessary to carry out the CBA. Instead, it can be performed with any multi-use flow cytometer.

The xMAP technique comes with conjugation-ready microspheres and commercially available kits containing antibody-coupled microspheres and detection antibodies. The CBA technique only operates with commercially available kits. Kits are available for e.g. cytokines, phosphoproteins, cancer markers, lipoproteins and endocrine markers in human, mouse and rat samples.

Since Carson & Vignali (1999) reported simultaneous detection of 15 murine cytokines in a single sample by the use of the xMAP technique, numerous studies on cytokines, inflammatory mediators, antibodies, growth factors and apoptotic

markers in various biological fluids in health and disease, have been performed with particle based flow cytometric assays (Cook *et al.*, 2001; Kuller *et al.*, 2005; Elshal & McCoy, 2006). Most studies have been focused on cytokines in human samples (Camilla *et al.*, 2001; de Jager *et al.*, 2005), but measurements have also, apart from the work in this thesis, been conducted in pigs, rodent and horses (Johannisson *et al.*, 2006; Zhong *et al.*, 2007; Go *et al.*, 2008).

The technique has become more versatile since the microspheres can be purchased carrying avidin, carboxyl groups or oligonucleotides. Studies on for instance genotyping, endocrine markers, allergens, enzymes and tumor markers in different species have been reported (Thraillkill *et al.*, 2005; Dolezalova *et al.*, 2007; Haasnot & de Pré, 2007; Sun *et al.*, 2007; Zhu & Salmeron, 2007).

All new laboratory techniques must be evaluated on the reproducibility, precision and accuracy. This is usually performed by comparison to existing methods. Microsphere based flow cytometric assays have been compared to ELISA (Prabhakar, Eirikis & Davis, 2002; Thraillkill *et al.*, 2005; Pang *et al.*, 2005; Elshal & McCoy, 2006; Lash *et al.*, 2006). Conclusions from those studies are good correlation but poor concurrence of quantitative values between the assays. This is probably due to different antibody clones, blocking agents and detection systems in the different assays.

#### *The ex vivo whole blood stimulation assay (WBA)*

Cytokines are potent mediators of the immune system, and they are often only secreted transiently in low concentrations even during inflammatory states (Eskay, Grino & Chen, 1990; Bemelmans, van Tits & Buurman, 1996; Kelso, 1998). This makes detection of them in body fluids difficult. By stimulating whole blood samples with specific pathogens or fragments of pathogens *ex vivo* the immunological responsiveness in an individual can be monitored (Finch-Arietta & Cochran, 1991; Wouters *et al.*, 2002).

In whole blood stimulation assays (WBA), leukocytes are used in an environment mimicking their natural one. This has advantages compared to both *in vitro* and *in vivo* studies. Monocytes extracted from blood to be used in studies *in vitro* tend to become activated by handling (Desch *et al.*, 1989; Allen *et al.*, 1992). Also, when monocytes are cultured in a medium, cytokines, receptors, hormones, metabolites and other components in the blood that naturally interact with the monocytes are lost. In the *ex vivo* assays, repeated challenges with different doses of endotoxin in samples from the same individual are possible, something that cannot be performed as easily during *in vivo* studies.

*Ex vivo* WBA with LPS as the stimuli have been useful for measurements of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 responsiveness in healthy individuals of several species (Finch-Arietta & Cochran, 1991; Zangerle *et al.*, 1992; Wouters *et al.*, 2002; Carstensen, Røntved & Nielsen, 2005; Røntved *et al.*, 2005). Several studies suggest that the severity of clinical symptoms during *E. coli* mastitis is correlated to the cows' capacity of pro-inflammatory mediator production (reviewed by *e.g.* Burvenich *et al.*, 2007). Investigations of the pro-inflammatory cytokine responsiveness to LPS could be valuable in terms of susceptibility analyses to Gram-negative bacterial infections.

During acute infections and active inflammatory diseases the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 has a pivotal role in initiating the immunological protection processes. It is however important that the production is closely regulated. High blood plasma concentrations of TNF- $\alpha$  have been associated with severe clinical mastitis and septic shock (Sordillo & Peel, 1992; Sordillo, Pighetti & Davis, 1995). Cows with a balanced immune system stand a better chance to successfully eliminate pathogen invasion, for instance in the udder. Whether the differences in capacity to produce TNF- $\alpha$  after various stimuli in healthy individuals have a connection to the genetic background has been studied. Louis *et al.* (1998) showed that an individuals' degree of TNF- $\alpha$  production is predisposed by genetic background, while de Jong *et al.* (2002) on the contrary showed that it is not. In a study performed on cows by Elsasser, Blum & Kahl (2005), the degree of TNF- $\alpha$  production after LPS stimulation was shown to be genetically regulated and also inheritable.

Bovine *ex vivo* studies on TNF- $\alpha$  responsiveness to LPS have strengthened the WBAs' usefulness for monitoring of the *in vivo* innate immune response (Elsasser, Blum & Kahl, 2005; Røntved *et al.*, 2005). Therefore extended information about the immune function connected to mammary *E. coli* infections in cows could be gained by quantifying the responsiveness of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 to LPS and *E. coli*. Also, if correlations between the responsiveness to TNF- $\alpha$ , IL-1 $\beta$  or IL-6 *ex vivo* and the severity of clinical *E. coli* mastitis could be established, the WBA may be used as a predictive tool for the severity of clinical mastitis in cows. The ability to predict the intensity of a response to different immunological challenges in livestock could also improve breeding programs, where TNF- $\alpha$  response data could be used to identify a genetic predisposition for a problem that could develop in calves of specific breeding lines.

## Aims

The overall aim of the present study was to develop new techniques for quantification of bovine pro-inflammatory markers, with emphasis on cytokines and APPs, and to apply the techniques, using mastitis as a disease model.

The specific aims were:

- to investigate the ability of commercially available antibodies against human TNF- $\alpha$  to cross-react with bovine TNF- $\alpha$  using the xMAP technique.
- to investigate if the xMAP technique is suitable for quantification of the cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in singleplex and multiplex assays in bovine samples.
- to apply the xMAP technique for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in studies on bovine milk and blood plasma samples from cows with mastitis.
- to evaluate if *ex vivo* LPS and *E. coli* induced production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in blood samples from healthy cows can predict their immunological response after intramammary *E. coli* inoculation.
- to evaluate if concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in milk and blood plasma after intramammary *E. coli* inoculation are correlated to each other and to the severity of clinical signs.
- to investigate if the xMAP-technology is applicable for multiplex detection of the cytokine IL-1 $\beta$  and the acute phase proteins SAA and LBP in bovine biological samples.

## Materials and methods

In this section of the thesis material, methods and experimental procedures are summarized and commented. Details about the materials and methods used in Papers I-IV are described in each paper, respectively.

### Animals and infection models

All experimental handling with animals were approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden or by the Danish Animal Experiments Inspectorate. Milk somatic cell count (SCC) and milk yield was observed daily during all the experimental protocols. Systemic and local clinical signs were monitored in the cows. Systemic signs included general attitude, appetite, saliva secretion, rectal temperature, heart rate, respiration frequency, rumen contraction frequency, and signs of diarrhoea. The udder was inspected for signs of inflammation and the milk composition was analysed. Fresh milk was aseptically collected for bacterial examinations and SCC determination. Milk samples for analyses of APPs and cytokines were collected in test tubes, aliquoted and frozen in -20°C until use. Blood samples used for analyses of APPs and cytokines were aseptically aspired in EDTA vacuettes from the jugular veins of the cows and centrifuged at 4°C so plasma could be collected, aliquoted and frozen in -80°C.

#### *Paper I*

Samples with known concentrations of TNF- $\alpha$  from two different experimental studies were included in Paper I. Milk samples were obtained from a study in which six clinically healthy cows of the Swedish Red and White, and Swedish Holstein breeds had been intramammary inoculated with *S. aureus* (Grönlund *et al.*, 2003). Blood samples were obtained from another study, in which five Danish Holstein Friesian cows had been intravenously challenged with LPS from *E. coli* (Røntved *et al.*, 2005).

#### *Paper II – III*

Milk and blood samples from ten high-yielding clinically healthy Danish Holstein cows in the midst of their first or second lactation, intramammary inoculated with live *E. coli* were used. The cows had a daily mean  $\pm$  SD milk yield of 35  $\pm$  5 kg and a SCC < 84000 cells/ml at the start of the experiment. In Paper II, blood and milk from six of the cows were included, while blood and milk samples from all ten cows were included in Paper III.

#### *Paper IV*

Blood plasma samples from two Danish Black and White cows were used. The udder of one cow had been inoculated with LPS from *E. coli* first and PGN from *S. aureus* two weeks later. The other cow was treated in the reverse order. The cows had a daily milk yield of 30 – 35 kg and a SCC < 110000 cells/ml at the start of the experiment.



## Laboratory analyses

### *Bacterial growth and SCC in milk (Papers I – III)*

Bacterial analyses of milk were performed according to standard procedures at the Section of Mastitis, National Veterinary Institute, Uppsala, Sweden, and according to standard procedures at the Department of Animal Health, Welfare and Nutrition, Research Centre Foulum, Tjele, Denmark. SCC was determined in fresh milk samples by analysis with a fluoro-optical method on a Fossomatic instrument (Foss Electric Ltd, Hillerød, Denmark).

### *The xMAP assays*

#### Microspheres

Different subsets of polystyrene microspheres ( $\text{\O} 5.6 \mu\text{m}$ ) with carboxyl groups on the surfaces, internally dyed with far red and infrared fluorophores, were used in all xMAP assays. In Papers I-II the microspheres were purchased from Luminex Corporation (Austin, TX, USA) and in Papers III - IV the microspheres originated from Bio-Rad Laboratories (Hercules, CA, USA).

#### Antibodies and recombinant proteins

In the xMAP assays, monoclonal antibodies against human TNF- $\alpha$  (Paper I), bovine TNF- $\alpha$  (Papers II –IV), ovine IL-1 $\beta$  (Papers II – IV), bovine IL-6 (Papers II – III), bovine SAA (Paper IV) and bovine LBP (Paper IV) were used. The polyclonal antibodies were directed against the same species except for IL-6 which was directed against ovine IL-6. As standards were recombinant bovine TNF- $\alpha$ , ovine IL-1 $\beta$ , bovine IL-6, bovine SAA and human LBP applied.

The anti-bovine LBP antibodies used in Paper IV are validated by the manufacturer (HyCult Biotechnology, Uden, The Netherlands) to cross-react with the recombinant human LBP used as standard. In addition, the same antibody clones have previously been employed in sandwich-format for detection of LBP in bovine samples (Bannerman *et al.*, 2003). The antibodies against ovine IL-1 $\beta$  are guaranteed by the manufacturer (AbD Serotec, Oxford, England) to detect bovine IL-1 $\beta$ . The recombinant ovine IL-1 $\beta$  has earlier worked successfully as standard for IL-1 $\beta$  detection in bovine samples (Persson Waller *et al.*, 2003).

#### Coupling of antibodies to microspheres

Before an xMAP assay was performed, antibodies were coupled to microspheres. The coupling was performed in two steps: (1) activation of the carboxyl groups on the microspheres using EDC and sulfo-NHS in sodium phosphate buffer (2) covalent coupling of antibodies to the microspheres. The antibody-coupled microspheres were kept protected from light at 2 – 8 °C, until use.

#### The Luminex 100 System

In all xMAP assays, the Luminex 100 instrument and the Luminex XY platform from Luminex Corporation were utilized. The XY platform allows analyses to be performed in 96-well-plates instead of in single tubes. The settings for the

classification and quantification lasers were calibrated daily, using special calibration microspheres of known fluorescent light intensities (Bio-Rad Laboratories), when the laboratory work in Papers I – IV was performed. Validation of the optics, fluidics, reporter- and classification systems was performed every third month, as suggested by the manufacturer, also with special validation microspheres bought from Bio-Rad Laboratories.

In Paper I, the software Luminex Data Collector 1.7 (Luminex Corporation) was used, while BioPlex Manager 4.0 (Bio-Rad Laboratories) was employed in Paper II - IV. Both softwares provide information on mean and median fluorescence intensity, standard deviations and number of microspheres of each subset in combination with data regression analysis. Analyte concentrations were obtained by interpolation from standard curves calculated with a five parametric regression model.

#### Validation

All singleplex and multiplex (duplex and triplex) xMAP assays used in the studies were optimised and validated before use. The concentrations of capture antibodies on the microspheres and the antibodies used for detection- and reporting were determined by titration. Limits of detections (LOD), linear ranges of the standard curves and intra- and inter-assay variations were calculated for the assays. Also, the recovery percentage of an analyte in milk and plasma as compared to buffer and cross-reactivity between the different analytes in the multiplex assays were studied.

#### Quantification of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, SAA and LBP (Paper I - IV)

In Paper I, four milk and four serum samples from the *S. aureus* mastitis study and the LPS challenge study, respectively, were used. They were integrated as standards in a commercially available Fluorokine MAP kit (R&D Systems, Minneapolis, MN, USA) directed towards human TNF- $\alpha$ . In addition, the samples were used to test cross-reactivity with antibodies against human TNF- $\alpha$  in our lab.

Milk and plasma samples collected from six cows at one occasion before, and one occasion after inoculation were used to validate singleplex and multiplex xMAP assays for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in Paper II. In Paper III, the singleplex assays developed in Paper II were used for quantification of IL-1 $\beta$  and IL-6 in the samples collected before and after inoculation of *E. coli*. In Paper IV, the plasma samples were analysed for their content of IL-1 $\beta$ , SAA and LBP using a triplex xMAP assay developed and validated in our lab.

