The Milk Proteome and the Acute Phase Response during Bovine Mastitis

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Abstract

The principal aims of the work presented in this thesis were to further investigate the pathophysiology of the acute phase proteins in bovine mastitis and to document the changes in the protein composition of milk during mastitis using modern proteomic methods.

Mastitis is one of the most important diseases affecting cattle worldwide. It has adverse effects on the economics of milk production through reducing the quality and quantity of milk. Acute phase proteins (APPs) are valuable markers of inflammatory lesions and are widely used in the veterinary field for disease diagnosis. This study further investigated the presence of APPs, haptoglobin (Hp) and mammary- associated serum amyloid A3 (M-SAA3) in the milk of cows with mastitis and evaluated the possibility of using these proteins to detect mastitis.

An Enzyme linked immunosorbent assay (ELISA) was developed and validated for the measurement of milk Hp. A commercially available ELISA was used for the detection of M-SAA3 in milk. Both Hp and M-SAA3 showed similar kinetics to somatic cell counts and can be considered as sensitive and reliable markers of mastitis.

Advanced proteomic methods were developed and used to further characterise the changes taking place in the proteins expressed in normal and mastitic milk. The methodology for analysis of the bovine milk proteome was successfully established. This part of the work concluded that the patterns of protein expression of clinically mastitic milk showed clear differences from that of normal milk. Further analysis of milk samples from a mild subclinical model of *Staphylococcus aureus* mastitis showed that alterations to the milk proteome were only minimal. However this study was useful in identifying areas worthy of future research.

Recent publications on APP research speculate upon the local production of M-SAA3 in the bovine mammary gland. The final objective of this study was therefore to investigate the expression of M-SAA3 in bovine mammary tissue. Our studies confirm the local production of M-SAA3 in the bovine mammary gland and its up-regulation during bovine mastitis. This finding may provide further information on a possible role for this protein.

Overall the findings detailed in this thesis indicate that APPs in milk are valuable markers of mastitis and additionally that the ease of collection of milk by non-invasive methods suggest that milk has potential for the discovery of disease biomarkers.

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List of Abbreviations

%	Percent
µl	Microlitre
α-LA	Alpha lactalbumin
° C	Degrees Celsius
2-DE	2-Dimensional electrophoresis
A	Adenosine
A-apoSAA	Acute phase apolipoprotein serum amyloid A
ACTH	Adrenocorticotrophic hormone
AD	Alzheimers disease
AMS	Automatic milking systems
Anti-Hp	Antiserum against haptoglobin
AP-1	Activating protein 1
ApoA-1	Apolipoprotein A-1
ApoSAA	Apolipoprotein serum amyloid A
APP	Acute phase protein
APR	Acute phase response
BMSCC	Bulk milk somatic cell count
Bp	Basepair
BSA	Bovine serum albumin
BTSCC	Bulk tank somatic cell count
C	Cytosine
Ca^{2+}	Calcium ion
C-apo SAA	Constitutive isoform apolipoprotein serum amyloid A
cDNA	Complementary deoxyribonucleic acid
CHAPS	3[(cholamidopropyl)dimethylammonio]-1-propane sulphonate
CHCA	Cyano-4-hydroxycinamic acid
Cl^-	Chloride ion
CMT	California mastitis test
CRH	C releasing hormone
CRP	C-reactive protein
CSF	Colony stimulating factor
CZE	Capillary zone electrophoresis
DIGE	Difference gel elctrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidephosphate
DTT	Dithiotriethol
E. coli	*Escherichia coli*
EC	Electrical conductivity
EC	European Community
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
E-selectin	Epithelium selectin
ESI	Electrospray ionisation
g	Gram
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H	Hour
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin

Hb^+	Methaemoglobin
HbSR	Haemoglobin scavenger receptor protein
HCl	Hydrochloric acid
HDL3	High density lipoprotein
HGF	Hepatocyte growth factor
Hp	Haptoglobin
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
ICSCC	Individual cow somatic cell count
IEF	Isoelectric focussing
IL	Interleukin
IL-1R	Interleukin 1 receptor
IL-1Ra	Interleukin 1 receptor antagonist
INF-γ	Interferon gamma
IPG	Immobilized pH gradient
IQSCC	Individual quarter cow somatic cell count
JAK	Janus kinase
KDa	Kilo Dalton
LPO	Lactoperoxidase
LPS	Lipopolysaccharide
M	Molar
MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen activated protein kinase
Mg_2Cl	Magnesium chloride
MHC	Major histocompatibility complex
Min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
M-MLV	Molony-murine leukaemia virus
MOWSE	MOecular Weight SEarch
Mr	Molecular mass
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
M-SAA3	Mammary-associated serum amyloid A 3
Na^{2+}	Sodium ion
Na_2CO_3	Sodium carbonate
NAGase	N-acetyl beta-D-glucosamide
$NaHCO_3$	Sodium bicarbonate
NCBI	National Centre for Biotechnology Information
NF	Nuclear factor
NF-κB	Nuclear factor kappa B
$(NH_4)_2SO_4$	Ammonium sulphate
nm	Nanomolar
OD	Optical density
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PCR	Polymerase chain reaction
PDB	Protein data bank
pI	Isoelectric point
PIR	Protein information resource
PKC	Protein kinase C
PMF	Peptide mass fingerprint

pmol	Picomol
PO_4^{2-}	Phosphate ion
PTM	Post-translational modification
r^2	Correlation coefficient
RBC	Red blood cells
RGD	Arginine-glycine-aspartic acid sequence motif
RID	Radial immunodiffusion
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediate
RPE	Retinal pigment epithelial cells
RT	Reverse transcriptase
S	Second
S. aureus	*Staphylococcus aureus*
S. uberis	*Streptococcus uberis*
SAA	Serum amyloid A
SAC	Scottish Agricultural College
SB-7	Chromogen reagent cocktail
SCC	Somatic cell count
SCN	Thiocyanate
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SELDI	Surface-enhanced laser desorption/ionization
SMCC	Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate
SRID	Single radial immunodiffusion
STAT	Signal transducer and activator of transcription
Taq	*Thermus aquaticus*
TBS	Tris buffered saline
TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor beta
Th-1	T-helper 1 lymphocytes
Th-2	T-helper 2 lymphocytes
Tm	Annealing temperature
TMB	Tetra methyl benzidine
TNF-α	Tumour necrosis factor alpha
TOF	Time-of-flight
UTR	Untranslated region
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VIDA	Veterinary Investigation Diagnosis Analysis Database
v/v	By volume
w/v	By weight

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Caroline Hogarth, June 2004

Author's Declaration

The work presented in this thesis was performed solely by the author except where the assistance of others has been acknowledged.

Caroline J Hogarth, June 2004

Publications and Presentations

Some of the work contained in this thesis has been the subject of the following publications or presentations:

Papers

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Ulrika Grönlund, Cecilia Hulten, Peter D Eckersall, Caroline J Hogarth and Karin Persson Waller 'Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis' *J Dairy Research, **2003**, 70:379-386*

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'Proteomic investigation of bovine milk - Are α-lactalbumin and β-lactoglobulin negative acute phase proteins?' *Doorn, The Netherlands*

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2002 **Association Veterinary Teachers Research Workers, (AVTRW)**
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2001 **The Second European Colloquim on Animal Acute Phase Proteins**
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2001 **Celsus Autumn Meeting**
Glasgow, UK

2001 **Association Veterinary Teachers Research Workers, (AVTRW)**
Scarborough, UK

Dedication

At Sorbie Farm where I was born
Looking to Arran's sunsetting sky
And in the fields of luscious grass
Lies a herd o' contented kye

David C. Hogarth, June 2004

Chapter I
Introduction

1.1 Background

Bovine mastitis, defined as 'inflammation of the mammary gland' has been described as the single most common cause of death in adult dairy cows (De Graves and Fetrow 1993; Menzies et al 1995; Kossaibati and Esslemont 1997), and usually arises as a result of intramammary infection by bacteria (Leigh 1999). Inflammation is induced in response to udder infection, by the release of inflammatory mediators, as part of the host defence mechanism, and can produce visible abnormalities in the milk accompanied by any of the following clinically recognisable signs of heat, pain, swelling and inflammation of the infected quarter within the udder (Blowey 1990). Many intramammary infections however produce no obvious abnormalites in the milk or udder and are termed by definition, sub-clinical mastitis. In severe cases, this inflammatory response can cause irreversible damage to the milk secretory tissue of the udder, often before signs of the disease become obvious to the dairyman. Mastitis is a complex disease and has a considerable impact on the welfare of individual animals and on the economics of the dairy industry. Mastitis is, in theory, a preventable disease, however effective control strategies remain a major challenge to the farmer, veterinarian and research scientist. Early diagnosis of mastitis is of principal importance and achievement of this requires the availability of an accurate, sensitive and robust diagnostic test. Recent evidence has suggested that acute phase protein (APP) in milk could provide the analyte for such a test. The aetiology of bovine mastitis is reviewed in detail in chapter 3, which deals with investigation of the naturally occurring disease. Here the acute phase proteins (APP) are introduced, then the physiology of the bovine udder and milk production are discussed.

1.2 Acute Phase Response

The acute phase response (APR) is a non-specific inflammatory response affecting various host organs, which commences within a few hours of any tissue injury and can last for several days (Eckersall 1992). Stimuli, which commonly give rise to the APR, include bacterial infection, surgical or other trauma, bone fracture, neoplasms, burn injury, tissue infarction, various immunologically mediated inflammatory states, and child birth

(Kushner 1982). All of these conditions induce a local inflammatory response and the early release of soluble mediators, which in turn leads to a systemic APR encompassing metabolic and physiological changes, which provide the host with an optimal infection-fighting environment. The purpose of the APR is to prevent ongoing tissue damage, to isolate and destroy the infective organisms, to remove harmful molecules and debris, and to activate the repair processes that are necessary to return the organ to normal function (Baumann and Gauldie 1994). The APR is a flexible response that helps host survival and aims to restore physiological homeostasis, as deviations from this stable situation impose a serious threat to the health of the living animal. The APR may be relatively transient, subsiding over 24-48h, with the host returning to normal function within a few days, demonstrating the protective and homeostatic nature of this host response. However, in chronic disease, the APR can be persistent (Baumann and Gauldie 1994). Among the changes in homeostatic mechanisms during the APR are fever, somnolence, anorexia, increased synthesis of a number of endocrine hormones, decreased erythropoiesis, thrombocytopaenia, alterations in plasma cation concentrations, inhibition of bone formation, negative nitrogen balance (largely resulting from proteolysis and decreased protein synthesis in skeletal muscle) with consequent gluconeogenesis, and alterations in lipid metabolism (Kushner and Mackiewicz 1987).

The hepatocytes of the liver have a key role in the response to inflammation (Pepys 1989). They express a variety of receptors for cytokines, growth factors, and other inflammatory mediators, such as prostaglandins and are therefore the target of a multiple set of mediators involved in both the systemic and the local host response. Pro-inflammatory cytokines enter the liver via the bloodstream through the portal vein, and consequently reach the non-parenchymal liver cells, such as the endothelial cells and the Kupffer cells, that express receptors for the main cytokines.

One of the major components of the APR is the alteration in concentration of a large number of plasma proteins synthesized by the liver. These are termed acute phase proteins (APPs)(Kushner and Mackiewicz 1987), the focus of the studies detailed within this thesis.

1.3 Acute Phase Proteins

An APP has been defined as one whose concentration increases (positive APPs) or decreases (negative APPs) by at least 25% during inflammatory disorders (Morley & Kushner, 1982). Following the initiation of the APR, the hepatocytes undergo dramatic

upregulation in the synthesis of positive APPs: a family of approximately 30 plasma proteins produced in increased amounts by the liver in inflammation.

C-reactive protein (CRP) was the first APP to be described. It was identified in humans suffering from infections and named after its ability to precipitate pneumococcal C-polysaccaride (Tillett and Francis 1930).

Positive APPs can be subdivided based on their response pattern to inflammatory stimuli. Major APPs have very low or undetectable levels in serum of healthy animals, but increase more than 100 fold during an APR. Minor or moderate APPs are present in the serum of healthy animals and their concentrations do increase during an APR, but only an increase of up to 10 fold is observed (Conner et al 1988b; Gruys et al 1994; Eckersall 1995b). In man, the concentration of APPs serum amyloid A (SAA) and CRP, can increase up to 1000-fold during acute disease states (Gruys et al 1993). Consequently these proteins are of diagnostic interest in clinical chemistry. However CRP is not considered an acute phase reactant in cattle. The major bovine acute phase proteins are haptoglobin (Hp) and SAA (Table 1.1).

1.3.1 Haptoglobin

Haptoglobin is a major plasma glycoprotein synthesized in the liver. It binds free haemoglobin (Hb) in the circulation of humans and other mammals. The binding of Hb to Hp is the strongest noncovalent interaction known among the transport proteins in plasma and is not reversible (Yang et al 1993). After Hp has bound free Hb and delivered it to the liver, the complex is degraded by lysosymes, leading to a decrease of Hp concentration in plasma following haemolysis. For example Hp was found to decrease in plasma to a non-detectable level in severe haemolysis (Yang et al 2003).

1.3.1.1 Structure of haptoglobin

Haptoglobin is a genetically determined α_2 – acidic glycoprotein with Hb-binding capacity, present in most body fluids (serum, urine, saliva, cerebrospinal fluid, amniotic fluid, ascites etc.) of humans and other mammals. As a positive acute phase reactant, its plasma level is increased during inflammation, infections of different aetiology, trauma, tissue damage and malignancy (Dobryszycka 1997).

CLASSIFICATION	ACUTE PHASE PROTEIN
Major	Serum amyloid A Haptoglobin
Moderate	α1-acid glycoprotein α1-proteinase inhibitor
Minor	Fibrinogen Ceruloplasmin LPS-binding protein α2-macroglobulin Complement C3
Negative	Albumin
Constitutive	C-reactive protein

Table 1-1 Classification of acute phase protein in cattle (after Eckersall and Conner (1988), Gruys et al. (1994) and Eckersall (1995). Major = 10 to several hundred times increase, moderate = 5 to 10 times increase, minor = 1 to 5 times increase, negative = decrease, constitutive = no change

The protein is synthesized as a single chain, which is processed posttranslationally into an amino-terminal α-chain and a carboxy-terminal β-chain by proteolytic cleavage (Gordon 2001). Mature Hp is composed of four polypeptide subunits, two α and two β (α_2 β_2), which are linked by disulphide bridges (Kim et al 2001) resulting in the Hp $\alpha\beta$ tetramer.

Variations in the α subunit of Hp exists in humans, and gives rise to three major Hp phenotypes (Yee and Brown 1999). Several functional differences between Hp phenotypes have been demonstrated and appear to have important biological and clinical consequences (Langlois and Delanghe 1996). In the past decade, a direct link has been identified between Hp phenotype and susceptibility to cardiovascular diseases (Langlois and Delanghe 1996; Delanghe et al 1999; Bamm et al 2004). Individuals with the Hp 2-2 phenotype suffer increased iron-related vascular pathologies in comparison to individuals with the Hp 1-1 phenotype (Bamm et al 2004).