#### *The ex vivo* WBAs (Paper III)

The WBA for bovine blood was developed and described by Røntved *et al.* (2005). In Paper III, a modified version where duplicate blood samples were incubated with Dulbecco's Modified Eagle's Medium (DMEM), phosphate buffered saline (PBS), LPS, heat-killed or live *E. coli* for 3.5 or 24 hours, was conducted. The WBA was carried out on two consecutive days before the start of the *E. coli* mastitis experiment and mean cytokine concentrations of the two days were calculated. Based on the concentrations of TNF- $\alpha$ , IL-1 $\beta$  and SAA after stimulation with LPS and live *E. coli*, the cows were grouped as high or low

responders for each cytokine. The cows were ranked 1-10, and the cow with the highest cytokine response was ranked nr 1. For each cytokine, the cows ranked 1-5 were grouped as high responders and the cows ranked 6-10 were grouped as low responders. The groups were used for further evaluation of differences in susceptibility to *E. coli* infection, severity of clinical symptoms, and cytokine concentrations in milk and plasma between the high and low responders.

#### *ELISA assay* (Paper III)

The ELISA used for quantification of TNF- $\alpha$  in blood samples from the *ex vivo* WBA and in all milk and plasma samples from the *E. coli* mastitis study was originally described by Ellis *et al.* (1993) and we used it with the modifications described by Røntved *et al.* (2005).

### **Statistical analyses**

Data obtained from the Luminex 100 were evaluated with BioPlex Manager 4.0 (Bio-Rad Laboratories) and data are presented as mean value  $\pm$  standard deviation (SD) as interpolated from standard curves calculated with a five parametric regression model. The statistical calculations of obtained results were performed with Minitab 15 (Minitab Inc., Coventry, England) and Statistical Analysis Systems (SAS) 9.1 (SAS Institute Inc., Cary, NC, USA).

A p-value  $< 0.05$  was regarded as statistically significant in all studies, and all results shown are significant, unless something else is stated.

#### *Paper I*

The limit of detection (LOD), defined as the lowest concentration of each analyte that can be detected, was established for the different antibody clones in the xMAP and Fluorokine MAP assays. The mean fluorescence intensity (MFI) for six replicates containing microspheres coupled with capture antibodies against each analyte, detection antibodies against each analyte, and assay buffer was calculated, and LOD was defined as average MFI + 3 SD, as suggested by the International Conference on Harmonization (1994).

Recovery percentages in serum and milk were determined by setting the MFI from experiments conducted in buffer to 100% and calculating the results from analyses of the same analytes in serum and milk as percentages  $\pm$  SD.

#### *Papers II and IV*

LOD was established for all analytes in singleplex and multiplex xMAP assays, as described for Paper I with the modification that eight replicates was used. Differences between the LODs for each analyte were evaluated by paired Student's t-test. Intra- and inter-assay coefficients of variation (CV's) were calculated for each analyte by the formula: SD/mean x 100.

Cross-reactivity between reagents included in the multiplex xMAP assays were evaluated by assays where different antibody-coupled microspheres directed against the different analytes in the multiplex assay were incubated with only one

antibody at the time. Paired Student's t-tests were applied on these data. In Paper II, paired Student's t-test was also applied on the data to examine whether equivalent results were achieved using singleplex and triplex xMAP assays.

### *Paper III*

In the WBA, the effects of different stimuli *ex vivo* on the cytokine concentrations in blood samples were analysed with a non-parametric multiple comparison test with randomized block design, the Friedman's test. Differences in cytokine concentrations due to incubation time and different *E. coli* doses were evaluated with a non-parametric two-sample rank test, a Mann-Whitney U test in the WBA. Based on the results obtained in the WBA, the cows were grouped as high (H) and low (L) responders for each cytokine, and differences in severity of clinical symptoms and concentrations of cytokines in milk or plasma were analysed with Mann-Whitney's U test.

Pre-inoculation mean values  $\pm$  SD was determined for heart rate, respiration, milk yield and SCC. The pre-inoculation mean value  $\pm$  2 SD was considered normal and values above or below were considered abnormal. The effect of time post inoculation (pi) on concentrations of milk and blood cytokine concentrations were analysed with the Friedman test. A general linear model analysis of covariance (ANCOVA) where time pi and within-cow covariance were compensated for was used for determination of associations between cytokine concentrations in milk and plasma pi and clinical signs, and between cytokine concentrations in milk and plasma, respectively. In order to do statistical analyses of the data, which not was normally distributed, data was transformed to a Poisson distribution prior to analysis.

## Results

### **Development of xMAP assays** (*Papers I, II and IV*)

Singleplex xMAP assays for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, SAA and LBP were developed and successfully applied for detection of the analytes in milk and blood samples from cows with mastitis, using ovine and bovine reagents. In addition, duplex assays for simultaneous detection of IL-1 $\beta$  and IL-6, TNF- $\alpha$  and IL-1 $\beta$ , or TNF- $\alpha$  and IL-6 as well as triplex assays for simultaneous detection of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 or IL-1 $\beta$ , SAA and LBP were developed and used for detection of the analytes in bovine milk and blood samples.

#### *Antibody clones cross-reacting between species*

All antibody clones directed against human TNF- $\alpha$  evaluated in Paper I could detect recombinant human TNF- $\alpha$  (rhuTNF- $\alpha$ ) using the xMAP assay. Two antibody clones, Mab11 (BD Pharmingen, San Diego, CA, USA) and 6401.1111 (BD Biosciences, San Diego, CA, USA), could also detect recombinant ovine TNF- $\alpha$  (rovTNF- $\alpha$ ) in concentrations greater than 2.5ng/ml. None of the antibody clones tested could, however, detect TNF- $\alpha$  in bovine milk or serum samples. The Fluorokine MAP-kit from R&D Systems detected very low concentrations (about 4 pg/ml) of rhuTNF- $\alpha$  from two different sources, R&D Systems and AbD Serotec. RovTNF- $\alpha$  could, however, not be detected with the Fluorokine MAP-kit, nor could TNF- $\alpha$  in the bovine milk and serum samples with known concentrations of TNF- $\alpha$ .

#### *Singleplex and multiplex xMAP assays*

In Paper II singleplex, duplex and triplex xMAP assays for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were developed, validated and used for detection of cytokines in milk and plasma from cows with mastitis. In singleplex assays the LODs for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were 0.5, 0.08 and 0.2 ng/ml, respectively. Corresponding LODs in the triplex assay were 3.5, 2.0 and 6.5 ng/ml.

In Paper IV, singleplex and triplex xMAP assays were designed, validated and applied for detection of IL-1 $\beta$ , SAA and LBP in plasma samples from cows with mastitis. LODs were for IL-1 $\beta$ , SAA and LBP 0.1, 2.0 and 1.0 ng/ml, respectively, in the singleplex assays, and 0.4, 3.8 and 0.8 ng/ml in the triplex assay. A selection of the intra- and inter-assay coefficients of variation (CV's) obtained for the different xMAP assays developed in Paper II and IV are shown in Table 2.

Table 2. Intra- and inter-assay variations of singleplex (A) and triplex (B) xMAP assays of roviIL-1 $\beta$ , rboIL-6, rboTNF- $\alpha$ , rboSAA and rhuLBP. Samples with 25, 100 or 200 ng/ml of recombinant analyte were analysed.

A	Intra- assay			Inter-assay		
	Analytes	Mean (ng/ml)	SD	CV (%)	Mean (ng/ml)	SD
rboTNF- $\alpha$	114.2	1.7	1.5	218.2	11.1	5.1
roviIL-1 $\beta$ <sup>1</sup>	123.1	4.8	3.9	24.6	3.5	14.2
roviIL-1 $\beta$ <sup>2</sup>	23.7	1.0	4.1	28.3	4.1	14.6
rboIL-6	86.9	9.3	10.7	185.0	37.2	20.1
rboSAA	29.1	5.3	18.0	24.4	5.2	21.4
rhuLBP	25.7	4.0	15.5	31.5	6.4	20.4

B	Intra- assay			Inter-assay		
	Analytes	Mean (ng/ml)	SD	CV (%)	Mean (ng/ml)	SD
rboTNF- $\alpha$	127.4	7.9	6.2	195.3	17.2	8.8
roviIL-1 $\beta$ <sup>1</sup>	131.1	27.8	21.2	21.5	8.5	39.6
roviIL-1 $\beta$ <sup>2</sup>	25.6	0.8	3.2	23.9	1.6	6.6
rboIL-6	92.6	21.5	23.2	174.2	66.2	38.0
rboSAA	24.7	1.5	6.0	25.8	5.1	19.8
rhuLBP	27.7	2.1	7.7	20.1	4.2	20.7

<sup>1</sup>IL-1 $\beta$  evaluated in Paper II, <sup>2</sup>IL-1 $\beta$  evaluated in Paper IV.

In Paper I, rhuTNF- $\alpha$  was added to bovine milk and serum samples and the recovery could be detected using the Fluorokine MAP-kit. In milk, the recovery was  $59.8 \pm 2.0\%$  while it in plasma was  $36.4 \pm 1.6\%$ . That suggests that components of the matrix interfere with the antibody assay. Also during the development work for Papers II and IV interference due to matrices was observed.

Reliable standard curves could be produced for all the above mentioned analytes in both singleplex and multiplex assays. The linear ranges of the curves varied between the analytes and for all of them the range was wider in singleplex assays than in multiplex assays. Standard curves from the two triplex assays (TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-1 $\beta$ , SAA, LBP, respectively) are shown in Figure 5.

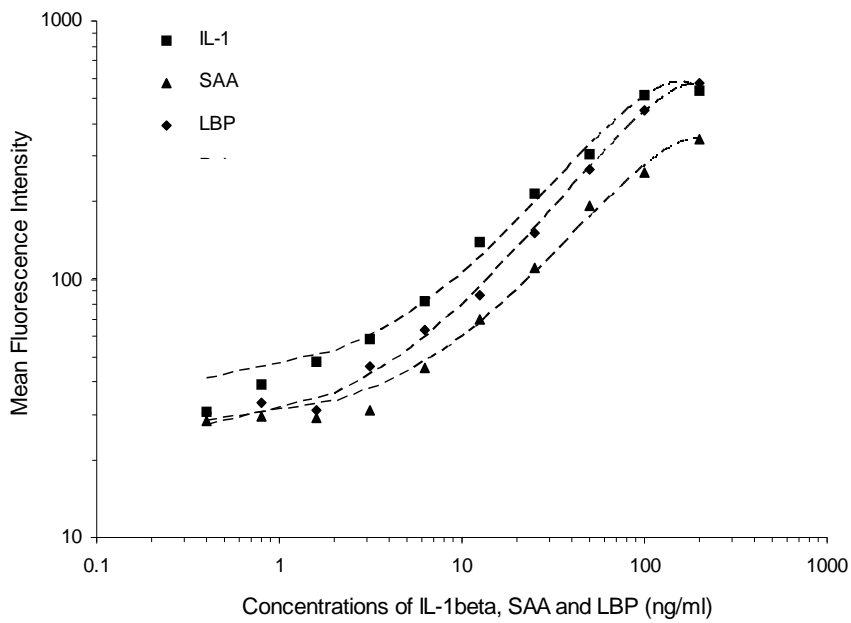
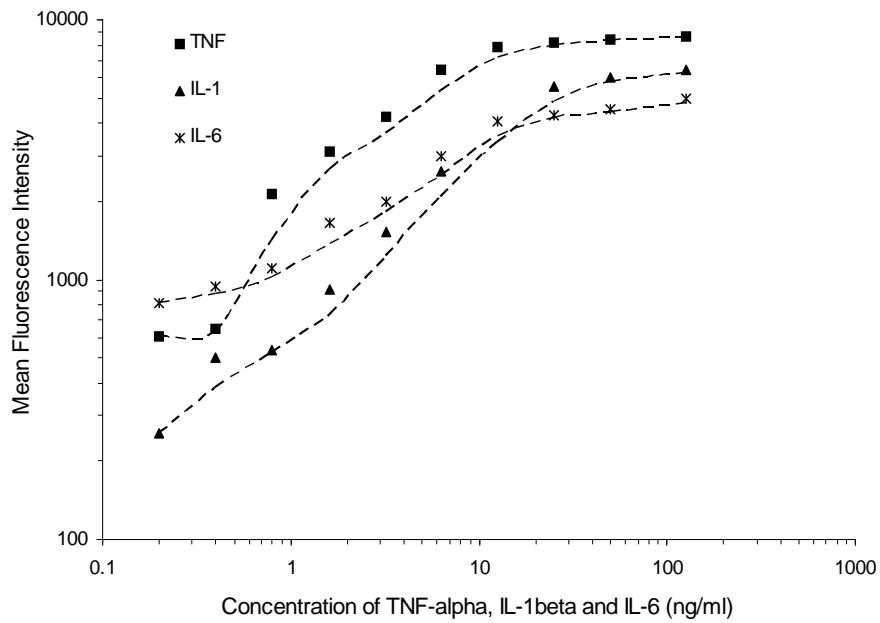


Figure 5. Mean fluorescence intensities of representative standard curves of triplex xMAP assays for recombinant bovine TNF- $\alpha$ , recombinant ovine IL-1 $\beta$ , and recombinant bovine IL-6 at the top. At the bottom are standard curves for recombinant ovine IL-1 $\beta$ , recombinant bovine SAA, and recombinant human LBP shown.

### **Experimental *E. coli* mastitis (Paper III)**

Six of ten cows became infected and developed moderate clinical mastitis after the *E. coli* inoculation. Detectable concentrations of *E. coli* bacteria were found in all infected mammary gland quarters pi. Fever and reduced daily milk yield were observed in all infected cows. Looser faeces consistency was observed at least once in all cows, both infected and non-infected. Local symptoms of the udder, such as redness, swelling, elevated SCC, changed milk colour and composition, were recorded in all infected cows from 18 hours pi until the end of the study. Also the non-infected cows showed sporadically elevated SCC pi.

#### *Concentrations of TNF- $\alpha$ , IL-1 $\beta$ and IL-6 in milk and plasma*

The variation in cytokine response after the *E. coli* inoculation was large, but significantly elevated concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were found in milk and plasma from all the infected cows. Except for the mean peak concentration of IL-1 $\beta$  in milk (72 hours pi), the mean peak concentrations of all cytokines were observed within the first 24 hours pi in both milk and plasma. High concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in plasma, and TNF- $\alpha$  in milk during the *E. coli* mastitis were associated with more severe clinical signs.

### **The *ex vivo* WBAs (Paper III)**

Incubation with LPS and *E. coli* in the *ex vivo* WBA stimulated the leukocytes to produce TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Elevated levels were observed in blood samples after both 3.5 and 24 hours, with highest concentrations of all cytokines at 3.5 hours. Two cows were ranked high responders for all three cytokines, two were ranked low responders for all three cytokines and six were ranked differently for the different cytokines.

Two cows ranked low for all cytokines, one cow ranked high for all cytokines and three cows ranked differently for the different cytokines became infected by the *E. coli* inoculation. One cow ranked high for all cytokines, and three cows ranked differently depending on cytokine were not infected by the *E. coli* inoculation. No significant differences between the pre-inoculation high and low responders and the TNF- $\alpha$ , IL-1 $\beta$  and IL-6 production in milk and plasma pi were observed. The diarrhoea score and the heart rate pi differed between the pre-inoculation high and low groups for IL-1 $\beta$  and IL-6, respectively.

### **Experimental *E. coli* LPS and *S. aureus* PGN mastitis (Paper IV)**

Detectable concentrations of IL-1 $\beta$ , SAA and LBP were found in all plasma samples analysed. In both cows the LPS inoculation induced higher concentrations of SAA and LBP than the PGN inoculation. IL-1 $\beta$  was found in highest plasma concentrations after LPS inoculation in one cow and after PGN inoculation in the other cow. LPS induced a peak in the LBP plasma concentration in both cows while PGN only induced LBP production in one cow and not the other.



## Discussion

This thesis is focused on development of microsphere based flow cytometric assays for detection of bovine markers of inflammation and infection. The reasons for this was that well-functioning immunoassays was lacking for immunologically interesting markers in different domestic animal species, and that flow cytometric microsphere assays previously had been proven successful in studies on rodents and humans. In this thesis, the xMAP technique was shown to function in bovine samples, allowing simultaneous detection of cytokines and APPs. The usefulness of the assays designed for bovine samples would be increased with further work on assay development. With the continuously expanding range of commercially available reagents, this technique has great potential of replacing less versatile techniques used today.

### Methodological considerations

#### *Antibody clones cross-reacting between species*

In the field of veterinary immunology, an important problem is the lack of reagents for immunoassays. In several cytokine and APP studies on cattle, antibodies against ovine or human homologues of the analytes are utilized, and human recombinant proteins are often applied as standards (Horadagoda *et al.*, 1993; Shuster, Kehrl & Stevens, 1993; Bannerman *et al.*, 2003; Grönlund *et al.*, 2003; Lahouassa *et al.*, 2007; Sohn *et al.*, 2007). Biological cross-reactivity between antibodies and cytokines or APPs of different species is evident, for instance several commercially available assays are validated for use in multiple species (SAA-ELISA, Tridelta Ltd, Maynooth, Ireland; LBP-ELISA, HyCult Biotechnology, Uden, Netherlands). The cross-reactivity between antibodies against a protein homologue in another species is often not as strong as within a species. Therefore, absolute concentrations achieved in such assays should be interpreted with caution. The sensitivity is, however, often enough for illustration of large concentration changes within animals.

In Paper I, all antibodies tested against human TNF- $\alpha$  could detect rhuTNF- $\alpha$  when applied in the xMAP technique, indicating that the technique worked satisfactorily. Other studies conducted in our laboratory have proven that the xMAP method can be used with reliable results. Successful cytokine measurements have been performed in human blood plasma and serum, brain tissue from mice and in porcine blood serum and lung lavage (Stricklin & Arvidsson, 2004; Johannisson *et al.*, 2006; Tyvold *et al.*, 2008).

Two of the antibody clones in Paper I identified rovTNF- $\alpha$  while none of the evaluated clones detected TNF- $\alpha$  in bovine samples. The detection level of rovTNF- $\alpha$  using anti-human TNF- $\alpha$  antibodies was, however, not satisfactory, given that cytokines are powerful signal molecules that induce effects at low concentrations (Kelso, 1998). The results from Paper I are probably explained by inability of the antibody clones to cross-react between species. Antibodies against

bovine TNF- $\alpha$  have become available, but this is not yet the case for antibodies against many other animal proteins.

Another study where cross-species reactivity of antibodies was investigated was conducted by Pedersen *et al.* (2002). In that study, monoclonal antibodies against different human, ovine or bovine cytokines were investigated for their ability to detect homologous cytokines produced by blood cells from different species. Antibodies directed against ovine TNF- $\alpha$  detected not only ovine TNF- $\alpha$  but also TNF- $\alpha$  produced by bovine, caprine, porcine, mink and human cells. The antibody clone against IL-6 also cross-reacted between species and could detect ovine, bovine, caprine, porcine, canine, mink and human IL-6, although to lesser extent than the anti-ovine TNF- $\alpha$  clone.

The reason that cross-species reactivity between antibodies and homologous immunological proteins can occur depends on their high conservation through evolution, thus they have similar structures in different species (Syversen *et al.*, 1994; Scheerlinck, 1999; Soller *et al.*, 2007). In the two latter reports, the origin of cytokines in human, mouse, sheep, cow, pig, rat, dog, cat and horse were investigated. The conclusions were that more than 60% amino acid homology between cytokine homologues usually allowed for cross-reactivity between species, and that cross-reactivity was seen more frequently with higher amino acid homology. When the coding sequences of IL-1 $\beta$  were compared between the species mentioned above, about 70 to 84% identity was observed. Corresponding values for TNF- $\alpha$  were 80 to 94%. In the report by Syversen *et al.* (1994), a high degree of homology between SAA from humans, sheep, cow, horse, dog, cat and mink, was found. Within the different cytokine and APP genes, some sequences are more well-preserved through evolution than others. If these well-preserved regions function as active site for antibody binding, proteins with low amino acid homology can still have a strong tendency to cross-react.

### *The xMAP assays*

#### Sensitivity

LODs of the singleplex assays for TNF- $\alpha$ , IL-1 $\beta$ , SAA and LBP, the duplex assay format for IL-1 $\beta$  and the triplex assay for IL-1 $\beta$ , SAA and LBP were lower or comparable with other immunoassays used for detection of these analytes in bovine samples (Goto *et al.*, 1997; Bannerman *et al.*, 2003; Grönlund *et al.*, 2003; Rambeaud *et al.*, 2003; Lehtolainen, Røntved & Pyörälä, 2004). Other assays with even lower detection limits for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 have also been reported (Rainard & Paape, 1997; Whelan *et al.*, 2003; Molina, 2005).

During inflammatory responses the pro-inflammatory cytokines are secreted transiently and in low concentrations (pg-ng/ml). Therefore, sensitive immunoassays with low LODs are necessary. The singleplex assays for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 developed in Paper II had satisfactory LODs, but that was not the case for the duplex and the triplex assays. One reason for this could be that the singleplex assays for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 had to be modified in order to design a functional multiplex assay. For instance, MFI signals generally increase with longer incubation time but at the same time the background noise increases. In the

duplex and triplex analyses the incubation time for IL-6 had to be shortened and this meant that the sensitivity for TNF- $\alpha$  and IL-1 $\beta$  were lost due to proportionally higher background fluorescence.

APPs can, unlike cytokines, be found in low but detectable levels (ng- $\mu$ g/ml) in milk and blood plasma from healthy cows (Bannerman *et al.*, 2003; Lehtolainen, Røntved & Pyörälä, 2004). In this study the LODs of the APP assays were satisfactory and we could show that the triplex xMAP assay for IL-1 $\beta$ , SAA and LBP was useful for detailed studies on APPs during inflammatory responses.

#### Linear range

During our studies we generally observed wider linear ranges in singleplex assays than in duplex or triplex assays, a phenomenon earlier reported for xMAP assays (Carson & Vignali, 1999). The linearity of the standard curves for IL-1 $\beta$  was similar in the different assays developed in Paper II and IV, supporting the robustness and reproducibility reported for the xMAP technique. Multiplex flow cytometric assays usually have broader linear ranges than ELISAs (Kellar & Iannone, 2002; Jenmalm *et al.*, 2003). Working ranges of ELISAs normally spread over one or two orders of magnitude while xMAP assays can have working ranges over three or four orders of magnitude. The linearity of the standard curves in the different singleplex assays agreed with those findings, although the linear ranges for the singleplex assays in Paper IV were not fully explored due to lack of sufficient quantities of recombinant proteins.

In multiplex assays where cytokine and APP detection is combined, the linear ranges of the individual assays are important. APPs can be abundant in biological fluids and would allow large dilution while cytokines are found only in low concentrations. To avoid dilution problems the sensitivity of the different assays included in the multiplex assay could be altered. The sensitivity of the APP assays could be lowered by coupling fewer antibodies to each microsphere (Dasso *et al.*, 2002; Sun *et al.*, 2007), making it possible to analyse them together with cytokines.

#### Intra- and inter-assay variation

Intra- and inter-assay variations of an assay are important measures of the precision and reproducibility. Low intra-assay variation allows for less replicates on the same plate, which is very valuable when the sample volume is small, and also saves time and consumption of reagents. Low inter-assay variation is important for comparisons of analysis results performed on different days and in studies where repeated analyses are performed over a long time span.

In the xMAP assays validated here, most CV's were comparable with values in guidelines for analytical procedures and other reports on assay development for soluble analytes (Validation of analytical procedures, 1996; Wadhwa & Thorpe, 1998; Morgan *et al.*, 2004). In some of the multiplex assays, the inter-assay CV's was however higher than recommended. Problems due to high intra- and inter-assay variation can be reduced by running samples in duplicates and by including internal control samples and standard curves in each assay.

#### Cross-reactivity between reagents

Significant cross-reactivity was not found between any reagents in the multiplex xMAP assays developed in this thesis. This allows for reliable quantification of several analytes in the same sample, with low risk of false positive results. Cross-reactivity is often a problem in assays where several antibodies and antigens are combined in the same solution (Rosner, Grassman & Haas, 1991). This problem is especially prominent in veterinary immunology where reagents developed for specific species are rare (Shuster, Kehrli & Stevens, 1993; Bannerman *et al.*, 2003; Lahouassa *et al.*, 2007; Sohn *et al.*, 2007). Yet, the choice of antibody clones is most important. In addition, assay buffers, incubation times and temperatures, blocking components and sample diluents also have a large influence (de Jager & Rijkers, 2006). In a sandwich immunoassay, the specific binding of antibodies to an analyte is utilized. Monoclonal antibodies are often used for the detection of an analyte as distortions due to other molecules become minimized. The concentration of bound analyte is often determined by polyclonal antibodies, as their broad range of epitopes increases the chances of detecting all analytes bound to the monoclonal antibodies.

#### Matrix effects

In the recovery experiment performed in Paper I, rhuTNF- $\alpha$  added to bovine milk or serum samples was detected to a lower degree compared to quantifications in buffer, indicating a quenching effect of the milk and serum. In Paper II, a general reduction of MFI was observed when samples were analysed in multiplex assays compared with singleplex assays. Similar findings were seen in Paper IV, but to a lesser extent than in Paper II. Those findings concur with results acquired *e.g.* by Carson & Vignali (1999). The reduced MFI is probably due to cross-reactivity between different host derived antibodies, the recombinant proteins and other components in the biological samples.

Quenching of fluorescent signals has previously been observed in immunoassays (Mire-Sluis, Gaines-Das & Thorpe., 1995; Selby, 1999; Phillips *et al.*, 2006). When immunoassays are performed in *e.g.* milk and blood plasma samples, endogenous antibodies, soluble receptors and anti-cytokine antibodies may interfere and disturb the results of the assay, leading to both false positive and false negative values. The composition of milk from cows with mastitis may diverge depending on the phase of the inflammation (Pyörälä, 2003; Berglund *et al.*, 2007), which means that recovery of analytes may differ widely. This could be one explanation why the recovery of rhuTNF- $\alpha$  was low in Paper I.

Recovery of recombinant human cytokines added to blood serum have been found to be concentration dependent, and high non-physiological concentrations were recovered to a larger extent than concentrations in a physiologically expected range (Prabhakar *et al.*, 2004). These and many other data show the importance of validating each assay using the intended matrix. During mastitis, the composition of milk becomes strongly altered, and validation of a general assay for use in milk from cows with mastitis can be difficult.

False positive signals are often caused by heterophile antibodies making bridges between capture and detection antibodies (Phillips *et al.*, 2006). When an analyte binds to soluble receptors or auto-antibodies in biological samples the concentration of an analyte is underestimated (Boscato & Stuart, 1988; Phillips *et al.*, 2006). Such false negative results have been reported for of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in human blood plasma and serum, and in synovial fluid (Svensson *et al.*, 1993; de Jager & Rijkers, 2006), but could also be expected to occur in bovine samples. Since physical barriers between vessel walls and the surrounding tissue are destroyed during many inflammatory responses, components of plasma can be found for instance in milk during mastitis (Rainard, 2003). Problems with soluble receptors and heterophile antibodies could therefore be expected in milk samples from cows with mastitis. Problems with matrix effects can to some extent be reduced by dilution.