Hp in bovine serum is highly polymerized Biochemical studies have shown that Hp is composed of two chains, an α-chain and a β-chain. The molecular weight of the α-chain varies between 16Kda (Morimatsu et al 1991) and 23Kda (Eckersall and Conner 1990). The molecular weight of the β-chain varies between 35 Kda (Eckersall and Conner 1990) and 40 Kda (Morimatsu et al 1991). The two α-chains and two β-chains are linked by a disulphide bond, forming the basic tetrameric subunit. The basic tetrameric subunit of Hp associates in polymers with albumin (Eckersall and Conner 1990), in bovine serum resulting in large and heterogeneous molecular sizes (Morimatsu et al 1991).

1.3.1.2 Association of haptoglobin with haemoglobin

Haptoglobin is classified as a scavenger protein, indicating its involvement in the clearance of inflammatory products (Whicher and Evans 1992). In the normal situation, ageing red blood cells (RBC) are degraded in the bone marrow, liver or spleen however in certain circumstances, such as infection or inflammatory disorders, red cells may burst within blood vessels. Haemoglobin (Hb) released from ruptured RBC is toxic unless rapidly cleared from the circulation (Dennis 2001). The plasma protein Hp, is thought to be involved in promoting the clearance of plasma Hb because it strongly binds free Hb and is depleted during haemolysis (Kristiansen et al 2001). Haptoglobin binds Hb in a 1:1 ratio with an association constant of greater than 10^{-15} mol/L (Dobryszycka 1997). Efficient removal of free Hb is essential since Hb can accelerate lipid peroxidation, leading to production of toxic molecules. In case of highly accelerated intravascular hemolysis, as seen in various inherited, infectious, and autoimmune disorders, Hp may be depleted from

plasma, resulting in an excess of circulating Hb leading to tissue damage (Kushner and Mackiewicz 1987).

The mechanisms leading to the removal of the Hp-Hb complex from the blood remained elusive until the recent discovery of a novel Hb scavenger receptor protein. The Hb scavenger receptor (HbSR) named CD163, was isolated as a virtually pure protein from solubilized membranes from liver, spleen, and placenta using Hp-Hb affinity and identified by mass spectrometry (Kristiansen et al 2001). CD163 is a transmembrane protein displaying an extracellular cysteine-rich domain (Graversen et al 2002), and has been identified as a receptor that engulfs Hb by mediating endocytosis of Hp-Hb complexes. CD163 will only bind Hp and Hb in complex, suggesting that a neo-epitope is presented by this interaction. The receptor-ligand interaction is calcium dependent, and of high affinity. CD163 is expressed in monocytes/macrophages. Internalization of Hp-Hb in macrophages is followed by lysosomal proteolysis of globin and conversion of haem to iron and bilirubin. It has been suggested that approximately 10% of all Hb is cleared via this pathway. CD163/HbSR is under acute phase regulation with mediators like IL-6 and glucocorticoids strongly inducing its expression (Graversen et al 2002). During inflammatory conditions the Hp-Hb-CD163 system might be coordinately induced, thereby enhancing the Hb clearance capacity in response to injury (Gordon 2001).

Elucidation of the mechanism for Hb clearance through identification of CD163 provides new perspectives for understanding the potential immunological properties of Hp during inflammatory conditions.

1.3.2 Serum Amyloid A

Serum amyloid A, a 14KD apolipoprotein is the precursor of the Amyloid A protein, which is the principal component of secondary amyloid plaques, which may be deposited in major organs as an occasional consequence of chronic inflammatory disease (Husebekk et al 1986). During inflammation SAA associates predominantly with the third fraction of high-density lipoprotein (HDL3) (Benditt et al 1979), and constitutes as much as 30-50% of HDL3 protein during the APR. Serum amyloid A is synthesized primarily by the liver and although the major role of this protein is as an acute phase reactant, 'constitutive' SAAs have been described in two species, human and mouse. Serum amyloid A is now more commonly referred to as being part of a family of differentially expressed apolipoproteins, which have been characterized in many species including human, mouse, hamster, rabbit, dog, mink, cow, sheep and horse (Uhlar and Whitehead 1999).

A conclusive structure for SAA has not yet been reported, however early work based on predictive methods suggested that it is likely to contain two regions of α-helix in addition to β-sheet regions, the latter of which are common to all amyloid proteins (Uhlar and Whitehead 1999). It has since been reported that SAA forms a hexamer in solution, containing a putative central channel (Wang et al 2002).

Several different isoforms of SAA have been shown to exist in acute phase serum from a number of species including horse (Hulten et al 1999), pig (Toussaint et al 1995) and cattle. In cattle, the different SAA isoforms seem to be induced by different disease states, however no functional significance of this has yet been derived (Alsemgeest et al 1995). An additional isoform of SAA termed mammary-associated or milk-associated SAA (M-SAA3) has recently been detected in the bovine mammary gland (McDonald et al 2001) possibly due to local synthesis of this protein. The functions of M-SAA3 have yet to be established, however speculations on this subject and a more detailed account of this protein will be discussed in chapters 3 and 6.

Few attempts have been made to elucidate the role of SAA; thus the normal physiological function of SAA remains unclear. However the high degree of conservation of the SAA genes and proteins, which has been maintained throughout the evolution of mammals, suggests the likelihood of an important biological role, perhaps in survival of the species (Hulten et al 1999) in the face of infection by pathogens. One member of the SAA family, SAA3, is constitutively expressed in a number of diseased tissues, in particular endothelial cells (Meek et al 1994; Urieli-Shoval et al 1998). This suggests that SAA plays an important role in the local defence mechanisms against invading microorganisms.

1.3.3 Negative Acute Phase Proteins

Another distinct class of liver proteins are the negative APPs, the concentration of which is down-regulated during the APR (Gruys et al 1994). The major negative acute phase protein in cattle is serum albumin. During the APR the demand for amino acids for APP synthesis is markedly increased, which necessitates reprioritization of the hepatic protein synthesis: albumin synthesis is down-regulated and amino acids are shunted into synthesis of APPs and other proteins needed for immediate survival (Aldred and Schreiber 1993). *In vivo* studies in rats with *Streptococcus pneumoniae,* have been used to study the effect of the APR on muscle metabolism and amino acid uptake by the liver (Powanda and Beisel 2003). It has been proposed that during the APR, 30 to 40% of the hepatic protein synthesizing capacity is used for production of positive APPs (Whicher and Dieppe 1995),

and household functions thus need to be diminished. For example, drug metabolism in the liver is decreased during the APR (Van Miert 1995; Monshouwer and Witkamp 2000).

1.3.4 Interspecies Variation in Acute Phase Proteins

It is now recognised that there is considerable variety among species in the pattern of APP produced following stimulation (Eckersall 1995b). An early indication of this difference was identified when it was observed that not all APPs originally identified in man give a similar response in animals. Thus, while CRP is a major APP in man, dog and pig, in cattle the presence of infection or inflammation causes little change in its serum concentration. In contrast, Hp is a major APP in cattle with levels increasing from being undetectable in normal animals, to over 100 fold on stimulation (Eckersall 1995a). However, in dogs, Hp acts as a constitutive serum protein and is a moderate APP (Conner et al 1988a; Eckersall et al 1996).

1.3.5 Functions of Acute Phase Proteins

Although several functions have been ascribed to the APPs, numerous speculations still surround this topic. Generally, the APPs have a variety of roles in inflammation acting as mediators and inhibitors, as well as scavengers of cell derived products released from damaged tissue or macrophages. In addition, some members of the family may influence the immune response, which often accompanies inflammation and the release of autologous antigens, through direct protection of the host (Thompson et al 1992). For example the binding of free Hb by Hp, restricts the availability of free iron to invading bacteria (Dobryszycka 1997). An *in vivo* experiment in mice has shown that α1-acid glycoprotein increases resistance of the host to gram-negative bacteria (Hochepied et al 2000). The fact that the APPs have been conserved throughout evolution, and that they are induced in large amounts in response to harmful stimuli, infers that they have a positive input to inflammatory and tissue repair processes. The different actions of various APPs are summarised in table 1.2.

1.3.6 Possible Applications of Acute Phase Proteins in Veterinary Clinical Biochemistry

The possible diagnostic use of APPs in veterinary clinical chemistry have been comprehensively reviewed, (Eckersall and Conner 1988a; Kent 1992; Gruys et al 1994; Eckersall 1995b; Pedersen et al 2004; Murata et al 2004). In the clinical field as well as

PROTEIN	FUNCTION
C-reactive protein	Opsonin, complement activation, immunomodulation, binding of chromatin
Serum amyloid A	Cholesterol scavenger, leukocyte activation, chemotaxis, phagocytosis
Haptoglobin	Haemoglobin scavenger, antioxidant, bacteriostatic effect, immunomodulatory effect, stimulation of angiogenesis
α_1-antitrypsin	Protease inhibitor, major inhibitor of elastase and trypsin
α_2-antiplasmin	Protease inhibitor, major inhibitor of fibrinolysis
α_1-acid glycoprotein	Transport protein, steroid binding
Ceruloplasmin	Copper transport protein, Fe^{2+} oxidation
Ferritin	Iron transport protein
Complement components: C1, C2, C3, C4, C5, C9	Opsonization, chemotaxis, mast cell degranulation
Factor VIII Fibrinogen Prothrombin	Clotting formation of fibrin matrix for repair
Plasminogen	Proteolytic activation of complement, fibrinolysis and clotting
Plasminogen activator inhibitor-1	Inhibits the activation of plasminogen
Fibronectin	Cell attachment, fibrin clot
LPS-binding protein	LPS binding to phagocytes via CD14

Table 1-2. Summary of acute phase proteins and their functions

being markers of infection or inflammation APPs may serve as indicators of prognosis and progress of the animal in response to treatment. In cattle as well as humans the scale and duration of the APP response reflect the severity of infection and underlying tissue damage (Conner et al 1988b; Hirvonen et al 1999). Bovine APPs have been utilized as nonspecific markers of clinical and subclinical disease (Saini and Webert 1991; Karreman et al 2000; Winter and Colditz 2002), to discriminate between acute and chronic disease (Horadagoda et al 1999) and for prognostic purposes, since the duration and magnitude of the response reflects severity of disease (Skinner et al 1991; Hulten et al 1997).

Many studies have indicated the significance of Hp as a clinically useful parameter for measuring the incidence and severity of inflammatory responses in cattle with experimentally induced or naturally occurring infections, including mastitis, pneumonia, peritonitis, endometritis and foot and mouth disease (Conner et al 1986; Skinner et al 1991; Morimatsu et al 1992; Hofner et al 1994; Horadagoda et al 1994; Hirvonen et al 1996; Wittum et al 1996). In pigs, Hp has been used to monitor the occurrence and development of inflammatory conditions following both natural and experimental infection, and pre-treatment with turpentine (Lampreave et al 1994; Eckersall et al 1996; Lipperheide et al 1997; Lipperheide et al 1998; Lipperheide et al 2000; Hulten et al 2003). Haptoglobin concentration has the ability to differentiate between healthy thriving pigs and those showing lack of weight gain due to the presence of sub-clinical infection (Lipperheide et al 1998). Therefore, Hp assays may be applied in the pig production field to determine the health status of the herd and identify individuals suffering compromised welfare. Haptoglobin has also been used as a marker of various inflammatory conditions in equine veterinary medicine (Kent and Goodall 1991; Taira et al 1992; Hulten et al 2002).

The application of SAA assays in veterinary diagnosis has not been as widespread as that of Hp assays, probably because of the difficulties surrounding the measurement of serum SAA (Eckersall 2000). However the measurement of SAA has proved a useful analyte in diagnosing cattle with infection and inflammation (Alsemgeest et al 1993; Horadagoda et al 1994; Horadagoda et al 1999; Heegaard et al 2000; Eckersall et al 2001).

Until recently, the use of APPs in veterinary medicine required the measurement of the specific protein in the serum of the animal. Hence although APPs are useful tools for identifying the presence and status of inflammation, they remain non-specific markers of infection and should not be relied upon to make a specific diagnosis. However recently Hp and SAA have been detected in the milk from cows with clinical and subclinical mastitis (Eckersall et al 2001; Pedersen et al 2003; Gronlund et al 2003a; Nielsen et al 2004),

suggesting the possibility of using APPs in milk as specific markers for the presence of mastitis within the udder.

The value of APP measurement has been severely hampered by the lack of readily available test systems that would allow the detection of the specific APP both quickly and cheaply and could preferably be used outwith the laboratory, i.e: in the field. Before APP measurement can be implemented into routine veterinary diagnostics, such test systems need to be developed. Furthermore, an international standardization of APP assays is essential if quantification of APPs is to become recognised as a reliable diagnostic tool by the worldwide veterinary community (Eckersall 1995b; Eckersall et al 1999b).

1.4 Initiation and Progression of Acute Phase Response

Following the discovery of CRP in the 1930s, APPs were simply used as markers of inflammation for many years, however, from the 1980's, research concentrated on the regulation of APP biosynthesis (Kushner 1982). *In vitro* model systems confirmed that the circulating mediators, capable of hepatocyte stimulation following local tissue injury, were small hormonal proteins named cytokines. Cytokines are intercellular signalling polypeptides produced by a variety of cell types, but the most important sources are tissue macrophages and blood monocytes at sites of inflammation. Mononuclear cells are most commonly associated with initiating the cascade of events during the APR. Pro-inflammatory cytokines including interleukin-1 (IL-1); interleukin-6 (IL-6) and tumour necrosis factor – α (TNF-α) are released from activated macrophages and monocytes at the site of inflammatory lesions or infection (Heinrich et al 1990) and are the major mediators of the APR in the liver. During the hepatic APR, these pro-inflammatory cytokines enhance the expression and secretion of plasma proteins, namely the positive APPs and decrease the expression and secretion of the negative APPs. The early release of these pro-inflammatory cytokines acts to 'alarm' the immune system, through both local and distal activity. At the local reaction site, these cytokines activate stromal cells, including fibroblasts and endothelial cells, to cause a secondary release of cytokines (Baumann and Gauldie 1994). This results in the systemic release of cytokines and initiates the large, complex, signal transduction cascade leading ultimately to the systemic APR. Many cytokines can regulate the production of other cytokines and cytokine receptors, so that cells are seldom exposed to only a single cytokine. Combinations of cytokines have been found to have additive, inhibitory, synergistic, or cooperative effects (Gabay and Kushner 1999).