#### *The WBA as a predictive tool*

The looser consistency of faeces and higher heart rate pi found in the pre-inoculation low responders for IL-1 $\beta$  and IL-6, were not enough to suggest that those groups were more susceptible to severe forms of *E. coli* mastitis than the high responders for IL-1 $\beta$  and IL-6. Differences in kinetics of bacterial clearance in milk, other clinical symptoms and *in vivo* cytokine concentrations pi were not observed between any of the pre-inoculation high and low groups, thus the WBAs, performed in their present form, could not be considered useful predictive tools for the clinical outcome of a following *E. coli* mastitis.

A reason that the TNF- $\alpha$ , IL-1 $\beta$  and IL-6 WBAs did not work as predictive tools of the severity of *E. coli* mastitis could be the LPS doses and incubation times which were used. In previous WBA studies, the aim has been to induce maximum response of the cytokines, most prevalently TNF- $\alpha$ , *ex vivo*, for comparisons with processes *in vivo* (Hutchinson *et al.*, 1999; Wurfel *et al.*, 2005). In order to determine the maximum response, studies on doses have been performed, and the TNF- $\alpha$  response seems to be dose dependent up to the level where the maximum response is reached (Finch-Arietta and Cochran, 1991; Foster *et al.*, 1993; Nakamura, Nitta & Ishikawa, 2004; Røntved *et al.*, 2005). In human, bovine and porcine blood samples, the maximum TNF- $\alpha$  response to LPS has been observed after 2 to 8 hours stimulation (Allen *et al.*, 1992; Carstensen *et al.*, 2005; Røntved *et al.*, 2005). In the WBA performed in Paper III, a LPS dose previously determined to induce maximum TNF- $\alpha$  response in bovine blood (Røntved *et al.*, 2005) was used, and our results corroborate the earlier studies, as higher TNF- $\alpha$  concentrations were found after 3.5 hours than after 24 hours stimulation. In order to determine the time point and LPS concentrations for maximum IL-1 $\beta$  and IL-6 responsiveness to LPS, further studies are needed. Thereafter, customized studies using the maximum response of IL-1 $\beta$  and IL-6 in the WBA method might be used as predictive tools for the immunological response to an infection.

The cytokine responsiveness may also be influenced by physiological status of the cow. Parturition, metabolic status, and lactation phase have been observed to affect the production of pro-inflammatory cytokines (Doherty *et al.*, 1994; Sordillo, Pighetti & Davis, 1995). Impaired immunological changes in cattle

around parturition have been demonstrated in both *in vivo* studies (Mallard *et al.*, 1997) and *ex vivo* TNF- $\alpha$  responsiveness studies (Røntved *et al.*, 2005). The latter study implicates that results from *ex vivo* WBAs can be useful for monitoring the *in vivo* innate immune system of cows if the right conditions and design is used.

Although *ex vivo* WBAs are designed to mimic the natural environment for the immune system as whole blood is used, data from studies performed outside an animal should always be interpreted and translated to *in vivo* situations with caution. The regulation of immunological responses is complicated and can not solely be explained by the cytokine levels in blood samples. The production rate and source of the cytokines, clearance dynamics, presence of circulating soluble receptors for cytokines and LBP, and membrane receptor distribution and their activity all contribute to the impact, shape, magnitude and duration of cytokine responses to LPS (Koj, 1996; Krishnaswamy *et al.*, 1999).

### *Experimental infection designs*

To reach the aims of this thesis, milk and blood samples from cows with clinical mastitis were needed, and samples from three different experimental mastitis studies have been utilized. In Papers I-II and IV, the focus was on development and application of the xMAP technique in bovine samples, therefore those experimental designs will not be discussed further.

In Paper III, however, the focus was on monitoring the effects of the experimentally induced *E. coli* infection in the cow in relation to the WBAs performed before the infection was induced. However, only six of ten cows became infected by the *E. coli* inoculation and developed clinical mastitis. With that small number of cows it is difficult to draw conclusions from the results of the *ex vivo* WBA experiments as a predictive tool of severity of the *E. coli* infection.

## **Cytokines and APPs in bovine milk and plasma**

Secretion of pro-inflammatory cytokines in biological fluids has been reported in a range of bovine mastitis studies, and TNF- $\alpha$ , IL-1 $\beta$  and IL-6 have been suggested as important mediators (Shuster, Kehrl & Baumrucker, 1995; Shuster *et al.*, 1997; Riollet, Rainard & Poutrel, 2000; Bannerman *et al.*, 2004; Sohn *et al.*, 2007). Major APPs and other inflammatory markers, *e.g.* SAA, haptoglobin, LBP, ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) and C5a, have also been detected in biological fluids in cows with mastitis (Shuster *et al.*, 1997; Hirvonen *et al.*, 1999; Grönlund *et al.*, 2003; Bannerman *et al.*, 2004; Lehtolainen, Røntved & Pyörälä, 2004; Piñeiro *et al.*, 2004). Our results from the TNF- $\alpha$ , IL-1 $\beta$ , IL-6, SAA and LBP analyses in Paper II – IV corroborate earlier studies as all used analytes could be detected in milk or blood from cows with mastitis.

As mentioned earlier, the production of the cytokines and APPs vary between inflammatory responses and the physiological status of the host. More severe cases of *E. coli* mastitis are often reported around calving, in early lactation and at drying-off (reviewed by Burvenich *et al.*, 2003). High concentrations of TNF- $\alpha$  in serum were correlated with severe mastitis, sepsis and death in some studies

(Sordillo & Peel, 1992; Hirvonen *et al.*, 1999; Blum *et al.*, 2000; Hisaeda *et al.*, 2001), while other studies did not report such associations (Nakajima *et al.*, 1997; Hoeben *et al.*, 2000). Our results from Paper III agree with the latter studies as the cows which developed moderate mastitis had elevated TNF- $\alpha$  levels in both milk and plasma.

Other factors than the physiological status of a cow influence the cytokine and APP concentrations in milk and blood during experimentally induced mastitis. The choice and dose of stimuli is important. The clearance of structural components of bacteria, *e.g.* LPS, PGN or LTA, would presumably be easier than clearance of proliferating bacteria in the udder. Thus, live pathogens are expected to induce longer lasting immunological responses than bacterial components, which were supported by a comparative study on *E. coli* and LPS mastitis performed by Blum *et al.* (2000).

When bacteria were used to induce mastitis, increased mRNA expression or secretion of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, SAA and LBP in milk and blood have been reported (Shuster *et al.*, 1997; Eckersall *et al.*, 2001; Bannerman *et al.*, 2004; Vangroenweghe *et al.*, 2005; Hyvönen *et al.*, 2006; Lahouassa *et al.*, 2007). In studies where *E. coli* LPS, *S. aureus* LTA or  $\alpha$ -toxin were used to induce mastitis, pro-inflammatory cytokines and APPs were frequently detected in milk but seldom in blood (Shuster, Kehrl & Stevens, 1993; Rainard & Paape, 1997; Lehtolainen, Røntved & Pyörälä, 2004). The TNF- $\alpha$  response in milk and blood during LPS mastitis has been shown to be local and regulated within the mammary gland (Rainard & Paape, 1997; Paape *et al.*, 2002; Lehtolainen, Røntved & Pyörälä, 2004). Taken together, the results suggest that the immunological response is systemically activated and regulated to a larger extent during bacterial udder invasion than when only fragments of bacteria are present.

In samples from the experimental *E. coli* mastitis study in Papers II and III, IL-1 $\beta$  and IL-6 were detected at least once in milk and plasma samples from all six infected cows. TNF- $\alpha$  concentrations were found at least once in milk samples from all the infected cows and in plasma from four of the infected cows. Our data from Papers II-III indicate that TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are involved in the local inflammatory response to *E. coli* bacteria in the udder, as well as in the systemic immune response to the invading pathogen, which concur with the results discussed above. In Paper IV, plasma from only two cows were analysed, and conclusions are difficult to draw. However, in accordance with previous studies, SAA and LBP were detected in plasma samples collected after endotoxin inoculation (Bannerman *et al.*, 2003; Lehtolainen, Røntved & Pyörälä, 2004). Also the PGN inoculation induced elevated SAA and LBP levels. The SAA plasma concentration increased two- to fiftyfold during mastitis, while LBP concentrations became doubled after both inoculations.

The initiation and regulation of early immune responses are not fully explored but the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 seems to be closely related, and the cytokines can probably influence the production of each other (Moshage, 1997; Gruys *et al.*, 2005). Our results agree with that theory.

In Paper III, we saw that high plasma concentrations of IL-1 $\beta$  were associated with high IL-6 and TNF- $\alpha$  plasma concentrations within the cow. In addition, high concentrations of TNF- $\alpha$  in milk and plasma tended to be associated with each other. The mean peak concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in plasma and the mean peak concentration of TNF- $\alpha$  in milk were observed at the same time point as fever, elevated heart rate and changes in milk colour occurred in the infected cows. Other *E. coli* mastitis studies have been reported where peak concentrations of the pro-inflammatory cytokines in milk and plasma were concurrent with onset of clinically visible symptoms (Sordillo & Peel, 1992; Bannerman *et al.*, 2004). In a study by Lehtolainen, Røntved & Pyörälä (2004), the milk concentrations of TNF- $\alpha$  and SAA after intramammary LPS inoculation seemed to be associated with each other and with the clinical signs. The results in Paper III concur with results from that study, where elevated milk TNF- $\alpha$  concentrations were found to be associated with local udder symptoms and changed milk parameters. In addition, high plasma levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were associated with fever, elevated heart rate, looser faeces consistency, reduced milk yield, changed milk colour and swollen and red udder.



## Conclusions

- Commercially available antibodies against human TNF- $\alpha$  did not cross-react with bovine TNF- $\alpha$  when applied in the xMAP assay. Two clones of anti-human TNF- $\alpha$  antibodies cross-reacted with recombinant ovine TNF- $\alpha$  in concentrations above 2.5 ng/ml.
- TNF- $\alpha$ , IL-1 $\beta$  and IL-6 could be detected in bovine milk and plasma with singleplex, duplex and triplex xMAP assays using ovine and bovine reagents. The singleplex xMAP assays for TNF- $\alpha$ , IL-1 $\beta$  and IL-6 could be applied to monitor cytokines in milk and plasma from cows with *E. coli* mastitis. In order to be a valuable tool in studies where low concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are of interest, the triplex xMAP assay need further development work.
- *Ex vivo* WBAs using LPS and *E. coli* induced production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. The cytokine concentrations in the pre-inoculation blood samples, induced by the LPS and *E. coli* doses and incubation times used, could not predict the severity of an *E. coli* induced mastitis.
- In cows, which had developed mastitis after intramammary inoculation of *E. coli*, high blood plasma concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and high milk TNF- $\alpha$  concentrations were associated with more severe clinical symptoms.
- The cytokine IL-1 $\beta$  and the acute phase proteins SAA and LBP could be detected in bovine plasma from cows inoculated with LPS and PGN using singleplex and triplex xMAP assays.

## Future perspectives

The development of microsphere based flow cytometric assays for multiplex analysis of human analytes has been rapid. From being able to analyse six cytokines simultaneously in 1999 (Chen *et al.*, 1999), more than 50 cytokines were detected simultaneously seven years later (Elshal & McCoy, 2006). During the last year an equine anti-virus antibody xMAP assay has been reported (Go *et al.*, 2008). With this progress, it is plausible to believe that microsphere based flow cytometric assays will be applied in new areas of veterinary medicine during the next years.

Predicting in which areas of veterinary medicine the xMAP technique will be applied is difficult mainly because the accessibility to reagents is limited. Below, a few thoughts on how our existing xMAP panels could be expanded, and some examples on where development of xMAP panels would be valuable are presented and discussed.

In Paper IV, the triplex assay for IL-1 $\beta$ , SAA and LBP was validated for blood plasma but with further validation work, the assay can be applied in any biological fluid, *e.g.* saliva, lung lavage, blood or faeces, depending on the type of information wanted. LBP is only present in high concentrations during bacterial infections and therefore serves as a good marker of such infections. By adding a marker for virus, our assay would contribute more information about the origin of a disease. Production of Type 1 interferons (IFN- $\alpha$  and IFN- $\beta$ ) is induced as a response to presence of virus or double-stranded DNA in a host (Sandberg, Matsson & Alm, 1990; Haller, Kochs & Weber, 2006), making them good markers of viral infections. For example, serum concentrations of IFN- $\alpha$  and IL-6 were used as markers of ongoing viral and bacterial infections in pigs (Le Bonnardiere & Laude, 1981; Fossum *et al.*, 1998). Thus it is feasible to use the xMAP panel in other domestic species than cows. Other options for detection of viral infections could be IFN- $\gamma$  inducible protein (IP-10/CXCL10), which is directly induced by IFN- $\alpha$  and IFN- $\beta$  (Gangur, Birmingham & Thanavorakul, 2002), or Mx proteins which are produced in cells stimulated with IFN- $\alpha$  (Horisberger & Gunst, 1991). Simultaneous detection of IL-1 $\beta$ , SAA and IFN- $\alpha$  can be performed in our laboratory (Dernfalk, Persson Waller & Johannisson, 2006), but at present the lack of IFN- $\alpha$  reagents makes further work with that panel impossible.

Bacterial and viral infections traditionally elicit different immunological responses, but cross-talk between immunological pathways occurs and therefore immunological markers used to differentiate between viral and bacterial infections should be regarded with caution.