1.4.1 Regulation of Acute Phase Protein Production

One of the earliest detectable responses of cells to either IL-1 or IL-6 family of cytokines involves changes in the phosphorylation state of different cellular proteins (Baumann and Gauldie 1994). Four major classes of factors regulate APP production: the IL-6/gp130 family of cytokines, IL-1 type pro-inflammatory cytokines (e.g. IL-1, TNF), growth factors, (e.g. hepatocyte growth factor (HGF), transforming growth factor-β (TGF-β) and corticosteroids. A critical role for IL-6 and IL-1 in the APR has been demonstrated *in vivo,* in experiments with knockout mice (Suffredini et al 1999). The authors demonstrated in inflammatory models that IL-1 is the pivotal mediator of the APR. Following local injury the induction of a systemic acute phase response relies upon the initiation of IL-6 cascade, and this is dependent on IL-1 (Suffredini et al 1999).

Acute phase proteins can be divided into two groups, based on the cytokines that induce them. Type I proteins include SAA, CRP (human), complement C3, Hp (rat) and α1- acid glycoprotein. This group of proteins are induced by IL-1 type cytokines including TNF- α. Type II proteins include fibrinogen, Hp (human), α1-antitrypsin and α1-antichymotrypsin, and are induced by IL-6 like cytokines (Suffredini et al 1999).

1.4.2 Interleukin-1 Induced Signalling

Two types of IL-1 receptor (IL-1R) exist, the 80kD IL-1R type I, and the 60-68kD IL-1R type II, both of which belong to the immunoglobulin (Ig) superfamily. The type I receptor is responsible for the transmission of the IL-1 signal, including the induction of type I APPs. After ligand binding, the IL-1R is rapidly phosphorylated at serine/threonine residues, the ligand-receptor complex is then internalised and translocated to the nucleus (Moshage 1997). Receptors for TNF- α. are present on hepatocytes, where their basal level of expression is low. However, during acute or chronic liver inflammation their expression is upregulated. There are two forms of TNF- α receptors, both of which are members of the Ig superfamily of receptors, but which are not homologous with the IL-1 receptors. Activation of the IL-1 /TNF receptors initiates the conversion of membrane sphingomyelin to ceramide via sphingomyelinase. Subsequently, ceramide-activated protein kinases connect to several signalling pathways, which ultimately lead to activation and translocation to the nucleus of the transcription factors (activating protein), AP-1, c-jun/c-fos heterodimer and nuclear factor (NF)-κB, which stimulates the transcription of type I APP genes (Figure 1.1). Originally identified as a B-cell specific transcription factor that binds to the promoter of the κ light chain of Ig, NF- κB is now recognised as one of the

main cellular factors involved in the induction of gene transcription during inflammation and the APR (Jensen and Whitehead 1998). Activation of NF- κB is initiated by the dissociation from its inhibitory subunit Iκ β. In addition to the activation of the NF- κ β pathway, IL-1 type cytokines are also able to activate the Mitogen Activated Protein Kinase (MAPK) signalling cascade. The molecular mechanism whereby binding of IL-1 type cytokines to their specific membrane receptors enables the transduction of the intercellular MAPK pathway is not well understood. However, examination of the induction of APR by IL-6 type cytokines highlights the MAPK signalling pathway as an area of convergence/overlap between the two subgroups.

1.4.3 Interleukin-6 Induced Signalling

Cytokines of the IL-6 family signal through a distinct functional receptor complex, which shares a common signalling membrane glycoprotein, the gp130 receptor. The IL-6 receptor is homologous to other cytokine receptors and to the Ig family. The IL-6 /IL-6R complex, associates extra-cellularly with gp130 permitting downstream signalling events to take place (Figure 1.2).

The gp130 and its heterodimers do not possess intrinsic tyrosine kinase activity. This problem is solved through recruitment of cytoplasmic kinases. However, following dimerization of gp130, receptor-associated tyrosine kinases (Janus kinases or JAK) are activated, phosphorylating tyrosine residues in the cytoplasmic region of gp130. The JAK kinases subsequently modify latent signal transducers and activators of transcription (STAT proteins) by phosphorylation of tyrosine. After phosphorylation, the STAT proteins form homo- or heterodimers, the signal for nuclear translocation and for STAT dimer binding to target sequences in the promotor regions of type II acute phase protein genes, stimulating their expression. Stimulation of gp130 also activates the ras/raf /MAPK pathway, which in turn activates transcription factor, nuclear factor (NF) IL-6, which then recognises target sequences in the promotor region of type I and II acute phase proteins (type 1 IL-6 response element) (Suffredini et al 1999).

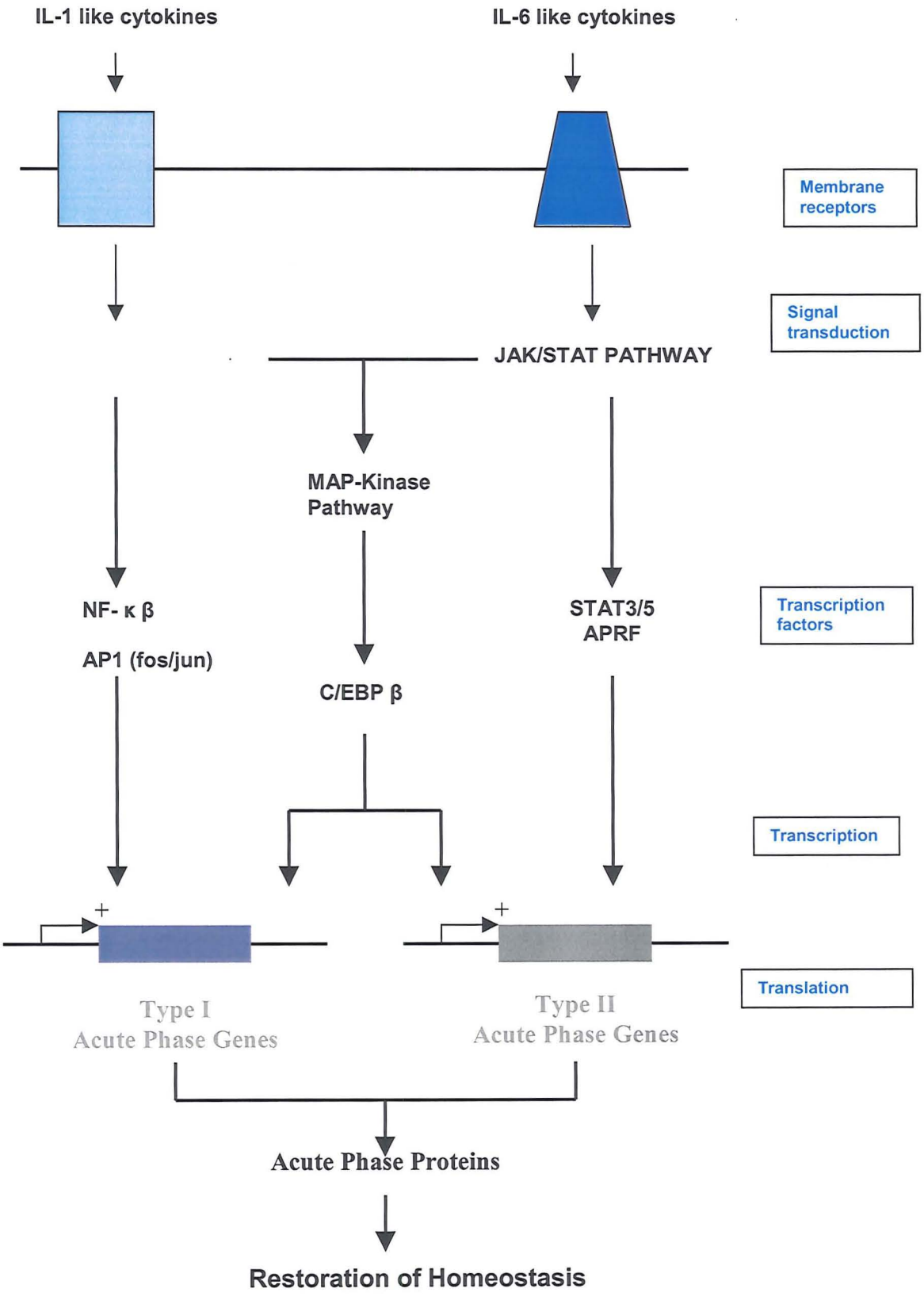


Figure 1-1: The signal transduction cascade leading to the acute phase response

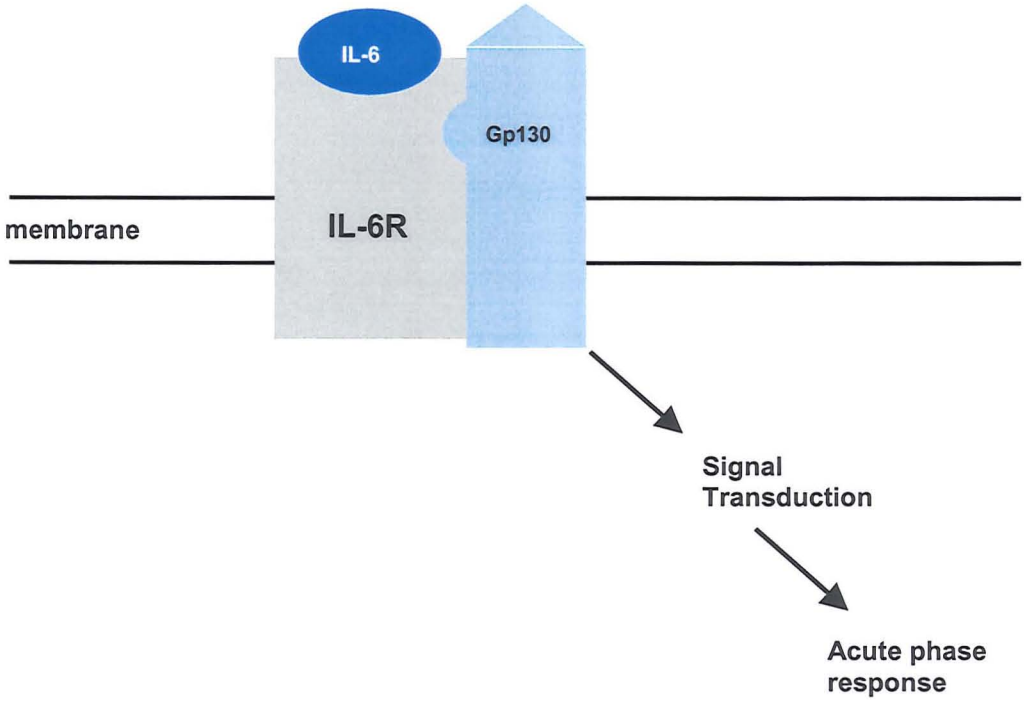


Figure 1-2: The cytokine Interleukin-6 triggers downstream signalling through the transmembrane receptor complex of the IL-6 and gp130 receptor complex.

1.4.4 Counter-regulation of the Acute Phase Response

Equally as intriguing and important as the initiation and propagation of the APR is the process whereby the APR is limited, as prolongation of this response and its accompanying inflammatory processes may have damaging consequences. Many of the cytokines involved in the APR have a short half-life, and hence prolonged stimulation of the response should not be a problem. However, active inhibition does still occur. Negative control mechanisms must be in place, to ensure that once the APR has fulfilled its host-protective functions, its expression can be rapidly returned to baseline levels. Many different regulatory mechanisms are involved in the tight control of APP biosynthesis, although the complete picture is still rather unclear (Jensen and Whitehead 1998). The pathways which have been elucidated involve transcriptional repressors, glucocorticoids, receptor antagonists and cytokines which either directly or indirectly mediate down-regulation of biosynthesis of APPs. Many cytokines have the ability to exert both positive and negative effects, depending on which cell types they interact with, and the context of the interaction. As described earlier, the pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) are heavily involved in the initiation of the APR and generally have up-regulating effects on aspects of inflammation, whereas the anti-inflammatory cytokines IL-4 and IL-10 generally have down-regulating effects acting mainly through interference with the production of pro-inflammatory cytokines. Interleukin-4 is primarily released by T helper-2 (Th-2) lymphocytes, and causes the down-regulation of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-8. Interleukin-10, produced by Th-2 lymphocytes, monocytes, macrophages and B cells, inhibits monocyte/macrophage synthesis of IL-1, TNF-α, IL-6, IL-8 and the colony stimulating factors (CSFs) (Baumann and Gauldie 1994).

The presence of cytokine receptor antagonists and soluble cytokine receptors, adds to the complexity of APR regulation. Soluble cytokine receptors have been described for IL-1, TNF- α, and IL-6 among others. Soluble IL-1 receptors have been detected in plasma, urine and inflammatory fluids (Moshage 1997). Soluble receptors are released during the late stages of an APR, to prevent further activation through posing as a decoy for the specific agonist, binding of which would prolong the response. The naturally occurring IL-1 receptor antagonist (IL-1Ra) acts as an extremely potent regulator of the APR by competing with IL-1 for binding to cellular IL-1 receptors, in particular IL-1R type 1. This interaction interferes severely with the ongoing cascade of inflammation, as binding of IL-1Ra to the IL-1R does not elicit a signal (Figure 1.3). Interleukin-4 and IL-10 have both been shown to upregulate the expression of IL-1Ra.

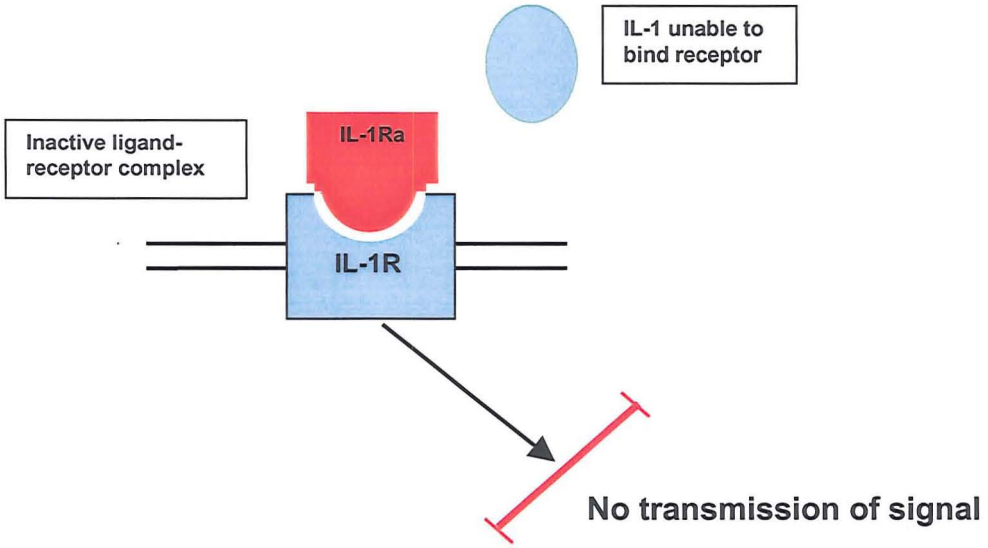


Figure 1-3: Counter-regulation of the acute phase response. Interleukin-1 receptor antagonist (IL-1Ra) competes with IL-1 for binding to the IL-1 receptor (IL-1R). IL-1Ra in complex with the IL-1R cannot transmit a downstream signal.

Glucocorticoids are synthesized in the adrenal cortex after induction by pro-inflammatory cytokines (e.g. IL-1) via the hypothalamus (CRH production) and the pituitary (adrenocorticotrophic hormone production) gland and transported to their 'effector sites', where they either enhance the effects of the pro-inflammatory cytokines in the liver, or provide a negative feedback loop by shutting down the inflammatory response and thereby preventing the continued generation of up-regulating signals to the hypothalamus, adrenal cortex, immune system and liver (Jensen and Whitehead 1998). Steroid hormones behave in an anti-inflammatory manner by the inhibition of the release of pro-inflammatory cytokines. IL-6 has been shown to regulate the anti-inflammatory response through the IL-1Ra. Recent evidence indicates that IL-6 counteracts the acute phase reaction in a negative feedback loop (Ramadori and Christ 1999). The pro-inflammatory cytokine is the major inducer of the hepatic acute phase reaction, but IL-6 simultaneously initiates a variety of shut-off mechanisms (Ramadori and Christ 1999). Control of the APR is therefore a complex network of up and down-regulating signals, involving numerous mechanisms.

1.4.5 Extra-hepatic Synthesis of Acute Phase Protein

For many years the liver has been considered to be the primary site of APP synthesis (Urieli-Shoval et al 1998). However numerous cases of extra-hepatic APP synthesis have also been reported. Yang et al (1995) carried out an experiment to investigate the presence of Hp mRNA in lung epithelium. This group reported results from an *in vivo* experiment in which an APR was stimulated in mice by treatment with lipopolysaccarride (LPS). Thirty hours after induction of inflammation with LPS, a four- fold increase in Hp mRNA was observed in the lung and was equivalent to the level observed in the normal unstimulated liver. *In situ* hybridization was used to investigate the cellular location of Hp mRNA expression. A strong signal for Hp mRNA was found on epithelial cells, while no significant signal was found in endothelium, smooth muscle cells, alveolar epithelium or macrophages. It was therefore concluded from this experiment, that Hp is synthesized in the respiratory epithelium. Another similar *in vivo* experiment examined the response of mouse adipose tissue subjected to LPS in order to induce inflammation, and demonstrated a significant increase in Hp mRNA. Thirty hours after LPS injection, a six-fold increase in the Hp mRNA was seen in the adipose tissue. *In situ* hybridization confirmed that the Hp expression within adipose tissue was confined to adipocytes (Friedrichs et al 1995). These findings suggest that adipose tissue may contribute to the presence of Hp in extravascular body fluids as well as in the serum.

The presence of amyloid-A mRNA has been observed in brain tissue of patients suffering from Alzheimers disease (AD), in addition to that of patients with conditions with a known inflammatory component, but not in that of patients with other neurological diseases (Liang et al 1997). ApoSAA in the human comprises the acute phase apoSAA (A-apoSAA), which is up regulated by the synergistic action of IL-1 and IL-6, but also a constitutive isoform (C-apo SAA), which appears to be produced by the liver in the absence of inflammation. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to analyse human brain tissue for the presence of A & C apoSAA mRNA. As local inflammation has been strongly implicated in AD brain, it was not surprising that only A-apoSAA mRNA was detected in the AD brain tissue.

The eye is very much exposed, making it highly susceptible to oxidative damage through the actions of reactive oxygen intermediates (ROI), including peroxides (Chen et al 1998). Other body tissues are protected from the detrimental effects of ROI through the production of protective proteins such as Hp and hemopexin synthesized by the liver. The previously described ability of Hp, to uptake free Hb in the circulation, enables Hp to act as an anti-oxidant, by preventing Hb from releasing its haem. However, the tight junctions between cells at the blood-retinal barrier leaves the retinal pigment epithelial cells (RPE) cut off from the circulation and hence inaccessible to plasma protective proteins synthesized by the liver. Thus local production of APP is essential. Haptoglobin was first identified as being present in the RPE through incubation of retinal sections with anti-human Hp antibodies. Immunofluorescence identified Hp distributed throughout the neural retina. RT-PCR confirmed the presence of Hp mRNA in the retina, and *in situ* hybridization localized the site of haptoglobin mRNA more specifically (Chen et al 1998).

Active endometriotic lesions exhibit an invasive growth pattern and intense vascularization. Peritoneal fluid from women with endometriosis contains growth factors and inflammatory mediators including IL-1, IL-6 and IL-8. While synthesis and secretion of a Hp-like protein (called ENDO-1) has been reported by these endometriotic lesions (Sharpe-Timms et al 1998). This study has demonstrated that ENDO-1 shares significant amino acid and cDNA sequence homology with the β chain of rat and human Hp. The fact that Hp is stimulated by the synergistic action of IL-1 and IL-6 and also has a known role in angiogenesis (Gabay and Kushner 1999), supports the case for ENDO-1 being a 'haptoglobin-like protein'. RT-PCR was used, as in all of the pre-mentioned experiments, to confirm the endometriotic origin of ENDO-1. Western blot analysis was used to demonstrate immunoreactivity of polyclonal anti-human Hp antibody with ENDO-1.

Urieli-Shoval et al (1998), demonstrated for the first time, that SAA mRNA and protein are widely expressed in many histologically normal human tissues, including stomach, small and large intestine, tonsil, breast, prostate, thyroid, lung, pancreas, kidney, skin epidermis, and brain neurons. SAA expression was localized predominantly to the epithelial components of these tissues. A strong *in situ* hybridization signal was observed in lobular epithelium and terminal duct epithelium in breast tissue. These findings suggest that, in addition to SAA release to the circulation by the liver during an acute phase response, constitutively expressed SAA may serve as an immunological defence molecule at local sites, thereby providing an immediate localized defence against inflammatory challenges during the time taken to mount a systemic response by increased hepatic synthesis.

Thus although the liver is the major site of APP synthesis, expression of both Hp and SAA in a wide range of tissues has been documented and it could be possible that local production of APP occurs in the bovine mammary gland. The udder is regularly exposed to pathogenic challenge, especially following the introduction of housing and milking cattle leading to the hypothesis that APPs may be constitutively expressed in the normal udder, functioning as a general housekeeping molecule, which may be upregulated by infection and inflammation resulting from the introduction of bacteria into the mammary gland.

1.5 The Bovine Udder

The bovine mammary gland is derived from a highly modified sweat gland, so that the inside lining of the teats and ducts of the mammary gland are essentially modified skin (Frandson 1992). The udder of a cow has four separate mammary glands, each with its own distinct teat. There is no flow of milk from one quarter to another, neither is there any significant direct blood flow from one quarter to another. The alveoli are the functional units of the mammary gland. The alveoli are kept together by a thin layer of connective tissue, and contain the nerves and blood vessels. A group of alveoli surrounded by connective tissue forms a lobule. A group of lobules, with a common collective duct, surrounded by connective tissue, forms a lobe. The large ducts have an epithelium of two or more layers. Surrounding the alveoli are myoepithelial cells. When the stimulus for milk let down occurs these cells contract, squeezing milk from the alveoli into the ducts, from where it travels to the udder and into the teat itself. The teat wall consists of four layers, each having an important function in milk let-down and mastitis control (Sordillo and Streicher 2002). The outer layer or epidermis is lined with a thick layer of keratinised cells which produce a hostile environment for bacterial growth (Sordillo et al 1997). The

underlying dermis is the tissue of the teat wall that carries the blood vessels and nerves. There are a variety of muscles that are set in transverse, oblique and longitudinal planes in the dermis of the teat wall. These muscles are responsible for the opening of the teat during milking but, more importantly, for maintaining a tight closure of the teat end between milkings, and hence hindering bacterial entry (Sordillo and Streicher 2002).

1.5.1 Innate and Specific Immune Mechanisms of the Udder

The bovine mammary glands have many forms of defence against invasion by pathogenic organisms, combining non-specific and specific systems, including the anatomical features of the gland and the humoral and cellular defence mechanisms (Outteridge and Lee 1988). In the mammary gland it is necessary for both innate and acquired immunity to coordinate in a highly interactive fashion in order to utilize the range of defence mechanisms available providing optimal protection of the gland.

1.5.1.1 Anatomical defences

Mastitis occurs when bacteria gain entrance to the udder via the teat canal. The anatomy of the udder is therefore, an extremely important first line of defence against infection, with the teat itself representing a physical barrier to the penetration of bacteria. The keratinised teat canal presents a waxy surface derived from stratified squamous epithelium that enables the trapping of invading bacteria. In addition, fatty acids with bacteriostatic properties such as myristic acid and linoleic acid are present have been identified within the teat lining (Sordillo et al 1997). These antibacterial properties are most effective when the teat canal is closed, when the contracted muscle has caused the adjoining folds of stratified squamous epithelium to interlock and form a tight seal (Nickerson 1985). The teat canal is of vital importance in the prevention of new cases of mastitis and clearly, it follows that any damage to the teat end will compromise the defence mechanisms.

1.5.1.2 Soluble defences

When bacteria successfully overcome the defence mechanisms of the teat canal, they are then confronted with the internal defences of the udder. A number of antibacterial soluble factors are present in milk, and have a concerted action. The functions of the main non-specific soluble components of the mammary defence mechanisms are outlined below. However it is difficult to judge the relative importance of an individual factor.

Lactoferrin, a protein of molecular weight of 76Kd is the most important iron binding protein of various secretions, including milk (Diarra et al 2003). In the healthy mammary gland, the concentration of lactoferrin is low but increases during involution and inflammation (Sordillo et al 1997). In the dry, non-lactating udder, lactoferrin prevents bacterial growth through removing iron from udder secretions, and hence limiting iron uptake of bacteria. Aerobic bacteria such as *Escherichia coli (E. coli)* in particular require an abundance of iron. However the bacteriostatic effects of lactoferrin can be abolished in the presence of citrate, a buffer produced by epithelial cells. Citrate concentration varies greatly depending on the stage of lactation, but is highest during lactation and lowest in the dry period (Kutila et al 2003). High citrate concentrations in milk compete with lactoferrin for iron, producing iron citrate that can readily be utilised for bacterial growth.

Transferrin, another iron binding protein is released from the liver in increased concentrations during infection. As a result of the increased tissue permeability during mastitis, transferrin is able to pass from blood to milk, thus increasing the iron binding capacity of milk. Thus the increase of lactoferrin and transferrin in milk demonstrates the immune system's attempt to prevent bacterial growth, through sequestering iron.

All milk contains the enzyme lactoperoxidase (LPO) (Kussendrager and van Hooijdonk 2000). Lactoperoxidase is a heam protein that prevents the growth of bacteria by catalysing the oxidation of thiocyanate to hypothiocyanate (Ostdal et al 2000). In the presence of thiocyanate (SCN) and hydrogen peroxide, LPO can inhibit the growth of some bacteria (gram-positive) and kill others (gram-negative). Lactoperoxidase is highly abundant in bovine milk, but the concentrations of H_2O_2 and thiocyanate in fresh milk are dependent on the plane of nutrition, which can cause variation in the effectiveness of LPO as an indigenous antimicrobial agent (Sordillo et al 1997).

Lysozyme is a low molecular weight (15KDa) basic protein, which causes osmotic lysis of bacterial cell walls through cleavage of peptidoglycans. In comparison with other secretions, the lysozyme content of bovine milk is low and its direct bacteriolytic effect in milk is weak, however lysozyme intensifies the bactericidal activity of lactoferrin (Sordillo et al 1997). The impact of these antibacterial soluble factors in the defence of the udder is not entirely clear, however they represent another hurdle for bacteria to overcome if an infection is to be successfully established.

1.5.1.3 Cellular defences

Specific immunological factors are also involved in the defence of the teat canal. Lymphocytes and plasma cells accumulate beneath and between the epithelium of the teat canal wall, indicating local immunological activity. Neutrophils directly penetrate the teat wall to the infected and inflamed teat canal.

Bacterial pathogens that are able to elude the anatomical defence provided by the teat end must then evade the further anti-bacterial activities within the mammary gland in order to establish an infection (Sordillo and Streicher 2002).

Mastitis researchers have long recognised the biological importance of the different types of cells found in bovine milk (Paape et al 2002) (Table 1.3). These consist primarily of white blood cells, including neutrophils, macrophages and lymphocytes and also a small percentage of epithelial cells (Nickerson 1985), and termed milk somatic cells. Milk somatic cells are probably the best recognised components of mammary gland immunity, and depending on the status of the gland, in terms of infection, Somatic Cell Counts (SCCs) vary in number and composition depending on the physiological status of the udder (Table 1.4).

Macrophages are the predominant cell type in milk tissues of healthy mammary gland and their primary function is phagocytosis and intracellular killing of invading microorganisms and also removal of milk fat from involuting glands (Outteridge and Lee 1981). Pro-inflammatory cytokines released from macrophages in response to infection have both local and systemic effects. As previously discussed, at the systemic level they mediate the acute phase reaction. At the local level, macrophages facilitate the migration and bactericidal effects of neutrophils, thereby amplifying the inflammatory response (Riollet et al 2000). Activated macrophages are triggered to release prostaglandins, leukotrienes, and cytokines that can greatly augment the local inflammatory process (Persson et al 1993; Kehrli et al 1999).

Neutrophils are non-specific leukocytes and the principal cell type present in mammary tissues and secretions during early inflammation (Sordillo and Streicher 2002). Following bacterial invasion, inflammatory mediators produced by cells in the infected gland elicit neutrophil recruitment. This can lead to neutrophil numbers rising from relatively low normal levels observed in the healthy udder to constitute greater than 90% of the total mammary leukocyte population during mastitis (Sordillo et al 1997).

FACTOR	BIOLOGICAL FUNCTION
Neutrophils	Phagocytosis and intracellular killing of bacteria; secretion of antibacterial factors
Macrophages	Phagocytosis and intracellular killing of bacteria; antigen presentation in conjunction with MHC
Natural killer cells	Nonimmune lymphocytes that secrete antibacterial proteins upon activation
T lymphocytes	
CD4+ (T helper)	Production of immunoregulatory cytokines following antigen recognition with MHC class II molecules; memory cells following antigen recognition
CD8+ (T cytotoxic)	Lysis of altered or damaged host cells when complexed with MHC class I molecules; production of cytokines that can down regulate certain leukocyte functions
γδ T lymphocytes	Biological role in the mammary gland is speculative
B lymphocytes	
Mature B cells	Display membrance bound antibody molecules to facilitate antigen presentation; memory cells following antigen interactions
Plasma cell	Terminally differentiated B lymphocytes that synthesize and secrete antibody against a specific antigen

Table 1-3: Summary of mammary gland cellular defences. Reproduced from (Sordillo and Streicher 2002)

	SCC (CELLS/ML OF MILK)	SCC COMPOSITION (%)			
		Neutrophils	Macrophages	Lymphocytes	Epithelial cells
Healthy Quarter	<200,000	0-11	66-88	10-27	0-7
Infected Quarter	>200,000	50-95	9-32	14-24	0-9

Table 1-4: Somatic cell count (SCC) in healthy and infected bovine mammary gland (Riollet et al 2000).