Health problems and economical losses due to infections are evident in many animal species. An example is bacterial dysentery infections which convey high mortality in growing pigs (Raynaud, Brunault & Philippe, 1980). In order to develop prophylactic measures and vaccination programmes for dysentery in pigs, an understanding of the host immune responses during the disease is essential. Johannisson *et al.* (2006) reported of development of a triplex xMAP assay for

simultaneous detection of TNF- $\alpha$ , IL-1 $\beta$  and IL-8 in porcine blood plasma and serum, which could provide useful information if applied in biological samples from pigs with dysentery. If this xMAP assay for porcine cytokines was expanded with IL-6 and SAA, also information about the acute phase response would be achieved.

Pigs are used as model animals in comparative studies with humans, for instance in the research on lung diseases. The porcine cytokine xMAP assay developed by Johannisson *et al.* (2006) was used to continuously monitor cytokines in bronchial epithelial fluid collected by microdialysis in pigs (Tyvold *et al.*, 2008). It is planned to expand the xMAP panel to include the pro-inflammatory cytokine IL-6, the anti-inflammatory cytokine IL-10 and the modulating cytokine IFN- $\gamma$  to increase the understanding of the inflammatory responses to lung injury or infection.

The majority of reports with xMAP assays are published within the field of immunology, although applications many other areas are possible (Morgan *et al.*, 2004; Elshal & McCoy 2006). An area attracting considerable interest in the human research field is at the moment obesity and its connections to development of type-2 diabetes, cardiovascular disease and correlations to increased risk of cancer (Lawrence & Kopelman, 2004). Studies on the interactions between nutrition, endocrinology and metabolism also become increasingly important in our domestic animals. For instance, race horses and dairy cows demand optimal nutrition, and obesity among pets is an increasing problem. Therefore an xMAP assay for simultaneous detection of proteins involved in hormonal metabolic signalling at circulation and at tissue levels could be designed for different animal species. Metabolic xMAP analyses are available for many human metabolic steroids and proteins (Yurkovetsky, 2007).

## Swedish summary

### Populärvetenskaplig sammanfattning

Infektioner hos nötkreatur är ett stort problem i hela världen. Mastit (juverinflammation) orsakas oftast av att juvret invaderas av bakterier och trots att det har gjorts otaliga försök att kartlägga orsakerna till och förloppet av mastit är sjukdomen fortfarande ett allvarligt problem. Studier för att fördjupa kunskaperna om mastit, utveckla förebyggande åtgärder, diagnostiska metoder eller hitta alternativa behandlingsvägar är därför ständigt aktuella.

När juvret invaderas av sjukdomsframkallande mikroorganismer startas ett immunsvaret i kon. Genom att mäta koncentrationsförändringar av de proteiner som reglerar immunsvaret kan man studera infektionsförloppet vid mastit. De reglerande proteinerna kallas cytokiner och är immunförsvarets budbärare. Olika grupper av cytokiner har olika funktioner, vissa aktiverar medan andra hämmar immunsvaret. Det är viktigt att det finns en balans mellan de aktiverande och de hämmande cytokinerna så att ett immunsvaret varken blir för kraftigt eller för svagt. Akutfasproteiner är en annan grupp viktiga proteiner i immunförsvaret. De tillverkas i levern när immunförsvaret aktiveras och de kan både stimulera och hämma immunsvaret. Att de kan användas som markörer för inflammation beror på att deras koncentrationer i blodserum ofta ökar dramatiskt vid inflammationer. Möjligheten att mäta koncentrationer av cytokiner och akutfasproteiner i biologiska prover är fundamental i forskning kring immunsvaret mot infektioner.

Ett exempel på hur man kan mäta immunologiskt viktiga proteiner är med den flödescytometribaserade xMAP-tekniken. Flödescytometri är en teknik där partiklars egenskaper bestäms via det ljus de sänder ut efter att ha blivit belysta av en laser. I xMAP-analyser fångas den analyt man vill mäta mellan två antikroppar riktade mot olika delar av analyten. De antikroppar som ska fånga in analyten kopplas till små plastkulor (mikrosfärer) som blandas med det biologiska provet. Om det finns analyter av intresse i provet binder de in till antikropparna på kulorna. Ett mått på mängden inbunden analyt fås genom att fluorescensmärkta antikroppar sätts till lösningen innan proverna analyseras i en flödescytometer. För att kunna mäta flera olika analyter samtidigt i samma prov används mikrosfärer med unika färgkombinationer. xMAP-tekniken har visat sig användbar inom många områden, bland annat för analys av cytokiner, antikroppar, ämnesomsättningsenzym, intracellulära proteiner och cancermarkörer.

Syftet med den här avhandlingen var att utveckla xMAP-analyser för inflammatoriska markörer vars koncentrationer ökar vid inflammationer i kor. Fokus låg på utveckling av analyser för cytokiner och akutfasproteiner i mjölk och blod från kor med mastit.

För att uppnå syftet designades xMAP-analyser för att studera en (singleplex), två (duplex), eller tre (triplex) analyter samtidigt. Analyser för cytokinerna tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  och IL-6 samt för akutfasproteinerna serum amyloid A (SAA) och lipopolysaccharide binding protein (LBP) utvecklades. Metoderna validerades enligt riktlinjer för immunologiska metoder.

För varje xMAP-analys bestämdes lägsta detektionsgräns, inom vilka koncentrationer analyserna var tillförlitliga samt variation inom och mellan olika analystillfällen. I de multiplexa (duplexa och triplexa) analyserna kontrollerades även eventuell korsreaktivitet mellan reagenser, dvs om detektion av en analyt ledde till att en ökad koncentration av en annan analyt felaktigt registrerades.

Att en analys har låg detektionsgräns är främst viktigt i cytokinstudier eftersom de är mycket kraftfulla mediatorer och därför bara utsöndras i låga koncentrationer under inflammatoriska svar. De singleplexa cytokinanalyserna hade tillfredsställande känslighet, men inte de multiplexa analyserna. När det gäller akutfasproteinanalyser krävs oftast inte lika låga detektionsnivåer, istället är analysens räckvidd viktigare eftersom akutfasproteiner kan förekomma i både låga och mycket höga koncentrationer. Räckvidden för tillförlitlig koncentrationsbestämning av SAA och LBP var tillfredsställande, men bör kunna förbättras ytterligare. Korsreaktivitet noterades inte mellan några av reagenserna i de duplexa och triplexa xMAP-analyserna.

De singleplexa och multiplexa analyserna utvecklade i den här avhandlingen kunde användas för detektion av analyterna i mjölk- och blodplasmaprover från kor med mastit. Komponenter i mjölk och plasma påverkade detektionen av analyter vilket är ett mycket vanligt problem som till viss del går att åtgärda med spädning av provet. I en av studierna visade vi att xMAP-tekniken kunde användas för att mäta ett cytokin och två akutfasproteiner i samma prov, något som kan ge ett tidsperspektiv på ett inflammationsförlopp, eftersom cytokiner främst utsöndras i början av en inflammation medan förhöjda koncentrationer av akutfasproteiner kan mätas under längre tid.

I en av avhandlingens studier inokulerades tio kor med bakterien *Escherichia (E.) coli* i juvret, vilket ledde till att sex av djuren fick mastit. Innan den experimentella mastitstudien genomfördes togs blodprover från korna. Dessa stimulerades med *E. coli* och en komponent som finns i bakteriens vägg kallad lipopolysackarid. När blodprover stimuleras kallas det för *ex vivo* stimulering. Efter *ex vivo* stimuleringen kunde TNF- $\alpha$ , IL-1 $\beta$  och IL-6 mätas i blodet. Kornas förmåga att producera cytokiner *ex vivo* jämfördes med deras immunförsvars förmåga att hantera inokulationen av *E. coli* i juvret. Vi såg att kor som producerade låga koncentrationer av IL-1 $\beta$  eller IL-6 *ex vivo* hade högre tendens att utveckla diarré respektive hade högre puls än vad grupperna som producerade höga nivåer av IL-1 $\beta$  eller IL-6 hade. Inga andra skillnader i kliniska symptom under infektionen sågs mellan kor som *ex vivo* hade producerat höga och låga koncentrationer av cytokinerna. *Ex vivo* stimuleringen utförd på det här sättet kunde därför inte anses vara ett värdefullt verktyg för att förutsäga svårighetsgraden av *E. coli*-mastiter.

Vid analyser av cytokiner i mjölk och plasma samlat från korna efter *E. coli* inokuleringen observerades att höga plasmanivåer av IL-1 $\beta$  hade samband med höga plasmakoncentrationer av TNF- $\alpha$  och IL-6. Dessutom kunde höga TNF- $\alpha$ , IL-1 $\beta$  och IL-6 plasmakoncentrationer och höga TNF- $\alpha$  koncentrationer i mjölk associeras med kliniska symptom både lokalt och systemiskt. Dessa resultat stämmer väl överens med tidigare cytokinstudier på kor med mastit och visar xMAP-metodernas användbarhet.

## References

- Aderem, A. & Ulevitch, R.J. 2000. Toll-like receptors in the induction of the innate immune response. *Nature* 406, 782 – 787.
- Akira, S., Taniuchi, T. & Kishimoto, T. 1993. Interleukin-6 in biology and medicine. *Advances in Immunology* 54, 1 – 78.
- Allen, J.N., Herzyk, D.J., Allen, E. D., Wewers, M.D. 1992. Human whole blood interleukin-1beta production: kinetics, cell source, and comparison with TNF-alpha. *The Journal of Laboratory and Clinical Medicine* 119, 538 – 546.
- Ames, T.R. 1997. Dairy calf pneumonia. The disease and its impact. *Veterinary Clinics of North America: Food Animal Practice* 13, 379 – 391.
- Andersson, M., Berg, M., Fossum, C., Jensen-Waern, M. 2007. Development of a microarray for studying porcine cytokine production in blood mononuclear cells and intestinal biopsies. *Journal of Veterinary Medicine* 54, 161 – 168.
- Bannerman, D.D., Paape, M.J., Hare, W.R., Sohn, E.J. 2003. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. *Journal of Dairy Science* 86, 3128 – 3137.
- Bannerman, D.D., Paape, M.J., Lee, J.W., Zhao, X., Hope, J.C., Rainard, P. 2004. *Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clinical and Diagnostic Laboratory Immunology* 11, 463 – 472.
- Barkema, H.W., Schukken, Y.H. & Zadoks, R.N. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *Journal of Dairy Science* 89, 1877 – 1895.
- Baron, S. *Medical Microbiology 4<sup>th</sup> Edition*, www.gsbs.utmb.edu/microbook. (Accessed Jan-11-2008).
- Barquet N. & Domingo P. 1997. Smallpox: the triumph over the most terrible of the ministers of death. *Annals of Internal Medicine* 127, 635 – 642.
- Bemelmans, M.H., van Tits, L.J. & Buurman, W.A. 1996. Tumor necrosis factor: function, release and clearance. *Critical Reviews in Immunology* 16, 1 – 11.
- Berglund, I., Pettersson, G., Östensson, K., Svennersten-Sjaunja, K. 2007. Quarter milking for improved detection of increased SCC. *Reproduction in Domestic Animals* 42, 427 – 432.
- Beutler, B. 2000. Endotoxin, toll-like receptor 4, and the afferent limb of the innate immunity. *Current Opinions in Microbiology* 3, 23 – 28.
- Blackburn, W.D.Jr. 1994. Validity of acute phase proteins as markers of disease activity. *The Journal of Rheumatology*. Supplement 42, 9 – 13.
- 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. *Domestic Animal Endocrinology* 19: 223 – 235.
- Boscato, L.M. & Stuart, M.C., 1988. Heterophilic antibodies: a problem for all immunoassays. *Clinical Chemistry* 34, 27-33.
- Bradley, A.J. 2002. Bovine mastitis: an evolving disease. 2002. *Veterinary Journal* 164, 116 – 128.
- Braeckmans, K., de Smedt, S.C., Leblans, M., Pauwels, R., Demeester, J. 2003. Encoding microcarriers: present and future technologies *Nature Materials* 2, 169 – 173.

- Bryson, D.G. 1985. Calf pneumonia. *The Veterinary Clinics of North America. Food Animal Practice* 1, 237 – 257.
- BSE Inquiry: The Report. 2000. Chapter 8, Cattle diseases and their control and management on farm, volume 12: Livestock Farming. Crown Copyright, London.
- Burvenich, C., Bannerman, D.D., Lippolis, J.D., Peelman, L., Nonnecke, B.J., Kehrl, M.E., Paape, M.J. 2006. Cumulative physiological events influence the inflammatory response of the bovine udder to *Escherichia coli* infections during the transition period. *Journal of Dairy Sciences* 90 (E-suppl), E39 – E54.
- Burvenich, C., van Merris, V., Mehrzad, J., Diez-Fraile, A., Duchateau, L. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. *Veterinary Research* 34, 521 – 564.
- Camilla, C., Mely, L., Magnan, A., Casano, B., Prato, S., Debono, S., Montero, F., Defoort, J.P., Martin, M., Fert, V., 2001. Flow cytometric microsphere-based immunoassay: analysis of secreted cytokines in whole-blood samples from asthmatics. *Clinical and Diagnostic Laboratory Immunology* 8, 776-784.
- Carson, R.T. & Vignali, D.A.A. 1999. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. *Journal of Immunological Methods* 227, 41 – 52.
- Carstensen, L., Røntved, C.M. & Nielsen, J.P. 2005. Determination of tumor necrosis factor- $\alpha$  responsiveness in piglets around weaning using an *ex vivo* whole blood stimulation assay. *Veterinary Immunology and Immunopathology* 105, 59 -66.
- Cecilian, F., Giordani, A., Spagnolo, V. 2002. The systemic reaction during inflammation: the acute-phase proteins. *Protein and Peptide Letters* 9, 211 – 223.
- Chen, K., Huang, J., Gong, W., Iribarren, P., Dunlop, N.M., Wang, J.M. 2007. Toll-like receptors in inflammation, infection and cancer. *International Immunopharmacology* 7, 1271 – 1285.
- Chen, R., Lowe, L., Wilson, J.D., Crowther, E., Tzeggai, K., Bishop, J.E., Varro, R. 1999. Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clinical Chemistry* 45, 1693 – 1694.
- Chinen, J., Shearer, W.T. 2007. Advances in basic and clinical immunology in 2006. *Journal of Allergy and Clinical Immunology* 120, 263 – 270.
- Cook, E.B., Stahl, J.L., Lowe, L., Chen, R., Morgan, E., Wilson, J., Varro, R., Chan, A., Graziano, F.M., Barney, N.P. 2001. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. *Journal of Immunological Methods* 254, 109 – 118.
- Dasso J., Lee J., Bach H., Mage R.G. 2002. A comparison of ELISA and flow microsphere-based assays for quantification of immunoglobulins. *Journal of Immunological Methods* 263, 23 – 33.
- de Jager, W., Prakken, B.J., Bijlsma, W.J., Kuis, W., Rijkers, G.T., 2005. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *Journal of Immunological Methods* 300, 124-135.
- de Jager, W. & Rijkers, G.T. 2006. Solid-phase and bead-based cytokine immunoassay: A comparison. *Methods* 38, 294-303.
- de Jong, B.A., Westendorp, R.G.J., Bakker, A.M., Huizinga, T.W.J. 2002. Polymorphisms in or near tumour necrosis factor (TNF)-gene do not determine levels of endotoxin-induced TNF production. *Genes and Immunity* 3: 25 – 29.

- Dernfalk, J., Persson Waller, K., Røntved, C.M., Johannisson, A. 2006. Development of a multiplex xMAP assay for quantification of bovine markers of inflammation and infection. Conference abstract *16<sup>th</sup> European Congress of Immunology*, Paris, France, PA-2095, page 266.
- Desch, C.E., Kovach, N.L., Present, W., Broyles, C., Harlan, J.M. 1989. Production of human tumor necrosis factor from whole blood *ex vivo*. *Lymphokine Research* 8, 141 – 146.
- de Schepper, S., de Ketelaere, A., Bannerman, D.D., Paape, M.J., Peelman, L., Burvenich, C. 2007. The toll-like receptor-4 (TLR-4) pathway and its possible role in the pathogenesis of *Escherichia coli* mastitis in dairy cattle. *Veterinary Research* 39, 5.
- Dinareello, C.A. 2000. Proinflammatory cytokines. *Chest* 118, 503 – 508.
- Doherty, J.F., Golden, M.H., Remick, D.G., Griffin, G.E. 1994. Production of interleukin-6 and tumour necrosis factor-alpha in vitro is reduced in whole blood of severely malnourished children. *Clinical Science (London)* 86, 347 – 351.
- Dolezalova, R., Lacinova, Z., Dolinkova, M., Kleiblova, P., Haluzikova, D., Housa, D., Papezova, H., Haluzik, M. 2007. Changes of endocrine function of adipose tissue in anoxia nervosa: comparison of circulating levels versus subcutaneous mRNA expression. *Clinical Endocrinology* 67, 674 – 678.
- Dosogne, H., Meyer, E., Sturk, A., van Loon, J., Massart-Leen, A-M., Burvenich, C. 2002. Effect of enrofloxacin treatment on plasma endotoxin during bovine *Escherichia coli* mastitis. *Inflammation Research* 51, 201 - 205.
- du Preez, J.H. 2000. Bovine mastitis therapy and why it fails. *Journal of the South African Veterinary Association* 71, 201 – 208.
- Dziarski, R. & Gupta, D. 2000. Role of MD-2 in TLR-2 and TLR-4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. *Journal of Endotoxin Research* 6, 401 – 405.
- Dziarski, R., Tapping, R.I. & Tobias, P.S. 1998. Binding of bacterial peptidoglycan to CD14. *The Journal of Biological Chemistry* 273, 8680 – 8690.
- Eberhart, R.J., 1984. Coliform mastitis. *Veterinary Clinics of North America, The Large Animal Practice* 6, 287 – 300.
- Eberhart, R.J. 1986. Management of dry cows to reduce mastitis. *Journal of Dairy Sciences* 69, 1721 – 1732.
- Ebersole, J.L. & Cappelli, D. 2000. Acute-phase reactants in infectious and inflammatory diseases. *Periodontology* 23, 19 – 49.
- Eckersall, P.D., Duthie, S., Toussaint, M.J., Gruys, E., Heegard, P., Alava, M., Lipperheide, C., Madec, F. 1999. Standardization of diagnostic assays for animal acute phase proteins. *Advances in Veterinary Medicine* 41, 643 – 655.
- Eckersall, P.D., Young, F.J., McComb, C., Hogarth, C.J., Safi, S., Weber, A., McDonald, T., Nolan, A.M., Fitzpatrick, J.L. 2001. Acute phase proteins in milk from dairy cows with clinical mastitis. *Veterinary Record* 148, 35 – 41.
- Ehrlich, P. 1892. Ueber Immunität durch Vererbung und Säugung. *Medical Microbiology and Immunology* 12: 183 - 203.
- Ellis, J.A., Godson, D., Campos, M., Sileghem, M., Babiuk, L.A. 1993. Capture immunoassay for ruminant tumor necrosis factor-alpha : comparison with bioassay. *Veterinary Immunology and Immunopathology* 35, 289 - 300.
- Elsasser, T.H., Blum, J.W. & Kahl, S. 2005. Characterization of calves exhibiting a novel inheritable TNF-alpha hyperresponsiveness to endotoxin: associations with increased pathophysiological complications. *Journal of Applied Physiology* 98, 2045 – 2055.



- Elshal, M.F. & McCoy, J.P. 2006. Multiplex bead array assays: performance evaluation and comparison to ELISA. *Methods* 38, 317 – 323.
- Eskay, R.L., Grino, M. & Chen, H.T. 1990. Interleukins, signal transduction, and the immune system-mediated stress response. *Advances in Experimental Medicine and Biology* 274, 331 – 341.
- Feery, B.J. 1976. The conquest of smallpox. *Australian Family Physician* 5, 720 – 733.
- Ferré, F. 1994. Polymerase chain reaction and HIV. *Clinical Laboratory Medicine* 14, 313 – 333.
- Finch-Arietta, M.B. & Cochran, F.R. 1991. Cytokine production in whole blood *ex vivo*. *Agents and Actions*. 34, 49 – 52.
- Fossum, C., Wattrang, E., Fuxler, L., Jensen, K.T., Wallgren, P. 1998. Evaluation of various cytokines (IL-6, IFN-alpha, IFN-gamma, TNF-alpha) as markers of acute bacterial infection in swine – a possible role for serum interleukin-6. *Veterinary Immunology and Immunopathology* 64, 161 – 172.
- Foster, S.J., McCormick, L.M., Ntolosi, B.A., Campbell, D. 1993. Production of TNF-alpha by LPS-stimulated murine, rat and human blood and its pharmacological modulation. *Agents and Actions* 38, Spec no: C77-79.
- Frengen, J., Kierulf, B., Schmid, R., Lindmo, T., Nustad, K. 1994. Demonstration and minimization of serum interface in flow cytometric two-site immunoassays. *Clinical Chemistry* 40, 420 – 425.
- Fulton, R.J., McDade, R.L., Smith, P.L., Kienker, L.J., Kettman, J.R.Jr. 1997. Advanced multiplexed analysis with the FlowMetrix system. *Clinical Chemistry* 43, 1749 – 1756.
- Gangur, V., Birmingham, N.P. & Thanavornakul, S. 2002. Chemokines in health and disease. *Veterinary Immunology and Immunopathology* 86, 127 – 136.
- Gilbert, R.O., Gröhn, Y.T., Miller, P.M., Hoffman, D.J. 1993. Effect of parity on periparturient neutrophil function in dairy cows. *Veterinary Immunology and Immunopathology* 36, 75 – 82.
- Go, Y.Y., Wong, S.J., Branscum, A.J., Demarest, V.L., Shuck, K.M., Vickers, M.L., Zhang, J., McCollum, W.H., Timoney, P.J., Balasuriya, U.B. 2008. Development of a fluorescent-microsphere immunoassay for detection of antibodies specific to equine arteritis virus and comparison with the virus neutralization test. *Clinical and Vaccine Immunology* 15, 76 – 87.
- Goldsby, R.A., Kindt, T.J., Osborne, B.A., Kuby, J. 2003. The inflammatory process. *Immunology 5<sup>th</sup> edition*. W.H. Freeman and Company, New York, 350-356.
- Goto M., Maruyama M., Kitadate K., Kirisawa R., Obata Y., Koiwa M., Iwai H. 1997. Detection of interleukin-1 $\beta$  in sera and colostrums of dairy cattle and in sera of neonates. *Journal of Veterinary Medical Science* 59, 437 – 441.
- Gross, C.P. & Sepkowitz, K.A. 1998. The myth of the medical breakthrough: smallpox, vaccination, and Jenner reconsidered. *International Journal of Infectious Diseases* 3, 54 – 60.
- Gruys, E., Toussaint, M.J.M., Niewold T.A., Koopmans, S.J. 2005. Acute phase reaction and acute phase proteins. *Journal of Zhejiang University SCIENCE* 6B, 1045 – 1056.
- Grøndahl-Hansen, J., Barfod, K., Klausen, J., Andresen, L-O., Heegard, P.M.H., Sørensen, V. 2003. Development and evaluation of a mixed long-chain lipopolysaccharide based ELISA for serological surveillance of infection with *Actinobacillus pleuroneumoniae* serotypes 2, 6 and 12 in pig herds. *Veterinary Microbiology* 96, 41 -45.

- Grönlund, U., Hultén, C., Eckersall, P.D., Hogarth, C., Persson Waller, K. 2003. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis. *Journal of Dairy Research* 70, 379 – 386.
- Haasnot, W. & du Pré, J.G. 2007. Luminex-based triplex immunoassay for simultaneous detection of soy, pea and soluble wheat proteins in milk powder. *Journal of Agricultural and Food Chemistry* 55, 3771 – 3777.
- Haller, O., Kochs, G. & Weber, F. 2007. Interferon, Mx, and viral countermeasures. *Cytokine & Growth Factor Reviews* 18, 425 – 433.
- Harmon, R.J. 1994. Physiology of mastitis and factors affecting somatic cell counts. *Journal of Dairy Science* 77, 2103 – 2112.
- Heegard, P.M.H., Godson, D.L., Toussaint, M.J.M., Tjørnehøj, K., Larsen, L.E., Viuff, B., Rønsholt, L. 2000. The acute phase response of haptoglobin and serum amyloid A (SAA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. *Veterinary Immunology and Immunopathology* 77, 151 – 159.
- Heinrich, P.C., Castell, J.V & Andus, T. 1990. Interleukin-6 and the acute phase response. *Biochemical Journal* 265, 621 – 636.
- Herlin, A., Hultgren, J. & Ekman, T. 2007. Smittskydd i stora mjölkbesättningar – rapport från två arbetskonferenser. Landskap Trädgård Jordbruk Rapportserie SLU, Rapport 2007: 1, Alnarp, Sweden.
- Hirvonen, J., Eklund, K., Teppo, A.M., Huszenicza, G., Kulcsar, M., Saloniemi, H. Pyörälä, S. 1999. Acute phase response in dairy cows with experimentally induced *Escherichia coli* mastitis. *Acta Veterinaria Scandinavica* 40, 35 – 46.
- Hisaeda, K., Hagiwara, K., Eguchi, J. Yamanaka, H., Kirisawa, R., Iwai, H. 2001. Interferon-gamma and tumor necrosis factor-alpha levels in sera and whey of cattle with naturally occurring coliform mastitis. *Journal of Veterinary Medical Science* 63, 1009 – 1011.
- Hoeben, D., Burvenich, C., Trevisi, E., Bertoni, G., Hamann, J., Bruckmaier, R.M., Blum, J.W. 2000. Role of endotoxin and TNF-alpha in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. *Journal of Dairy Science* 67, 503 – 514.
- Horadagoda, A., Eckersall, P.D., Alsemgeest, S.P., Gibbs, H.A. 1993. Purification and quantitative measurement of bovine serum amyloid-A. *Research in Veterinary Science* 55, 317 – 325.
- Horadagoda, N.U., Knox, K.M.G., Gibbs, H.A., Reid, S.W.J., Horadagoda, A., Edwards, S.E.R., Eckersall, P.D. 1999. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. *The Veterinary Record* 144, 437 – 441.
- Horisberger, M.A. & Gunst, M.C. 1991. Interferon-induced proteins: identification of Mx proteins in various mammalian species. *Virology* 180, 185 – 190.
- Horst Ibelgauf's COPE Cytokines & Cells Online Pathfinder Encyclopaedia, Version 19.4 (April 2007) <http://www.copewithcytokines.de> (Accessed 28-Jan-2008).
- Hutchinson, I.V., Pravica, V., Hajeer, A., Sinnott, P.J. 1999. Identification of high and low responders to allografts. *Reviews in Immunogenetics* 1, 323 – 333.
- Hyvönen, P., Suojala, L., Orro, T., Haaranen, J., Simola, O., Røntved, C., Pyörälä, S. 2006. Transgenic cows that produce recombinant human lactoferrin in milk are not protected from experimental *Escherichia coli* intramammary infection. *Infection and Immunology* 74, 6206 – 6212.