In order to reach the inflammatory site from the blood compartment, leukocytes have to evade the circulatory system. The endothelium is key to this process of extravasation. Endothelial cells have a potent inflammatory role, through their ability to secrete inflammatory mediators and modulate the adhesion of leukocytes to their surface. This process involves the interaction between adhesion molecules expressed by both endothelial cells and leukocytes. Following exposure to cytokines (IL-1, TNF and IL-6), vascular endothelial cells increase their expression of adhesion molecules including E-selectin, ICAM-1 and VCAM-1. These molecules interact specifically with neutrophils and other circulating leukocytes and act initially to slow their rate of flow. At this stage the leukocytes can be seen to 'roll' along the endothelial surface. The neutrophils then attach securely to the endothelial cells through tight binding between leukocyte integrins (LFA-1) and endothelial adhesion molecules (ICAM-1). This interaction increases the affinity of neutrophils for endothelial adhesion molecules enabling the leukocyte to squeeze between the endothelial cells in a process known as diapedesis (Janeway 2001). Finally, the leukocytes migrate through the tissue under the influence of chemokines such as IL-8.

Neutrophil recruitment from the circulation to the focus of infection is essential in the defence of the mammary gland against invading bacteria. If the APR facilitates elimination of the bacteria which act as inflammatory stimuli, then neutrophil recruitment ceases and SCC returns to healthy levels. This is observed during clinical mastitis where *E. coli* is the causative agent and a spontaneous cure of the infected gland occurs within a few days of the infection commencing (Shuster et al 1996). If the bacteria are able to survive this immediate host response, then inflammation and leukocyte migration continues (Kehrli et al 1999). Prolonged diapedesis of leukocytes causes damage to mammary parenchyma tissue resulting in decreased production of milk. This is the case in *S. aureus* mastitis which tends to be chronic and are often subclinical. Several months after the beginning of infection, neutrophils are still the major cells in the infected gland (Rainard and Riollet 2003).

The specific immune mechanisms of the mammary gland are generally less well understood than that of the innate immune mechanisms. Recently attention has been paid to the role of the specific immunity, particularly to the role of the lymphocyte population in the protection of the mammary gland against bacterial infections.

Lymphocytes are the only cells of the immune system that recognize antigens through specific membrane receptors (Sordillo et al 1997). There are two distinct subsets of lymphocytes, which differ in function and protein products, T and B lymphocytes. The T

lymphocytes can be classified further into αβ T lymphocytes, which include CD4+ (T-helper) and CD8+ (T-cytotoxic or T-suppressor) lymphocytes and γδ T cells. In the healthy lactating gland CD8+ T cells are the predominant phenotype of the T lymphocyte population, in contrast to infected mammary glands where CD4+ T cells prevail (Sordillo and Streicher 2002). The T-helper lymphocytes are activated to produce cytokines in response to recognition of antigen-major histocompatibility complex (MHC) class II complex on antigen presenting cells such as B cells or macrophages. Depending on the repertoire of cytokines produced, the T helper cell response can facilitate either a cell-mediated (Th1 type) or a humoral (Th2 type) response (Brown et al 1998). Interleukin-2 (IL-2) and interferon-γ (IFN-γ) are the major cytokines characterized during the Th1 response, and IL-4, IL-5 and IL-10 dominate during the Th-2 response. Interleukin-10 however can be produced by and regulate all, Th cells (Brown et al 1998).

CD8+ T cells can exert either a cytotoxic or suppressor function. It has been suggested that CD8+ cytotoxic cells may act as scavengers, removing old or damaged secretory cells, the presence of which could increase the susceptibility of the mammary gland to infections (Taylor et al 1994; Sordillo et al 1997). Suppressor T lymphocytes are thought to control or modulate the immune response to bacterial infections. Researchers have demonstrated the ability of CD8+ lymphocytes to alter or suppress the proliferative response of CD4+ lymphocytes, through evaluation of lacteal secretions from cows infected with *S. aureus* (Park et al 1993). The biological function of γδ T cells, is as yet still unknown, so their contribution to mammary gland immunity largely remains speculative, although they have been associated with the protection of epithelial surfaces (Sordillo et al 1997). The functional significance of the variation of lymphocyte subpopulations found in the mammary gland remains unclear (Riollet et al 2000).

The primary role for B lymphocytes is to produce antibodies against invading pathogens. Unlike macrophages and neutrophils, B lymphocytes utilize their cell surface receptors to recognize specific pathogens. Through their ability to act as antigen presenting cells, B lymphocytes stimulate the production of IL-2 from T-lymphocytes which in turn induces proliferation and differentiation of the B lymphocyte, into either plasma cells that produce antibody, or memory cells. Unlike T lymphocytes, the proportion of B cells among the total lymphocyte population remains fairly constant throughout the different stages of lactation and does not increase when infection occurs (Riollet et al 2000).

1.6 Bovine Milk

Milk is a food with excellent nutritional properties, the quality of which decreases markedly with mastitis. Of the five constituents of milk, the largest in quantity is water (87%), followed by lactose (5%), fats (3.9%) and proteins (3.2%) (Paquin 1998). It is the protein component of milk that is of most interest to this study. Approximately 80% of milk protein is casein and the remaining 20% is whey protein. Caseins are grouped together in micelles: spherical structures containing α, β, and κ casein, and calcium phosphate. It is the casein fraction that gives milk its white-blue colour. It can be removed from milk through acid precipitation at pH 4.6, and is essential to the manufacture of cheese (Wong et al 1996). The whey proteins are soluble globular proteins, transparent in solution. Beta-lactoglobulin (β-Lg) is the most abundant whey protein, followed by α-lactalbumin. β-lactoglobulin is a member of the lipocalin family, and although no biological role for this protein has yet been unveiled, its family origin and its identified similarity to retinol-binding protein have led to suggestions of it having a role in transport of retinal or fatty acids (Sawyer et al 1998; Kontopidis et al 2002). Present on the outer surface of this protein is a cavity lined with hydrophobic residues. This hydrophobic cleft is able to accommodate fatty acids like palmitate and stearate, a property which could be exploited for the removal of this protein from solution. Alpha-lactalbumin (α-LA) is a small (Mr 14200), acidic (isoelectric point (pI) 4-5), Ca^{2+} binding protein, which is essential for lactose biosynthesis. Alpha-lactalbumin is one of two components of lactose synthase, which catalyses the final step in lactose biosynthesis in the lactating mammary gland (Permyakov and Berliner 2000).

Inflammatory reactions in the udder alter the quantity of milk produced and change the composition of milk in terms of quality. Reduction in milk yield is one of the clearest results of mastitis (Hortet and Seegers 1998; Hortet et al 1999; Yalcin et al 2000). Table 1.5 outlines the total loss in milk throughout lactation in association with SCC. The ratio between the different proteins of milk change with an elevated SCC indicative of infection. Casein concentrations decrease greatly during mastitis (Munro et al 1984). This is a consequence of the increased enzymatic and biochemical activity in mastitic milk. The activity of the major oxidizing lipolytic and proteolytic enzymes is noticeably higher in mastitic milk than in normal milk. The casein is degraded by proteinases originating from bacteria, leukocytes or blood. β-casein is hydrolysed into smaller fragments by plasmin, resulting not only in a decrease of total casein, but also an abnormally high ratio of κ-casein to β-casein. As casein is essential for the coagulation of cheese, mastitic milk is

CELL COUNT ($X10^3$/ML)	DROP IN PRODUCTION (LITRES/LACTATION)
<250	
250-499	-191
500-749	-336
750-999	-768
>1000	-895

Table 1-5: An increase in milk somatic cell count (SCC) is associated with a decrease in yield. The estimated drop in yield per lactation is outlined above. This table was reproduced from the Department for Environment Food and Rural Affairs (DEFRA) web site: http://www.defra.gov.uk/animalh/welfare/farmed/advice/calendar/housing.asp?id=cells

useless for manufacture of this particular dairy product. Bearing in mind that casein constitutes 80% of the total protein, its degradation is of concern to producers in receipt of payment systems for milk based particularly on total protein concentration (Auldist and Hubble 1998).

The concentrations of β-LG and α-LA can drop by more than 70% of their normal level during mastitis (Auldist and Hubble 1998). The decrease in α-LA leads to a proportional decrease in lactose: the osmotic regulator of milk volume. The decrease in both these whey proteins is probably due to leakage of these proteins out of milk and into the extracellular fluid. As the degree of inflammation increases the chemical composition of milk approaches more and more that of blood. This is largely due to the disruption of the integrity of the mammary epithelia by bacterial toxins coupled with the customary increase in vascular permeability at inflammatory sites (Watanabe et al 2000). Depending on the severity of the blood-mammary breakdown, differing degrees (if any) of blood-milk mixing can occur, accounting for the presence of blood in mastitic milk. An increase in proteins of blood serum origin including lactoferrin and albumin has been reported in cows with mastitis.

The ionic composition of mastitic milk differs from that of normal milk, with Na^{2+} and Cl^{2-} ions both becoming more concentrated, in contrast to the levels of Ca^{2+} and PO_4^{2-} both of which are reduced. In addition to the biochemical changes, the physical properties of mastitic milk also change. The pH increases from 6.6 to over 7, mainly due to the transfer of bicarbonate ions from blood to milk. An increase in the cell numbers and serum protein content are responsible for an increase in the viscosity of mastitic milk.

1.7 Diagnosis of Mastitis

The dairy industry is challenged by the increasing demand to produce high quality dairy products from a milk supply that is inconsistent in its processing characteristics across the season. The relationship between bulk milk somatic cell counts (BMSCC) and the prevalence of mastitis has been the focus of numerous studies. Holdaway et al (1996) reported a correlation of 0.84 ($p<0.001$) between BMSCC and the percentage of infected quarters. Consequently BMSCC have become universally adopted as a screening test for mastitis in herds. The EC directive requires bulk milk to have a total bacteria count <100,000 bacteria /ml and SCC <400,000 cells/ml. Milk quality incentive schemes for BMSCC are routinely in place by dairy companies around the world, and are based on penalties and premiums. Payment schemes differ between milk companies. (Table 1.6)

SCC (CELLS/ML)	GAIN/LOSS (PENCE/L)
<150,000	+ 0.2
151-250,000	+ 0.1
250-350,000	nil
351 - 400,000	-1
>400,000	-5

Table 1-6: Financial penalities currently employed by Scottish Milk for high bulk milk somatic cell count (SCC) levels

A BMSCC of <250,000 cells/ml is now considered to be a realistic upper limit for UK herds with good mastitis control (Blowey and Collis 1992). Individual cow somatic cell count (ICSCC) has been identified as the most efficient way of identifying high SCC cows. Although not routinely used on all farms, some carry out monthly recordings of ICSCC, in an attempt to try and lower BMSCC through identifying infected animals within the herd. Much variation still remains over deciding on a threshold SCC at which cows should be classified either as normal or infected. Variation between pathogen type has been one of the main problems in setting a SCC threshold. Mean SCCs for *Streptococcus sp.* are much higher than they are for *Staphylococcus sp.* (Holdaway et al 1996). Various other factors influence SCC, including breed, nutrition, age, management, stage of lactation and also physiological stresses such as excitement, high temperatures and trauma (Holdaway et al 1996). Somatic cell count is known to increase towards the end of lactation. For farmers who employ a 'block calving' system, where a high percentage of the herd will simultaneously be at their lactation end, inevitably BMSCC will be raised at this time. Use of SCC as an indication of infection should be exercised with caution in such a situation. Bulk milk SCC is decreasing in several countries, but the decline in BMSCC has not been accompanied by a decrease in incidence of clinical mastitis (Peeler et al 2000). On the contrary, studies on clinical mastitis in herds with low BMSCC have led to the suggestion that low SCC may be associated with increased risk or severity of mastitis (Barkema et al 1998; Peeler et al 2000).

Automatic milking systems (AMS) are now commercially available and their growth in popularity is likely to have a considerable effect on udder health (Hamann 1999; Pyorala 2002). Due to the reduced contact time between stock and dairy personnel, physical diagnosis of mastitis by the herdsman in the milking parlour needs to be replaced by other systems. Automated detection of mastitis has become feasible, because several parameters which change in relation to mastitis such as milk production, milk electrical conductivity, N-acetyl β-D-glucosamide (NAGase), lactate and milk temperature, can be measured automatically by sensors during milking (http://www.sensortec.co.nz). The goal of automation is to detect in advance of the clinical signs, thereby allowing early intervention (Nielen et al 1995). Electrical conductivity is currently being used for automated detection of mastitis but is insufficiently accurate to be used as the sole criterion for mastitis detection and further treatment decisions (Mottram 1997; Biggadike et al 2002). Automatic milking systems offer an improved quality of life for the farmer and the possibility of increased performance of the cow, however deficiencies in fulfilling the milk hygiene regulations through identification of abnormal milk remain (Hamann 2001).

The recent detection of APPs, Hp and SAA, in the milk from cows affected with mastitis has led to the possibility of such proteins being applied to the development of an online sensor system for the automatic detection of mastitis in robotic milking systems (Eckersall et al 2001; Pedersen et al 2003; Gronlund et al 2003a; Nielsen et al 2004).

The overall objectives of the work presented in this thesis are 1) To establish the concentrations and earliest time point of appearance of APPs, Hp and SAA, in milk of cows with naturally occurring mastitis, and to determine their relationship with conventional markers of mastitis such as SCC. 2) To establish a protocol using advanced proteomic methodology allowing investigation of the total protein repertoire of normal bovine milk. 3) To use these established proteomic methods to further characterize the alterations in the bovine milk proteome as induced by mastitis. 4) To identify further biomarkers for bovine mastitis by analysis of protein spots differentially expressed in normal and mastitic milk. 5) To determine the origin of APPs in milk through examination of bovine mammary tissue.

Chapter II

Development of an Immunoassay for the Quantification of Bovine Milk Haptoglobin

2.1 Introduction

2.1.1 Haptoglobin in Cattle

The APR when measured in serum is non-specific and therefore measurement of an increased concentration of Hp in serum is not a specific diagnostic test for mastitis. Recently investigations have been focused on the local inflammatory process in the mammary gland and APP measurements in milk (Eckersall et al 2001; McDonald et al 2001; Pedersen et al 2003; Gronlund et al 2003a; Nielsen et al 2004).

Haptoglobin is a major APP in cattle in which it has a negligible circulating level in blood of normal animals (Conner et al 1988b), but increases up to 300 fold during the APR from a concentration of less than 0.01g/L to reach 2-3 g/L within 48hrs of infection (Eckersall 1999). Contrary to humans where Hp is a normal blood protein, it is absent from the plasma of healthy cattle (Eckersall and Conner 1988b). Previous investigation of a variety of diseases in cattle have shown that Hp has an APR in pasteurellosis (Conner et al 1989) (Horadagoda et al 1994); pneumonia (Wittum et al 1996); mastitis (Hirvonen et al 1996); foot and mouth disease virus (Hofner et al 1994); bovine respiratory disease (Godson et al 1996); fatty liver (Nakagawa et al 1997) and *salmonella* (Deignan et al 2000). In cattle and other ruminants, Hp has been the acute phase protein most commonly monitored as a marker of inflammation (Skinner et al 1991; Wittum et al 1996). Hp has the disadvantage of being reduced during haemolytic crises (Kent 1992), resulting in the need for caution in the use of Hp for the diagnosis or monitoring of disease involving inflammation if simultaneously there is haemolytic crisis in the animal. In such a situation where free Hb is present in the circulation, all available Hp binds to the Hb with the resulting complex being removed by the liver. In this case, even if production of Hp has been stimulated by inflammation, all the Hp binds to the excess Hb and Hp is effectively removed from the circulation (Eckersall and Conner 1988a). In veterinary clinical chemistry measurement of Hp is particularly important in assessing the health status of cattle, and has been reported

as a more specific variable to monitor animals with inflammatory lesions, than SAA (Gruys et al 1994).

2.2 Measurement of Acute Phase Proteins

The methods used to analyse APP have developed considerably over the past few years. Originally, most of the proteins were estimated by use of their biochemical activity, however with advances in technology, immunoassays have become the method of choice for the measurement of animal APP. Although the majority of assays for APPs lie within these two main groups, there are still a few exceptions, including the use of capillary zone electrophoresis (CZE), for the determination of bovine Hp (Alvarez et al 2001).

2.2.1 Activity Assays

By the early 1980s, it was recognized that Hp could be a useful diagnostic tool particularly in cattle but that the difficulties in quantitative determination by conventional methods was a limitation (Makimura and Suzuki 1982). Haptoglobin (Hp), which has the ability to bind to haemoglobin (Hb), thus preserving the peroxidase activity of the Hb from inactivation at an acidic pH, can be measured by quantification of this activity (Conner et al 1988b).

In veterinary diagnostics the original assays depended on the formation of the Hb-Hp complex altering the absorbance characteristics of Hb (as used in a study to assess the potential of Hp as a diagnostic indicator of feline disease), in proportion to the concentration of Hp in the serum sample (Harvey and Gaskin 1978). In this study, the concentration of Hp was determined by Hb binding method using cyanmethaemoglobin as a more stable derivative of Hb, and is widely accepted as the method of choice for the haemoglobinometry of whole blood (Harvey and Gaskin 1978). Makimura and Suzuki (1982) reported the use of the Hb-Hp complex in the quantification of Hp but modified the previously reported method (Tarukoski 1966), by using methaemoglobin (Hb^{+}) as a more stable alternative to oxy-haemoglobin. This method was employed as the assay of choice in veterinary diagnostic laboratories, and at the time held the advantage over immunoassays because of its convenience and ability to be performed on all species (Eckersall et al 1999a). An automated version of this assay was developed (Skinner and Roberts 1994), but a major drawback of this procedure was the necessity to use guaiacol, a reagent with a very strong odour. Hence the goal of developing an automated assay for Hp, using stable and convenient reagents, was not yet achieved. In 1999 Eckersall *et al* successfully developed a robust biochemical assay for serum Hp. A major finding in this development was that

serum albumin interfered with the assay system, particularly at low normal levels, which had led to the consistent problem in estimating zero control samples. The effects of albumin were overcome through the development of a chromogen reagent cocktail (SB-7), which inhibited this interfering effect of albumin. This assay is now available as a commercially available kit and is being widely used in the measurement of Hp in a range of species.

Using a subcutaneous injection of turpentine as inflammatory stimuli to produce an acute phase response results in an increase in the APPs: α_1-antitrypsin, ceruloplasmin, fibrinogen, Hp and seromucoid (Conner et al 1988b). α_1-antitrypsin, Hp and seromucoid were all measured using activity based assays. The anti-protease activity of α_1-antitrypsin is measured through its ability to inhibit the action of trypsin, on the synthetic substrate N-benzoyl-arginine-p-nitroanalide. Not only was this aforementioned method used for the measurement of serum APPs in this study, but α_1-antitrypsin was actually measured in mastitic milk (Sandholm et al 1984), but the authors concluded that this APP was not a reliable and sensitive marker of mastitis because not only was the percentage increase small but the levels also showed no dose-related variation.

2.2.2 *Immunoassays*

In the early to mid 1990s the trend moved towards the usage of antibody based systems for the measurement of APPs.

2.2.2.1 General

The term 'immunoassay' is used to describe a diverse range of techniques for the detection and quantification of the antibody-antigen interaction, encompassing a wide array of different labels and detection systems (Ronald and Stimson 1998). The basic principle of immunoassay is based on the reversible reaction between an antigen and its specific antibody. The introduction of monoclonal antibodies and advances in labeling methodologies, combined with the speed, simplicity and relatively low cost of immunoassays, have contributed to their employment for the detection of clinically important analytes (Gosling 1990). An advantage of these systems is that antibody-based methods allow development of immunoassays of differing format i.e: immunodiffusion, immunoturbidimetry, latex agglutination and enzyme linked immunosorbent assay (ELISA),(Eckersall 1995a). The preparation of monoclonal antibodies for bovine Hp

(Sheffield et al 1994), was the stimuli for numerous publications, reporting the use of various formats of ELISA for the quantification of APP.

A number of assays have been described where antiserum raised against one species has been used to quantify the same protein in a different species (Eckersall 1995a). This is not ideal but acceptable so long as the assay has been validated for the species under investigation.

The earliest immunoassays in the 1960s and 70s utilized primarily radioisoptope labels (Berson and Yalow 1971), which had disadvantages of their short half-life, potential health hazard and waste disposal problems. Continual developments in the field of immunoassay, all aimed towards improving the utility, sensitivity, accuracy and specificity of the assay. One of the first advances in this direction was the replacement of the radioisotope labels with alternative labels, primarily enzyme labels although a whole variety of different labels have now been developed, including fluorescent and chemiluminescent compounds. The advent of these new labels and the instrumentation to use them has given rise to a wide range of robust immunoassays with differing formats. The most commonly used assay formats are described below, with examples being given of their application in measurement of APP.

Single radial immunodiffusion (SRID) is a precipitation method employing diffusion of the antigen, in antibody containing agar gels (Mancini et al 1965). SRID has been developed as a simple, cheap and practical method for the quantitative determination of antigens. The method of Mancini et al (1965) was employed for the measurement of bovine serum Hp under various physiological and pathological conditions, and the levels obtained were found to be comparable to those obtained by methods based on haemoglobin binding activity (Morimatsu et al 1992). SRID was used to demonstrate increased levels of Hp in horses with clinical signs of inflammatory disease (Taira et al 1992). Single radial immunodiffusion has previously been demonstrated as an accurate and easy to use means of measuring serum Hp, however one drawback of this method is the requirement that the gel must be incubated for a period of 24-48hrs before reading the results. Another disadvantage of SRID is its sensitivity and the possibility of introducing error through manual measurement of the precipitin ring. Many of the studies using ELISA for determination of serum Hp (Young et al 1995; McNair et al 1997; Nakagawa et al 1997), report that SRID was first used but the levels of sensitivity required were not achieved, hence the need to develop an assay with increased sensitivity.

Ligand labelled assays. In this procedure, it is the analyte/ligand of interest that is conjugated to the label. Free, unlabelled ligand in the sample will compete with the labeled ligand for the limited number of available antibody binding sites, hence the reason why this ELISA format is commonly referred to as a 'competitive assay'. Removal of the free ligand with a washing step, and detection of the signal generated by the bound labeled analyte, will produce an inverse relationship. i.e The higher the concentration of the analyte of interest in the sample, the less labelled ligand will be able to bind. Competitive immunoassays have been evaluated for the measurement of bovine Hp by comparison with Hb binding methods (Young et al 1995; McNair et al 1997). The authors of these studies conclude improved sensitivity as the main advantage of this immunoassay format, but also the ability of this assay to measure Hp in the presence of haemolysed red blood cells. However, Young *et al*, also compared the method of competitive inhibition, with two other ELISA formats and although this assay format gave the best correlation with the Hb binding methods, it was concluded to be the most time consuming method and not suitable for routine processing of samples.

Antibody labelled assays. In these assays the specific antibody is conjugated with the label. The most common approach to this method is by direct detection, where the antigen in the sample is bound/captured onto the solid support, and subsequently detected by the addition of a labelled antibody. This gives a direct relationship of signal to levels of antigen. A variation of this method is where a limited amount of antigen is bound/captured onto the solid support and the binding of a labelled antibody is made to compete with free antigen in the sample. This competitive assay gives an inverse relationship between signal and the level of antigen in the sample.

Sandwich assays. This assay utilizes two antibodies, which bind independent epitopes on the antigen. The capture antibody is immobilized on a solid support, binding any antigen present in the sample. The antigen is then detected by addition of a second labeled antibody. A major advantage of using the two-site sandwich assay is that the specificity of the assay is improved, as signal generation requires the binding of both antibodies. The double antibody method has been employed in the measurement of APPs (Nakagawa et al 1997; Kanno and Katoh 2001; Schroedl et al 2001). A common aim in the experimental design of these studies is to develop a method with the sensitivity required to measure APP in the serum of healthy cattle, as earlier investigations failed to detect Hp in the serum of healthy cattle (Spooner and Miller 1971; Makimura and Suzuki 1982; Conner et al 1988b; Morimatsu et al 1992).

Ligand binding assays. Eckersall et al (1989) described an ELISA for canine CRP, that utilizes the binding affinity of CRP to phosphorylcholine (PC). The plate was coated with PC conjugated to albumin, and CRP from positive serum samples was hence bound to the plate. The concentration of CRP in the serum sample was then quantified through incubation with a primary antiboby (anti-CRP), followed by a secondary peroxidase labeled antibody (Eckersall et al 1989).

Immune complex particle assays. Assays in this group include precipitation, nephelometric, and turbidimetric immunoassays. The basic principle of these assays is the reaction of a specific polyclonal antibody with a multivalent antigen, which leads to the formation of a visible precipitate. Nephelometry and turbidimetry increase the sensitivity of the assay by utilizing the light scattering properties of the antigen-antibody complexes. An immunoturbidimetric method has been used to measure serum Hp in the horse (Kent and Goodall 1991). The authors of this study concluded that the immunoturbidimetric assay for equine Hp was quick and easy to perform, and showed a good correlation with the more traditional haemoglobin binding methods, but with better precision. An immunoturbidimetric assay developed for the measurement of canine CRP, correlated with results obtained using an ELISA method and was recommended by the authors of the study as the method of choice for the routine analysis of canine CRP (Eckersall et al 1991).

Another study used an automated immunonephelometric detection system, to determine the Hp concentration in the plasma of fattening pigs (Lipperheide et al 1998). Single radial immunodiffusion (SRID) was used as a reference method to determine the Hp concentration in 27 out of the 100 plasma samples and led to the conclusion that optimal accuracy was only obtained when an animal-relevant standard was used, reinforcing the need for an international standardization of all methods to quantify APPs in animal serum by use of antibody-based assays (Eckersall et al 1999b).

2.3 Measuring Hp in Milk

Serum Hp can be measured by peroxidase activity of Hp-Hb complex, electrophoresis, immunodiffusion, differential acid denaturation of Hb and Hp-Hb complex, immunonephelometry. However all of these methods lose precision when Hp is present in low amounts (Katnik and Dobryszycka 1990).

In preliminary studies attempts to measure the concentration of Hp in milk by the haemoglobin-binding method were not successful because the sample contained

peroxidase activity, probably due to lactoperoxidase, which interfered with the assay (Eckersall et al 2001).

The aim of this work was therefore to develop and validate an immunoassay for quantitative determination of bovine Hp in milk with a limit of detection to allow quantification of low concentration. The initial method for the measurement of haptoglobin in milk was by SRID assay, as used by Eckersall and others (2001) to measure the concentration of Hp in this fluid. However, Hp was only detectable in a few milk samples from cows with mastitis, a possible reason being that the limit of detection of the SRID assay was too high to allow low level changes to be detected, so a new more sensitive method had to be developed. An ELISA based method using a ligand binding approach was developed and validated and compared to SRID. The ELISA was then used in a collaborative study with the Swedish University of Agricultural Sciences, Uppsala, to determine the concentration of Hp in milk, from dairy cows experimentally challenged with *S. aureus.*

2.4 Materials and Methods

2.4.1 General Reagents and Equipment

Reagents were obtained from Sigma Chemical Company Ltd, unless otherwise stated. Microtitre plates used were from Nunc. Plate incubator/ shaker and plate reader connected to a computer, with Revelation^{TM} immunoassay software (Dynex Technologies Inc, Virginia, USA) installed.

2.4.1.1 Milk samples

The milk samples from this study were from the Swedish University of Agricultural Sciences, Faculty of Veterinary Medicine, Uppsala, Sweden. A model of an experimentally induced *Staphylococcus aureus* mastitis in six dairy cows, was used, in which an acute inflammation was transformed to a chronic phase by controlled use of antibiotics. Stripping milk samples were aseptically collected from the infected and the control quarters from each animal at, day 2 and day 1 before infection, and at 6, 24, 32, 48, 54, 72, 80, 96, 104, 120, 128, 144, 168, 192 hours post-infection (h.p.i), and on days 12, 15, 19, 22, 26, 29, 33 and 36 post-infection (p.i). For further information refer to Gronlund *et al* (2003a).

2.4.2 Single Radial Immunodiffusion

2.4.2.1 Antisera

The ovine antiserum against bovine Hp (anti-Hp) was selected from a group of antisera raised in sheep immunized against this antigen (Eckersall and Conner 1990). The antisera having the strongest cross-reaction with antigen or high acute phase serum, was selected by screening this cross-reaction using Ouchterlony plates.

2.4.2.2 Immunodifusion methods

Single radial immunodiffusion (SRID) was initially used to measure Hp in the milk samples from an experimental model of *S. aureus* mastitis. The standards and the internal controls for the final ELISA assay were quantified using SRID.

Single radial immunodifusion agarose plates (w/v 1.5%) were prepared in 0.05M tris buffered saline (TBS, pH 7.4), which contained 4% (v/v) antisera to bovine Hp. A milk sample with a high concentration of Hp was calibrated for use as a standard by comparison to a bovine serum Hp standard. For the SRID the milk standard was diluted in NaCl in 0.9% w/v sodium chloride to give standard concentrations over the range, of 680 to 5.3 μg/ml. Samples or standards (7μl) were transferred into wells (2.5mm diameter) in the antisera containing agarose, which was incubated in a humidity chamber for up to 48 hours at 4°C. The diameter of the circles of precipitation was measured and the results from the standards plotted against concentration on log-linear graph paper. The test results were read off the standard curve.