- International Conference of Harmonization, ICH. 1994. Q2(R1): Validation of Analytical Procedures: Text and Methodology.
- Isaacs, A. & Lindenmann, J. 1957. Virus Interference. I. The interferon. *Proceedings of the Royal Society of London, Series B* 147, 258 – 267.
- Jenmalm, M.C., Malit, M., Torrence, J., Zhang, A. 2003. Bio-plex cytokine immunoassays and ELISA: Comparison of two methodologies in testing samples from asthmatic and healthy children, Rev A. Technical Article, Bio-Rad Laboratories, Hercules, CA.
- Jensen, L.E. & Whitehead, A.S. 1998. Regulation of serum amyloid A protein expression during the acute phase response. *The Biochemical Journal* 334, 489 – 503.
- Johannisson, A., Jonasson, R., Dornfalk, J., Jensen-Waern, M., 2006. Simultaneous detection of porcine proinflammatory cytokines using multiplex flow cytometry. *Cytometry: Part A* 69, 391-395.
- Jones, C. 2005. *Spotlight: Bovine mastitis caused by Streptococcus uberis*, a longstanding problem. Animal Sciences, CABI Publishing, Wallingford, England.
- Kalaycioglu, A.T. 2007. Bovine viral diarrhoea virus (BVDV) diversity and vaccination. *The Veterinary Quarterly* 29, 60-67.
- Kellar K. L. & Iannone M. A., 2002. Multiplexed microsphere-based flow cytometric assays. *Experimental Hematology* 30, 1227-1237.
- Kelso, A., 1998. Cytokines: principles and prospects. *Immunology and Cell Biology* 76, 300 – 317.
- Kerro DeGo, O.K., van Dijk, J.E., Nederbragt, H. 2002. Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. *The Veterinary Quarterly* 24, 181 – 198.
- Knapp, W., Haaijman, J.J. Schuit, H.R., Rádl, J., van den Berg, P., Ploem, J.S., Hijmans, W. 1975. Microfluorometric evaluation of conjugate-specificity with the defined antigen substrate spheres (DASS) system. *Annals of the New York Academy of Sciences* 30, 94 – 107.
- Koj, A. 1996. Initiation of acute phase response and synthesis of cytokines. *Biochimica Biophysica Acta* 1317, 84 – 94.
- Krishnaswamy, G., Kelley, J., Yerra, L., Smith, J.K., Chi, D.S. 1999. Human endothelium as source of multifunctional cytokines: molecular regulation and possible role in human disease. *Journal of Interferon and Cytokine Research* 19, 91 – 104.
- Kuller, L., Watanabe, R., Anderson, D., Grant, R. 2005. Development of a whole-virus multiplex flow cytometric assay for antibody screening of a specific pathogen-free primate colony. *Diagnostic Microbiology and Infectious Disease* 53, 185 – 193.
- Lahouassa H., Moussay E., Rainard P., Riollet C. 2007. Differential cytokine and chemokine responses of bovine mammary epithelial cells to *Staphylococcus aureus* and *Escherichia coli*. *Cytokine* 38, 12 – 21.
- Lash, G.E., Scaife, P.J., Innes, B.A., Otun, H.A., Robson, S.C., Searle, R.F., Bulmer, J.N. 2006. Comparison of three multiplex cytokine analysis systems: Luminex, SearchLight and FAST Quant. *Journal of Immunological Methods* 309, 205 – 208.
- Last, J.M. 1998. The history, scope, and methods of public health. *Public Health and Human Ecology 2<sup>nd</sup> edition*. McGraw-Hill Professional, New York, 1 – 33.
- Lawrence, V.J. & Kopelman, P.G. 2004 Medical consequences of obesity. *Clinics in Dermatology* 22, 296 – 302.
- Le Bonnardiere, C. & Laude, H. 1981. High interferon titer in newborn pig intestine during experimentally induced viral infections. *Infection and Immunity* 32, 28 – 31.

- Ledue, T.B. & Rifai, N. 2003. Preanalytic and analytic sources of variations in C-reactive protein measurement: implications for cardiovascular disease risk assessment. *Clinical Chemistry* 49, 1258 – 1271.
- Lehtolainen T., Røntved C.M. & Pyörälä S. 2004. Serum amyloid A and TNF-alpha in serum and milk during experimental endotoxin mastitis. *Veterinary Research* 35, 651 – 659.
- Lisi, P.J., Huang, C.W., Hoffman, R.A., Teipel, J.W. 1982. A fluorescence immunoassay for soluble antigens employing flow cytometric detection. *Clinica Chimica Acta* 120, 171 – 179.
- Lofthouse, S.A., Andrews, A.E., Elhay, M.J., Bowles, V.M., Meeusen, E.N., Nash, A.D. 1996. Cytokines as adjuvants for ruminant vaccines. *International Journal of Parasitology* 26, 835 – 842.
- Lohuis, J.A.C.M., van Leeuwen, W., Verheijden, J.H.M., van Miert, A.S.J.P.A.M., Brand, A. 1988. Effect of dexamethasone on experimental *Escherichia coli* mastitis in the cow. *Journal of Dairy Sciences* 71, 2782 – 2789.
- Louis, E., Franchimont, D., Piron, A., Gevaert, Y., Schaaf-Lafontaine, N., Roland, S., Mahieu, P., Malaise, M., de Groote, D., Louis, R., Belaiche, J. 1998. Tumour necrosis factor (TNF) gene polymorphism influences TNF- $\alpha$  production in lipopolysaccharide (LPS)-stimulated whole blood cell culture in healthy humans. *Clinical and Experimental Immunology* 113, 401 – 406.
- Lun, S., Åström, G., Magnusson, U., Östensson, K. 2007. Total and differential leukocyte counts and lymphocyte subpopulations in lymph, afferent and efferent to the supramammary lymph node, during endotoxin-induced bovine mastitis. *Reproduction in Domestic Animals* 42, 126 – 134.
- MacCrindle, C., Schwenzer, K. & Jolley, M. 1985. Particle concentration fluorescence immunoassay: A new immunoassay technique for quantification of human immunoglobulins in serum. *Clinical Chemistry* 31, 1487 – 1490.
- Mallard, B.A., Dekkers, J.C., Ireland, M.J., Leslie, K.E., Sharif, S., Lacey Vankampen, C., Wagter, L., Wilkie, B.N. 1997. Alteration in immune responsiveness during the peripartum period and its ramification on dairy cow and calf health. *Journal of Dairy Science* 81, 585 – 595.
- Malle, E. & De Beer, F.C. 1996. Human serum amyloid A (SAA) protein: a prominent acute phase reactant for clinical practice. *European Journal of Clinical Investigation* 26, 427 – 435.
- Masseyeff, R.F. 1991. Antibody quantitation in human and veterinary medicine. *Scandinavian Journal of Clinical and Laboratory Investigation. Supplementum* 205, 63 – 69.
- Mire-Sluis, A.R., Gaines-Das, R. & Thorpe, R. 1995. Immunoassays for detecting cytokines: what are they really measuring? *Journal of Immunological Methods* 186, 157-160.
- Molina E. 2005. Serum interferon-gamma and interleukin-6 and -8 during infection with *Fasciola gigantica* in cattle and buffaloes. *Journal of Veterinary Science* 6, 135-139.
- Morgan, E., Varro, R., Sepulveda, H., Ember, J.A., Apgar, J., Wilson, J, Lowe, L., Chen, R., Shivraj, L., Agadi, A., Campos, R., Ernst, D., Gaur, A. 2004. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clinical Immunology* 110, 252 – 266.
- Morley, J.J., Kushner, I. 1982. Serum C-reactive protein levels in disease. *Annals of New York Academy of Sciences* 389, 406 – 418.

- Moore, B.R. 1996. Clinical application of interferons in large animal medicine. *Journal of the American Veterinary Medical Association* 208, 1711 – 1715.
- Moshage, H. 1997. Cytokines and the hepatic acute phase response. *Journal of Pathology* 181, 257 – 266.
- Nakajima, Y., Mikami, O., Yoshioka, M., Motoi, Y., Ito, T., Ishikawa, Y., Fuse, M., Nakano, K., Yasukawa, K. 1997. Elevated levels of tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) activities in sera and milk of cows with naturally occurring coliform mastitis. *Research in Veterinary Sciences* 62, 297 – 298.
- Nakamura, T., Nitta, H., Ishikawa, I. 2004. Effect of low dose *Actinobacillus actinomycetemcomitans* lipopolysaccharide pre-treatment on cytokine production by human whole blood. *Journal of Periodontological Research* 39, 129 – 135.
- Norcross, N.L. & Stark, D.M. 1970. Immunity to mastitis: a review. *Journal of Dairy Science* 53, 387 – 393.
- Opitz, B., Hippenstiel, S., Eitel, J., Suttorp, N. 2007. Extra- and intracellular innate immune recognition in endothelial cells. *Thrombosis and Haemostasis* 98, 319 – 26.
- Oviedo-Boयो, J., Valdez-Alarcón, J., Cajero-Juárez, M., Ocha-Zarzosa, A., López-Meza, J.E., Bravo-Patiño, A., Baizabal-Aguirre, V.M. 2007. Innate immune response of bovine mammary gland to pathogenic bacteria responsible for mastitis. *Journal of Infection* 54, 399 – 409.
- Paape, M.J., Bannerman, D.D., Zhao, X., Lee, J-W. 2003. The bovine neutrophil: Structure and function in blood and milk. *Veterinary Research* 34, 597 – 627.
- Paape, M.J., Mehrzad, J., Zhao, X., Detilleux, J., Burvenich, C. 2002. Defense of the bovine mammary gland by polymorphnuclear neutrophil leukocytes. *Journal of Mammary Gland Biology and Neoplasia* 7, 109 – 121.
- Paape, M.J., Shafer-Weaver, K., Capuco, A.V., van Oostveldt, K., Burvenich, C. 2000. Immune surveillance of mammary tissue by phagocytic cells. *Advances in Experimental Medicine and Biology* 480, 259 – 277.
- Paape, M.J., Wergin, W.P., Guidry, A.J., Pearson, R.E. 1979. Leukocytes – second line of defense against invading mastitis pathogens. *Journal of Dairy Science* 62, 135 – 153.
- Pang, S., Smith, J., Onley, D., Reeve, J. Walker, M., Foy, C. 2005. A comparability study of the emerging protein array platforms with established ELISA procedures. *Journal of Immunological Methods* 302, 1 – 12.
- Pedersen, L.G., Castelruiz, Y., Jacobsen, S., Aasted, B. 2002. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. *Veterinary Immunology and Immunopathology* 88, 111 – 122.
- Persson Waller, K., Colditz, I.G., Lun, S., Östensson, K., 2003. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. *Research in Veterinary Science* 74, 31-36.
- Persson Waller, K. & Unnerstad, H. 2004. Klebsiellamastit – ett potentiellt gissel för mjölkproducenten. *Svensk Veterinärtidning* 10, 11 – 17.
- Petersen, H.H., Nielsen, J.P. & Heegaard, P.M.H. 2004. Application of acute phase protein measurements in veterinary clinical chemistry. *Veterinary Research* 35, 163 – 187.
- Phillips, D.J., League, S.C., Weinstein, P., Craig Hooper, W. 2006. Interference in microsphere flow cytometric multiplexed immunoassays for human cytokine estimation. *Cytokine*, 36, 180-188.
- Phillips, D.J., Reimer, C.B., Wells, T.W., Black, C.M. 1980. Quantitative characterization of specificity and potency of conjugated antibody with solid-phase, antigen bead standards. *Journal of Immunological Methods* 34, 315 – 327.

- Piñeiro, M., Andrés, M., Iturralde, M., Carmona, S., Hirvonen, J., Pyörälä, S., Heegaard, P.M., Tjørnehoj, K., Lampreave, F., Piñeiro, A., Alava, M.A. 2004. ITIH4 (inter-alpha-trypsin inhibitor heavy chain 4) is a new acute-phase protein isolated from cattle during experimental infection. *Infection and Immunology* 72, 3777 – 3782.
- Prabhakar, U., Eirikis, E. & Davis, H.M. 2002. Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMAP assay. *Journal of Immunological Methods* 260, 207 – 218.
- Prabhakar, U., Eirikis, E., Reddy, M., Silvestro, E., Spitz, S., Pendely II, C., Davis, M.H., Miller, B.E. 2004. Validation and comparative analysis of a multiplexed assay for the simultaneous quantitative measurement of Th1/Th2 cytokines in human serum and human peripheral blood mononuclear cell culture supernatants. *Journal of Immunological Methods* 291, 27 – 38.
- Pyörälä, S. 2003. Indicators of inflammation in the diagnosis of mastitis. *Veterinary Research* 34, 565 – 578.
- Rainard, P. 2003. The complement in milk and defense of the bovine mammary gland against infections. *Veterinary Research* 34, 647 – 670.
- Rainard, P. & Paape, M.J., 1997. Sensitization of the bovine mammary gland to *Escherichia coli* endotoxin. *Veterinary Research* 28, 231-238.
- Rainard, P. & Riollet, C. 2006. Innate immunity of the bovine mammary gland. *Veterinary Research* 37, 369 – 400.
- Rajala-Schultz, P.J. & Gröhn, Y.T. 1999. Culling of dairy cows. Part I. Effects of diseases on culling in Finnish Ayrshire cows. *Preventive Veterinary Medicine* 41, 195 – 208.
- Rajala-Schultz, P.J., Gröhn, Y.T., McCulloch, C.E., Guard, C.L. 1999. Effects of clinical mastitis on milk yield in dairy cows. *Journal of Dairy Sciences* 82, 1213 – 1220.
- Rajala-Schultz P.J., Hogan J.S., Smith K.L. 2005. Short communication: Association between milk yield at dry off and probability of intramammary infections at calving. *Journal of Dairy Science* 88, 577 – 579.
- Ramadori, G. & Armbrust, T. 2001 Cytokines in the liver. *European Journal of Gastroenterology and Hepatology* 13, 777 – 784.
- Rambeaud M., Almeida R.A., Pighetti G.M., Oliver S.P., 2003. Dynamics of leukocytes and cytokines during experimentally induced *Streptococcus uberis* mastitis. *Veterinary Immunology and Immunopathology* 96, 193-205.
- Raynaud, J.P., Brunault, G. & Philippe, J. 1980. Swine dysentery. Comparison of experimental diseases produced by infection with colonic mucosa or with *Treponema hyodysenteriae*, French strains, and of 'natural' disease. *Annals de Recherchers Vétérinaires* 11, 68 – 87.
- Riollet, C., Rainard, P. & Poutrel, B. 2000. Cells and cytokines in inflammatory secretions of bovine mammary gland. *Advances in Experimental Medical Biology* 480, 247 – 258.
- Rosner, M.H., Grassman, J.A. & Haas, R.A. 1991. Immunochemical techniques in biological monitoring. *Environmental Health Perspectives* 94, 131 – 134.
- Ryan, G.B. & Majno, G. 1977. Acute inflammation. A review. *American Journal of Pathology* 86, 183 – 276.
- Røntved, C.M., Andersen, J.B., Dornfeld, J., Ingvarsen, K.L. 2005. Effects of diet energy density and milking frequency in early lactation on tumor necrosis factor-alpha responsiveness in dairy cows. *Veterinary Immunology and Immunopathology* 104, 171 – 181.