2.4.3 ELISA

An ELISA based on the affinity of Hp with Hb has been developed for the measurement of Hp in milk. In this method, Hb is initially coated onto the microtitre plates and Hp in milk specifically binds to Hb attached to the surface of the plate

2.4.3.1 Haemoglobin preparation

Canine erythrocytes were washed five times with 0.9% saline. An equal volume of the washed red cells was then added to an equal volume of distilled water, and toluene was layered on top (roughly 10% of total volume). The mixture was shaken vigorously for 15

mins, and then centrifuged for 15mins at 3500rpm. The lower layer obtained was the haemolysate: very dark red, almost black in colour, and should not contain any cell debris (Makimura and Suzuki 1982). The concentration of haemoglobin was determined according to manufacturers instructions using the Abbott 3500 Analyser (Abbott Diagnostics, Berkshire, UK). The haemolysate was aliquoted and stored at -20°C. Each aliquot was centrifuged for 2 mins before use.

2.4.3.2 Reagents and buffers

The different buffers used at various stages throughout the assay are detailed below. The coating buffer consisted of 10mM sodium bicarbonate ($NaHCO_3$) and 10mM sodium carbonate buffer (Na_2CO_3) at pH 9.6. Tris buffered saline was used to wash the plate between steps. In order to inhibit non-specific binding, a blocking buffer consisting of 5% w/v dried skimmed milk powder in TBS containing 0.1% sodium azide was used. The antibody that was used for the SRID (see section 2.4.2.1) was conjugated to streptavidin using heterobifunctional methods which utilize the cross-linker, succinimidyl (4-N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), to form a stable amide/thioether linkage between antibody and streptavidin. Conjugation of the antibody was carried out by Tridelta Development Ltd, Dublin, Ireland.

2.4.4 Assay Procedure

An optimisation procedure was performed to determine the best concentration for reagents. The following are the final 'optimised' conditions.

2.4.4.1 Coating

The Hb was diluted to a final concentration of 2.5mg/ml in bicarbonate buffer (10mM, pH 9.6) and then 100µl was dispensed into each well of a Nunc-Immuno™ 96 MicroWell™ Plate (Nunc International, Rochester,. NY) and incubated at 4°C overnight.

2.4.4.2 Washing

The ELISA plate was washed, by adding 300µl of TBS to each well, shaking the plate and the liquid decanted. This was repeated 3 times.

2.4.4.3 Blocking

Unoccupied binding sites were blocked by adding 300µl of 5% w/v dried skimmed milk in blocking buffer to each well and incubating the plate at 37°C with gentle shaking for 1hour.

2.4.4.4 Washing

The plate was again washed as in 2.4.4.2, but the TBS this time contained 0.1% (v/v) Tween-20 (TBST)

2.4.4.5 Sample dilution and loading volume

Standards, controls and test milk samples were diluted 1:20 in TBST containing blocking agent (1% w/v of dried skimmed milk) and 100µl of each sample was added to separate wells in duplicate and the plate was incubated at 37°C with gentle shaking for 1hour. The plate was then washed as in section 2.4.4.4. For further information refer to Gronlund et al (2003).

2.4.4.6 Addition of antibody

After washing, 100µl of conjugated antibody diluted 1:400 (in TBS-Tween + 1% w/v dried skim milk) was dispensed to each well and the plate was incubated at 37°C with gentle shaking for 1hour.

2.4.4.7 Addition of substrate

After decanting the plate was again washed 3 times as in 2.4.4.4. Tetra methyl benzidine (TMB) peroxidase substrate was freshly prepared according to manufacturers instructions (KPL, Maryland USA), and 200µl was added to each well. The plate was then left at room temperature to allow the colour to develop (approximately 20mins). The peroxidase-enzyme reaction was terminated by the addition of 50µl of 2M H_2SO_4 to each well.

2.4.4.8 Absorbance

The absorbance was measured at 450nm using an ELISA plate reader, and the results were analysed using the RevelationTM immunoassay software (Dynex Technologies Inc,

Virginia, USA), which employed a linear-logarithmic transformation in the calculation of the ELISA results.

2.4.5 Assay Optimisation

Assay validation was carried out as described by Sipe *et al* (1999) in several steps. Each step was carried out a minimum of 3 times and the mean values are presented in the following results section.

2.4.5.1 Titration of the horseradish peroxidase conjugated antibody

The optimal dilutions of antisera were determined by demonstration of the effect of varying antibody dilutions whilst keeping all other conditions constant. Three plates were reacted with all possible variables being kept constant, while HRP-conjugated antibody concentrations were varied. The objective was to identify the concentration of antibody that gave the maximum optical density for the highest standard while also giving a steep standard curve.

2.4.5.2 Optimisation of the standard and sample dilution

A standard curve was prepared by diluting the working standard in the coating buffer. The top standard with a known amount of Hp (340μg/ml), determined by radial immunodiffusion was diluted serially to give standards of: 340, 170, 85, 42, 21 and 10 μg/ml, respectively. In calibration of the standard, milk and serum were used in the beginning of development to demonstrate through parallelism of the standard curves, that the same analyte was being measured regardless of the matrix being used. Standards and samples were diluted 1:20 in TBST containing blocking agent (1% w/v of dried skimmed milk), to give an optimal balance between diluting out the matrix effect of the milk, without compromising the sensitivity of the assay.

2.4.5.3 Assessment of blocking agents

The ELISA was carried out, using, 5% dried milk, 5% dried milk and 0.1% bovine serum albumin (BSA); and also 5% porcine gelatin as blocking agents in TBS. Normal BSA was added to reduce any non-specific binding that may be present.

2.4.6 Assay Validation

Assay validation was carried out using methods described by (Sipe et al 1989) on the bovine Hp ELISA assay which was performed according to section 2.4.4.

2.4.6.1 Precision

The reproducibility of the ELISA was demonstrated by intra-assay and interassay coefficients of variation (CV%). Interassay precision was estimated by deriving the CV of low (25 μg/ml) and high (219 μg/ml) quality controls and was calculated by the standard deviation as a percentage of the mean of the results from 25 different assays. The intra-assay CV was determined by calculation of the CV of duplicate pairs for each immunoassay plate according to (Fraser 1986).

2.4.6.2 Accuracy

In analytical measurements accuracy is defined as how close the average measured value is to the true value. In the absence of purified Hp for recovery studies, the accuracy of the assay was assessed through a dilution experiment, to see whether or not the dilutions of sample lie parallel to the calibration curve.

2.4.6.3 The limit of detection

The limit of detection was determined as the lowest amount of Hp that could be distinguished from the zero standard. This was determined as the Hp concentrations at 2 SD away from the mean (mean ± 2 x SD) of the zero standard.

2.4.6.4 Comparison to other methods

In order to confirm that the assay developed for bovine Hp in milk offered increased sensitivity to existing methods, the concentration of Hp in 288 milk samples from infected and control animals which had been assayed first by SRID were also assayed by the ELISA. A regression analysis was carried out on the results of these assays.

2.4.6.5 Detection of pathophysiological change

The milk samples described in section 2.4.1.1 were assayed using the ELISA with assay optimisation as per section 2.4.5.

2.5 Results

2.5.1 Assay optimisation

2.5.1.1 Titration of the horseradish peroxidase conjugated antibody

Throughout the optimisation procedure a wide range of antibody dilutions were screened, however final optimisation covered a range of 1:400 to 1:1600. Figure 2.1 shows the antibody dilution of 1:400 gave the best results with the maximum standard giving a high optical density of 1.6 and a steep curve with still a reasonably low background.

2.5.1.2 Standard and sample dilution

Assay development included calibration of a suitable standard. Milk and serum were both diluted serially in assay buffer and are shown to display parallelism (Figure 2.2) indicating that the same analyte is being measured regardless of the matrix being used. For the final assay protocol milk, diluted in assay buffer was chosen as the working standard to keep variation to a minimum.

2.5.1.3 Influence of the blocking agents

The results of the OD of the zero standard with different blocking agents are shown in figure 2.3. The 5% w/v dried milk; 2% w/v BSA and 1% w/v porcine gelatin all give a suitable low OD. The porcine gelatin was discounted because it was more difficult to wash from the wells (due to its gel-like consistency), than the other two blocking agents. 5% w/v dried milk was chosen over BSA due to the convenience in preparation and lower cost.

2.5.2 Assay validation

2.5.2.1 Intra- and interassay precision and the assay repeatability

The inter-assay coefficients of variance were 9.8% and 26% at Hp concentrations of 219µg/ml and 25.5µg/ml, respectively in 25 assays. The intra-assay CV was determined by calculation of the CV of 40 duplicate pairs for each immunoassay plate according to Fraser (1986) and had a median value of 7.3% and a range of 3.4-10.1%.

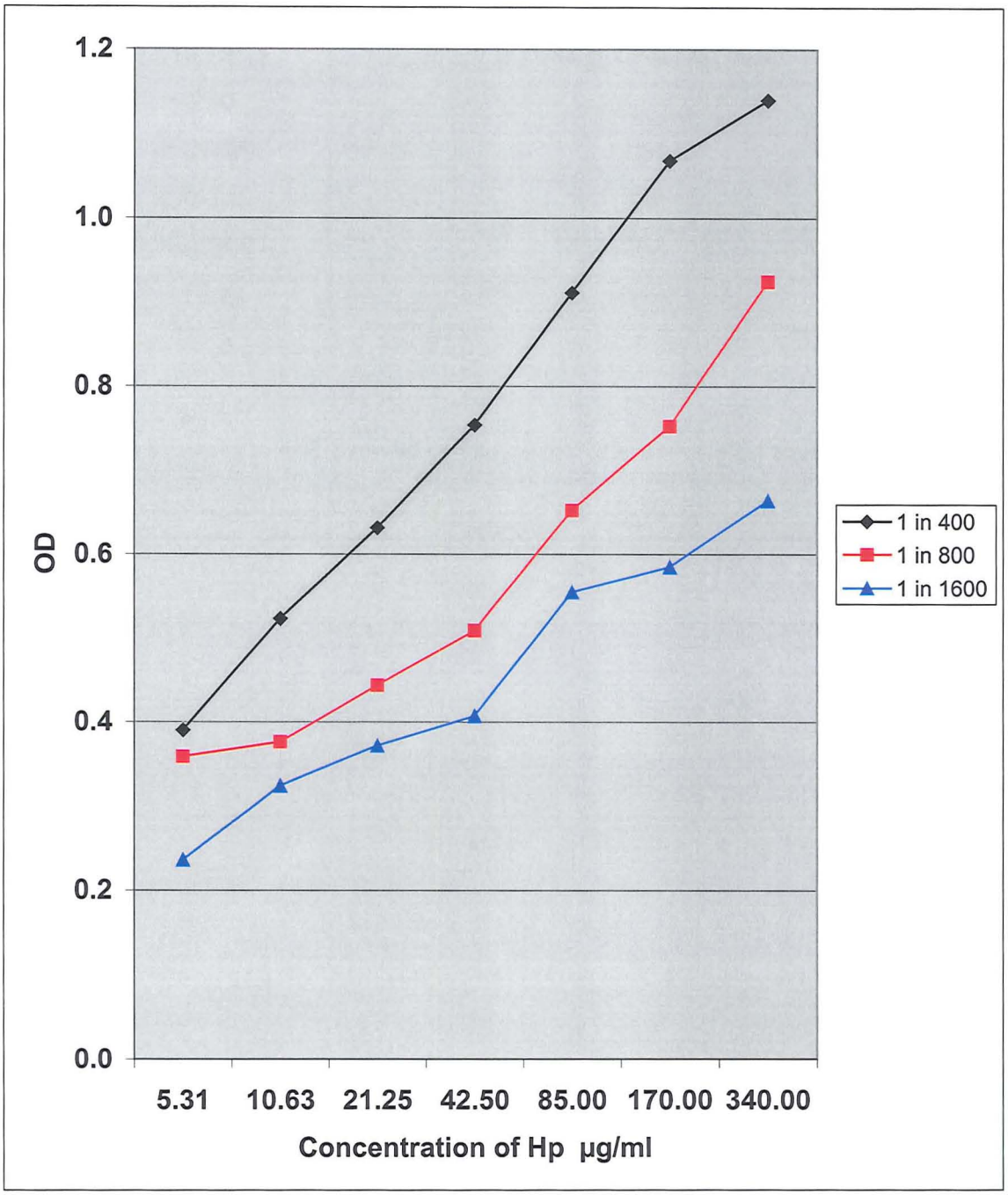


Figure 2-1: Optimisation of the ELISA for bovine Hp. The effect of the HRP conjugated antibody dilutions at 1:400; 1:800 and 1:1600 on the Hp standard curve. 1:400 dilution produced a steep standard curve and the highest optical density (1.595) of the 3 antibody dilutions

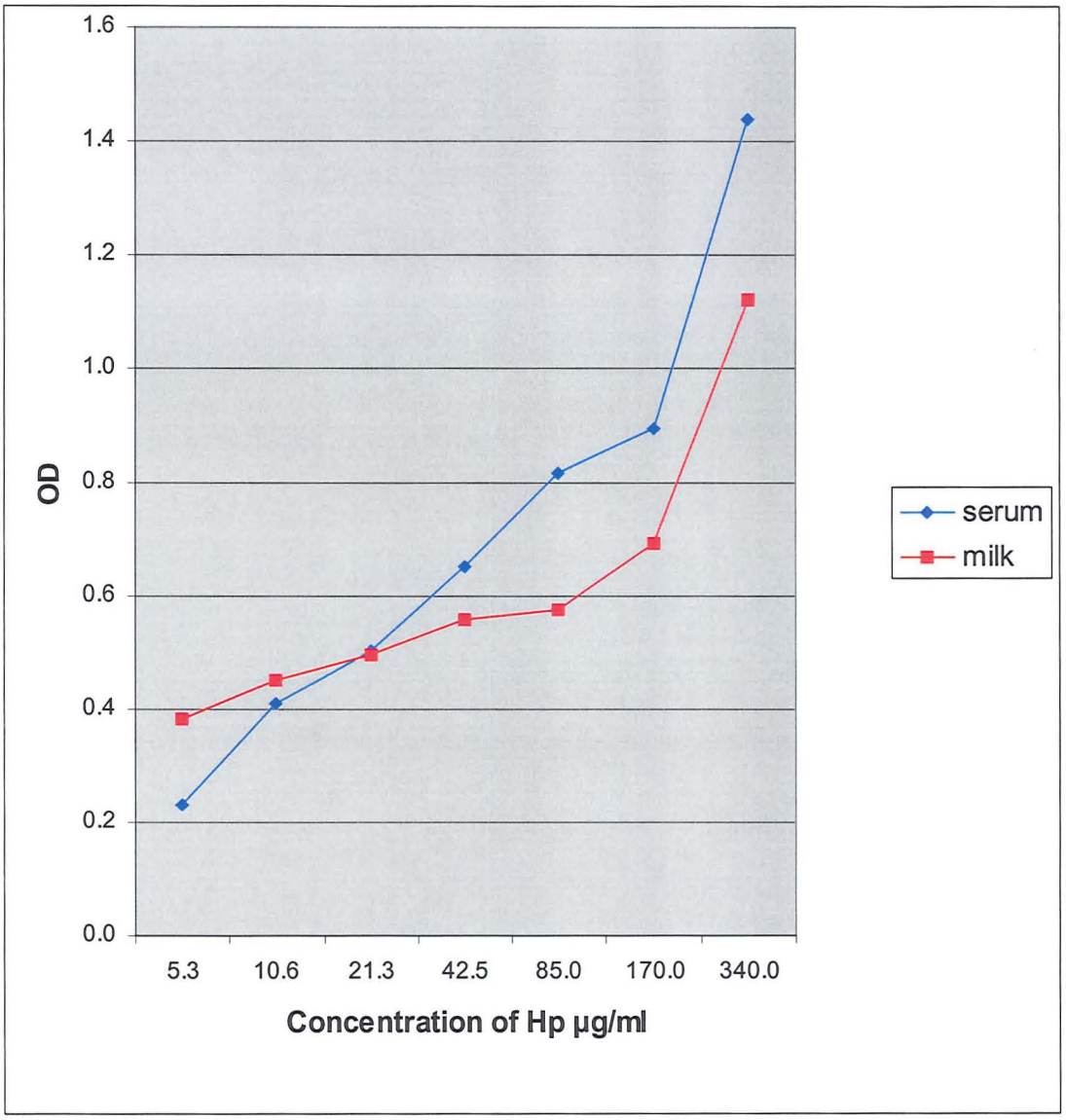


Figure 2-2: Serial dilutions of milk standard parallel serial dilutions of serum in preparation of standard curve