- Sachdeva, N. & Asthana, D. 2007. Cytokine quantitation: technologies and applications. *Frontiers in Bioscience* 12, 4682 – 4695.
- Sandberg, K., Matsson, P. & Alm, G.V. 1990. A distinct population of nonphagocytic and low level CD4<sup>+</sup> null lymphocytes produce IFN- $\alpha$  after stimulation by herpes simplex virus-infected cells. *The Journal of Immunology* 145, 1015 – 1020.
- Scheerlinck, J.P. 1999. Functional and structural comparison of cytokines in different species. *Veterinary Immunology and Immunopathology* 15, 39 – 44.
- Schultze, W.D., Stroud, B.H. & Brasso, W.B. 1985. Dairy herd problem with mastitis caused by a rapidly growing *Mycobacterium* species. *American Journal of Veterinary Research* 46, 42 – 47.
- Seegers H., Fourichon C., Beaudou F. 2003. Production effects related to mastitis and economics in dairy cattle herds. *Veterinary Research* 34, 475 – 491.
- Selby, C. 1999. Interference in immunoassay. *Annals of Clinical Biochemistry* 36, 704-721.
- Shapiro, H.M. 2003. What (and what good) is flow cytometry? *Practical Flow Cytometry 4<sup>th</sup> Edition*, Wiley-Liss Incorporated, New York, 1 – 2.
- Shuster, D.E., Harmon, R.J., Jackson, J.A., Hemken, R.W. 1991. Reduced lactational performance following intravenous endotoxin administration to dairy cows. *Journal of Dairy Science* 74, 3407 – 3411.
- Shuster, D.E., Kehrli, M.E. Jr. & Baumrucker, C.R. 1995. Relationship in inflammatory cytokines, growth hormone, and insulin-growth factor-1 to reduced performance during infectious disease. *Proceedings of the Society for Experimental Biology and Medicine* 210, 140 – 149.
- Shuster, D.E., Kehrli, M.E. Jr., Rainard, P., Paape, M. 1997. Complement fragment C5a and inflammatory cytokines in neutrophil recruitment during inflammatory infection with *Escherichia coli*. *Infection and Immunity* 65, 3286 – 3292.
- Shuster, D. E., Kehrli, M.E. Jr. & Stevens, M.G. 1993. Cytokine production during endotoxin-induced mastitis in lactating dairy cows. *American Journal of Veterinary Research* 54, 80 - 85.
- Sikora, K. 1980. Does interferon cure cancer? *British Medical Journal* 281, 855 – 858.
- Sohn E.J., Paape M.J., Connor E.E., Bannerman D.D., Fetterer R.H., Peters R.R. 2007. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF- $\alpha$ , IL-1 $\beta$ , IL-12 and IFN- $\gamma$ . *Veterinary Research* 38, 809 – 818.
- Soller, J.T., Murua-Escobar, H., Willenbrock S., Janssen, M., Eberle, N., Bullerdiek, J., Nolte, I. 2007. Comparison of the human and canine cytokines IL-1(alpha/beta) and the TNF-alpha to orthologous other mammals. *Journal of Heredity* 98, 485 – 490.
- Sordillo, L.M. & Peel, J.E. 1992. Effect of interferon- $\gamma$  on the production of tumor necrosis factor during acute *Escherichia coli* mastitis. *Journal of Dairy Sciences* 75, 2119 – 2125.
- Sordillo, L.M., Pighetti, G.M. & Davis, M.R. 1995. Enhanced production of bovine necrosis factor-alpha during the periparturient period. *Veterinary Immunology and Immunopathology* 49, 263 – 270.
- Staros, J.V., Wright, R.W. & Swingle, D.M. 1986. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Analytical Biochemistry* 156, 220 – 222.
- Stricklin, D. & Arvidsson, E. 2004. Application of rapid analysis methods of protein markers for exposure assessment. FOI-R—0911—SE, Scientific report, Umeå, Sweden.

- Sun, K., Wang, Q, Huang X.H., Zhen, M.C., Li, W., Zhang, L.J. 2007. Establishment of multiplexed, microsphere-based flow cytometric assays for multiple human tumor markers. *Acta Pharmacologica Sinica* 28, 2011 – 2018.
- Svensson, M., Hansen, M. B., Heegaard, P., Abell, K., Bendtzen K., 1993. Specific binding of interleukin 1 (IL-1) beta and IL-1 receptor antagonist (IL-1ra) to human serum. High-affinity binding of IL-1ra to soluble IL-1 receptor type I. *Cytokine* 5, 427-35.
- Swedish Dairy Association, 2007. Djurhälsövärd 2006/2007. Yearbook of the Swedish Association of Livestock Breeding and Production, Eskilstuna, Sweden.
- Syversen, P.V., Juul, J., Marhaug, G., Husby, G., Sletten, K. 1994. The primary structure of serum amyloid A protein in the sheep: comparison with serum amyloid A in other species. *Scandinavian Journal of Immunology* 39, 88 – 94.
- Thraillkill, K.M., Moreau, C.S., Cockrell, G., Simpson, P., Goel, R., North, P., Fowlkes, J.L., Bunn, R.C. 2005. Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex Multiplex technology. *Clinical Chemistry and Laboratory Medicine* 43, 1392 – 1399.
- Tizard, I.R. 2004. *Veterinary Immunology 7<sup>th</sup> edition*. W.B. Saunders Company, Philadelphia, 1 – 482.
- Tournant, B. 1995. Lymphocytic plasma cell mastitis. *Archives d'anatomie et de cytologie pathologiques* 43, 88 – 92.
- Tracey, K.J. 2002. The inflammatory reflex. *Nature* 420, 853 – 859.
- Trent, M.S., Stead, C.M., Tran, A.X., Hankins, J.V. 2006. Diversity of endotoxin and its impact on pathogenesis. *Journal of Endotoxin Research* 12, 205 – 223.
- Tyvold, S.S., Solligård, E., Gunnes, S., Lyng, O., Aadahl, P., Johannisson, A. 2008. Microdialysis for continuously monitoring cytokines in bronchial Epithelial lining fluid. Conference abstract, *The Society of Critical Care Medicine 37<sup>th</sup> Congress*, Honolulu, USA.
- Tråvén, M., Sundberg, J., Larsson, B., Niskanen, R. 1993. Winter dysentery diagnosed by farmers in dairy herds in central Sweden: incidence, clinical signs and protective immunity. *Veterinary Record* 133, 315 – 318.
- Underhill D.M. 2003. Toll-like receptors: networking for success. *European Journal of Immunology* 33, 1767 – 1775.
- Validation of analytical procedures: methodology. 1996. ICH Harmonised Guideline, National Institute of Health Sciences, Bethesda, MD, USA.
- Vangroenweghe, F., Duchateau, L., Boutet, P., Leukeux, P., Rainard, P., Paape, M.J., Burvenich, C. 2005. Effect of Carprofen treatment following experimentally induced *Escherichia coli* mastitis in primiparous cows. *Journal of Dairy Science* 88, 2361 – 2376.
- Vangroenweghe, F., Lamote, I. & Burvenich, C. 2005. Physiology of the periparturient period and its relation to severity of clinical mastitis. *Domestic Animal Endocrinology* 29, 283 – 293.
- Wadhwa M. & Thorpe R. 1998. Cytokine immunoassay: recommendations for standardisation, calibration and validation. *Journal of Immunological Methods* 219, 1 – 5.
- Wanda, P.E. & Smith, J.D. 1982. A general method for heterokaryon detection using resonance energy transfer and a fluorescence-activated cell sorter. *The Journal of Histochemistry and Cytochemistry* 30, 1297 – 1300.
- Weber, J.R., Freyer, D., Alexander, C., Schröder, N.W., Reiss, A., Küster, C., Pfeil, D., Tuomanen, E.I., Schumann, R.R. 2003. Recognition of pneumococcal peptidoglycan: an expanded, pivotal role for LPS binding protein. *Immunity* 19, 269 – 279.



- Wellenberg, G.J., van der Poel, W.H.M. & van Oirschot, J.T. 2002. Viral infections and bovine mastitis: a review. *Veterinary Microbiology* 88, 27 – 45.
- Wenz, J.R., Barrington, G.M., Garry, F.B., Dinsmore, R.P., Callan, R.J. 2001. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. *Journal of the American Veterinary Medical Association* 218, 567 – 572.
- Werling, D., Jungi, T.W. 2003. Toll-like receptors linking innate and adaptive immune response. *Veterinary Immunology and Immunopathology* 19, 1 – 12.
- Whelan, A.O., Hope, J.C., Howard, C.J., Clifford, D., Hewinson, R.G., Vordermeier H.M., 2003. Modulation of the bovine delayed-type hypersensitivity responses to defined mycobacterial antigens by a synthetic bacterial lipopeptide. *Infection and Immunity*, 71, 6420-6425.
- Wilson, H.L., Aich, P., Roche, F.M., Jalal, S., Hodgson, P.D., Brinkman, F.S.L., Potter, A., Babiuk, L.A., Griebel, P.J. 2005. Molecular analyses of disease pathogenesis: application of bovine microarrays. *Veterinary Immunology and Immunopathology* 105, 277 – 287.
- Wood, P.R. & Jones, S.L. 2001. BOVIGAM: an in vitro cellular diagnostic test for bovine tuberculosis. *Tuberculosis* 81, 147 – 155.
- Wouters, I.M., Douwes, J., Thorne, P.S., Heederik, D., Doekes, G. 2002. Inter- and intraindividual variation of endotoxin and beta (1->3) glucan-induced cytokine response in a whole blood assay. *Toxicology and Industrial Health* 18, 15 – 27.
- Wurfel, M.M., Park, W.Y., Radella, F., Ruzinski, J., Sandstrom, A., Strout, J., Bumgarner, R.E., Martin T.R. 2005. Identification of high and low responders to lipopolysaccharide in normal subjects: an unbiased approach to identify modulators of innate immunity. *Journal of Immunology* 175, 2570 – 2578.
- Yurkovetsky, Z., Ta'asan, S., Skates, S., Rand, A., Lomakin, A., Linkov, F., Marrangoni, A., Velikokhatanaya, L., Winans, M., Gorelik, E., Maxwell, G.L., Lokshin, A. 2007. Development of multimarker panel for early detection of endometrial cancer. High diagnostic power of prolactin. *Gynecologic Oncology* 107, 58 – 65.
- Zangerle, P.F., de Groote, D., Lopez, M., Meuleman, R.J., Vrindts, Y., Fauchet, F., Dehart, I., Jadoul, M., Radoux, D., Franchimont, P. 1992. Direct stimulation of cytokines (IL-1beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood: II. Application to rheumatoid arthritis and osteoarthritis. *Cytokine* 4, 568 – 575.
- Zeytun A., van Velkinburgh J.C., Pardington P.E., Cary R.R., Gupta G. 2007. Pathogen-specific innate immune response. *Advances in Experimental and Medical Biology* 598, 342 – 357.
- Zhong, H., Han, B., Tourkova, I.L., Lokshin, A., Rosenbloom, A., Shurin, M.R., Shurin, G.V. 2007. Low-dose paclitaxel prior to intratumoral dendritic cell vaccine modulates intratumoral cytokine network and lung cancer growth. *Clinical Cancer Research* 13, 5455 – 5462.
- Zhu, T. & Salmeron, J. 2007. High-definition genome profiling for genetic marker discovery. *Trends in Plant Science* 12, 196 – 202.
- Ziv, G. 1992. Treatment of peracute and acute mastitis. *The Veterinary Clinics of North America. Food Animal Practice* 8, 1 – 15.
- Åkerstedt, M., Björck, L., Persson Waller K., Sternesjö, A. 2006. Biosensor assay for determination of haptoglobin in bovine milk. *Journal of Dairy Science* 73, 299 – 305.

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