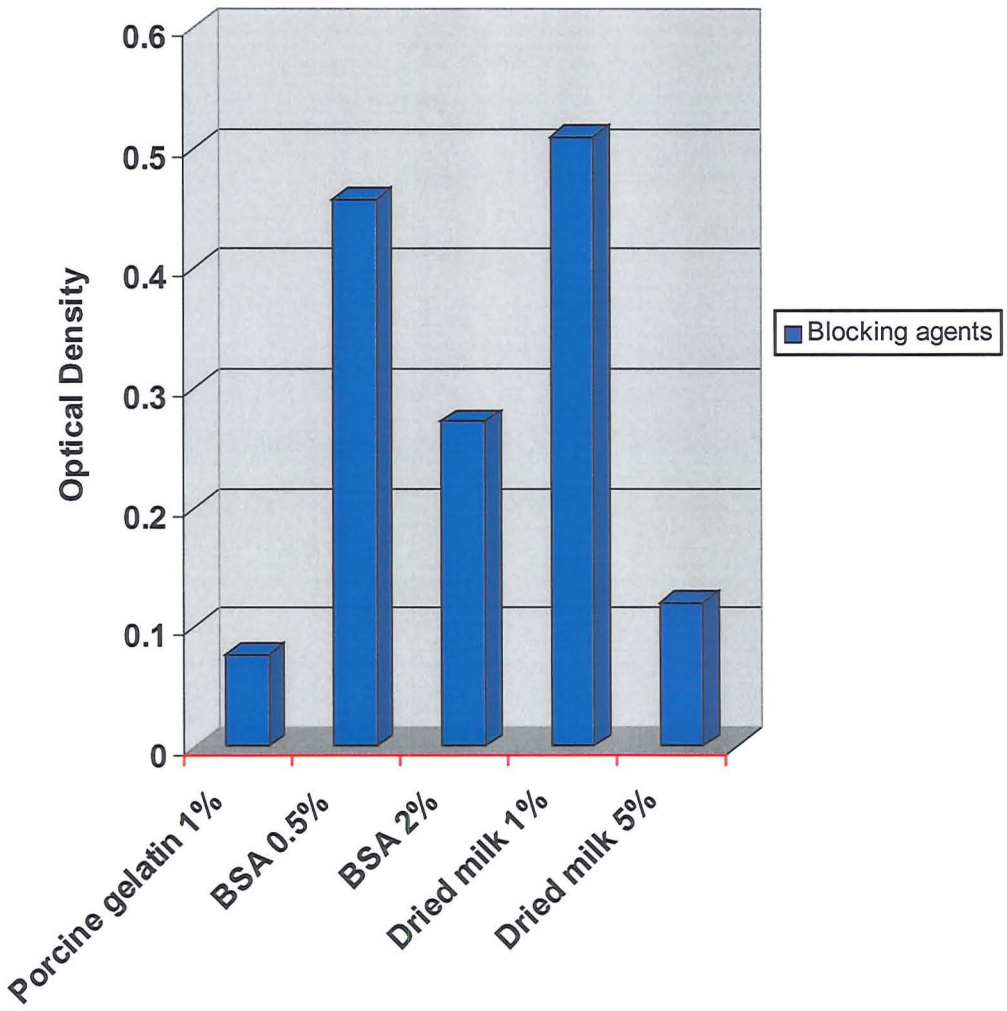


Figure 2-3: Effect of different blocking agents on the milk Haptoglobin standard curve

2.5.2.2 Accuracy

The accuracy of the assay was tested by diluting a Hp containing milk sample in 2 different analyte-free matrices (normal milk and buffer), to show that the results display parallelism (Figure 2-4)

2.5.2.3 The lower limit of detection

The minimum detection limit significantly different from the zero standard was determined as the Hp concentrations at 2 SD away from the mean (mean ± 2 x SD) of the zero standard resulting in a limit of detection for Hp in milk at a concentration of 1.2 μg/ml.

2.5.2.4 Comparison to previous method

The Hp concentrations of the bovine milk samples measured by both SRID and ELISA are displayed as a scatter graph in figure 2.5. A total of 288 milk samples were assayed by both SRID and ELISA, and Hp was detected in 39 and 109 samples respectively. The ELISA method was therefore shown to be 64% more efficient in detecting Hp in milk than SRID. This comparison give a correlation coeffiecient (r) of 0.69 which confirms a significant correlation between methods ($p<0.001$).

2.5.3 Milk Haptoglobin Concentration

The ELISA was used to measure Hp concentration in milk samples from both control and infected quarters from six animals during the acute and chronic phases of experimentally induced *S.aureus* mastitis. As this study was in collaboration with the Faculty of Veterinary Medicine, Uppsala Sweden, results from the other parameters measured (SAA in milk, Hp and SAA in serum, and SCC), including clinical observations of the animals, were provided by U. Gronlund (Uppsala, Sweden) and are included in the results section below, in order to aid interpretation of data at later stages in this chapter and in chapter three. The concentration of Hp in milk was determined in Glasgow as part of this thesis.

2.5.3.1 Milk and serum from infected quarters

The samples were taken from an experimental model that used antibiotic treatment to successfully transform acute clinical mastitis to chronic subclinical mastitis. Milk samples were analysed for bacteriology and SCC. In addition milk and serum samples were

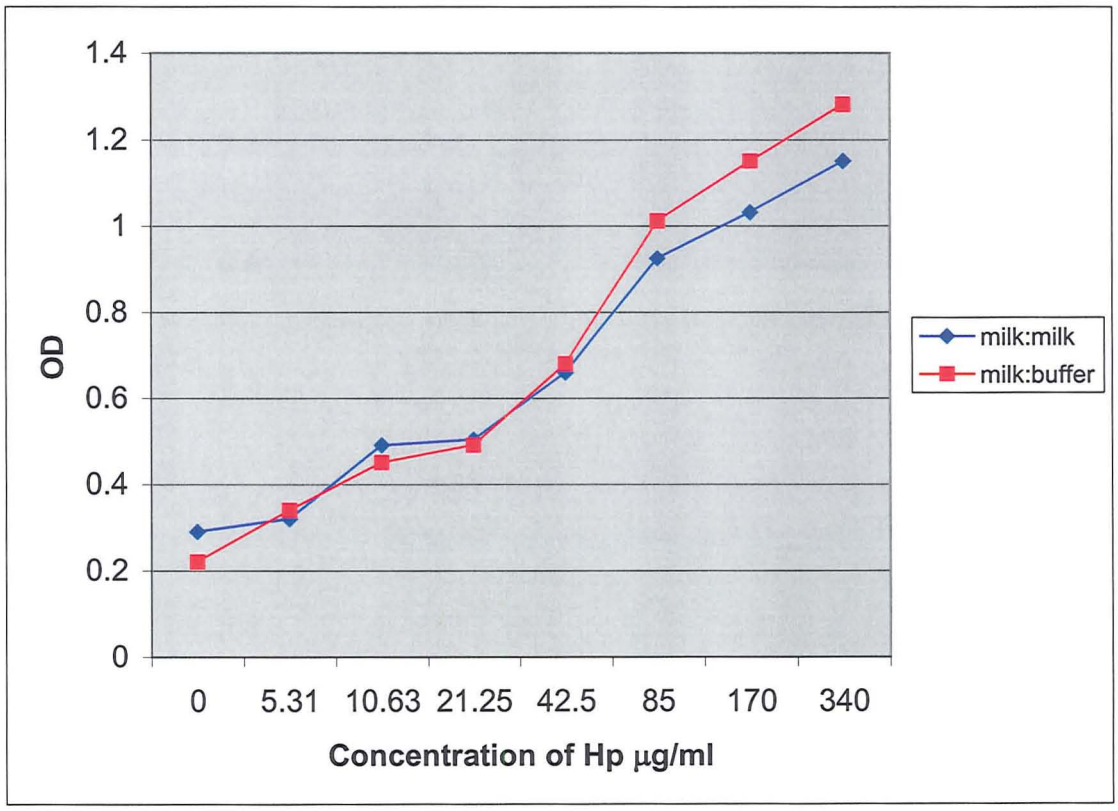


Figure 2-4: The accuracy of the ELISA was tested with a dilution experiment. The milk standard was diluted in a normal milk sample containing no haptoglobin (Hp) and also in buffer.

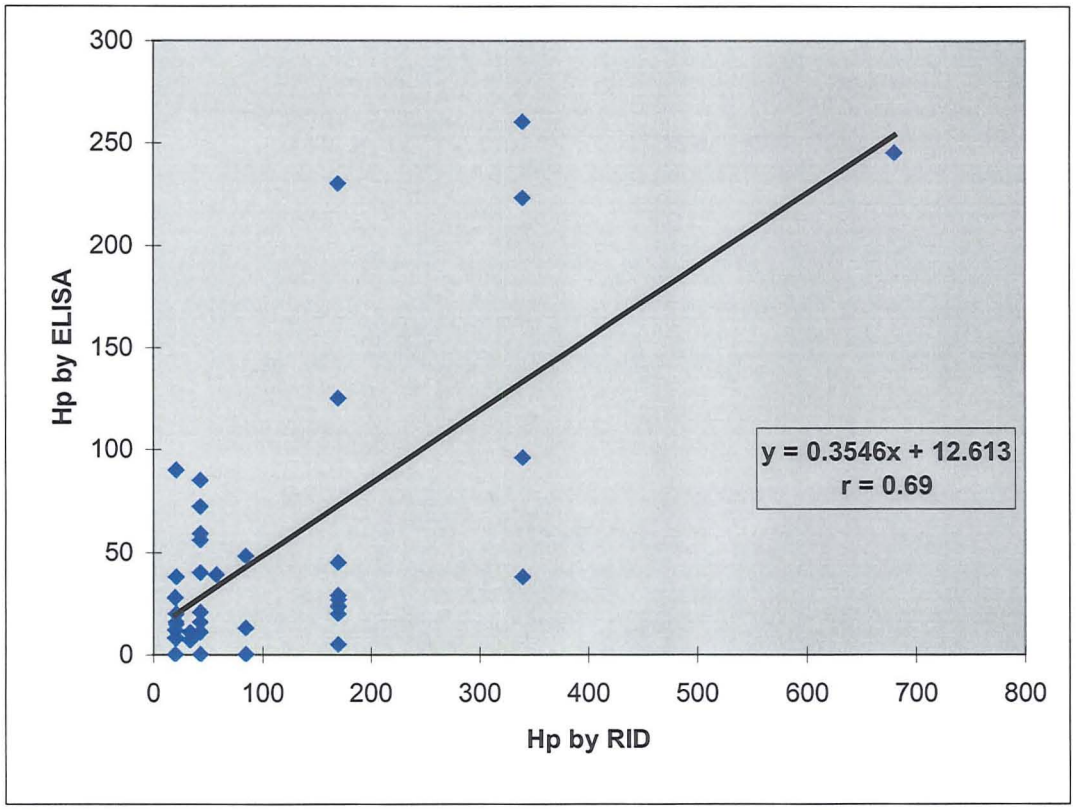


Figure 2-5: Correlation between parallel measurements of Hp in 108 milk samples using ELISA (y axis) and radial immunodiffusion (RID) (x axis).

analysed for the concentrations of Hp and SAA. In the acute phase, serum levels of Hp and SAA were higher than pre-infection and chronic phase levels (Figure 2.6). The highest concentrations of serum Hp and SAA were found at 72-80h p.i. and 54-104h p.i., respectively and ranged from 960 to 2190 mg/l for Hp, and from 487 to >1200 mg/l for SAA. Additionally, Hp and MAA were higher than both pre-infection and control samples in milk (Figure 2.7) during the acute phase.

In milk from udder quarters inoculated with *S. aureus* (n= 6 cows), Hp was detectable in a 100% of the samples at 24h p.i.. Maximal values of Hp and SAA were found in the milk samples during the acute phase of infection at 54-80 h p.i. and 48-120h p.i., respectively. They ranged from 52-323 mg/l for Hp and from 34 to 286 mg/l for SAA (Figure 2.7). Clinical signs including abnormalities in the milk and swelling was observed in all of the infected quarters during the acute phase of infection. Somatic cell counts in this phase ranged from 56 $x10^3$ to 22 $x10^6$ cells/ml. .

In the chronic phase all serum samples had supra-normal Hp concentrations while 77% had increased levels of SAA. Maximal chronic phase concentrations for Hp and SAA in serum ranged from 70 to 320 mg/l and 6.4 to 41.2 mg/l respectively (Figure 2.6), and were randomly distributed throughout the chronic phase. Sixty two percent of the infected milk samples had increased Hp during the chronic phase, 91% had increased concentrations of SAA. Maximal concentrations of milk Hp and SAA during the chronic phase ranged from 17 to 26 mg/l and 10.8 to 43 mg/l, respectively. Somatic cell counts during the chronic phase ranged from 607 $x10^3$ to 26 $x10^6$ cells/ml.

2.5.3.2 Milk from control quarters

Haptoglobin was detected by ELISA in only 6 out of 118 milk samples from control quarter samples, the concentration of which ranged from 8.3 to 63 μg/ml. Elevated SAA was found in 7 samples out of 123 samples and ranged from 1.8 to 20 μg/ml. Control udder quarters remained normal throughout the study, i.e, with no changes in the appearance of the milk or the udder. During the study their SCC ranged from 22 $x10^3$ to 445 x 10^3 cells/ml.

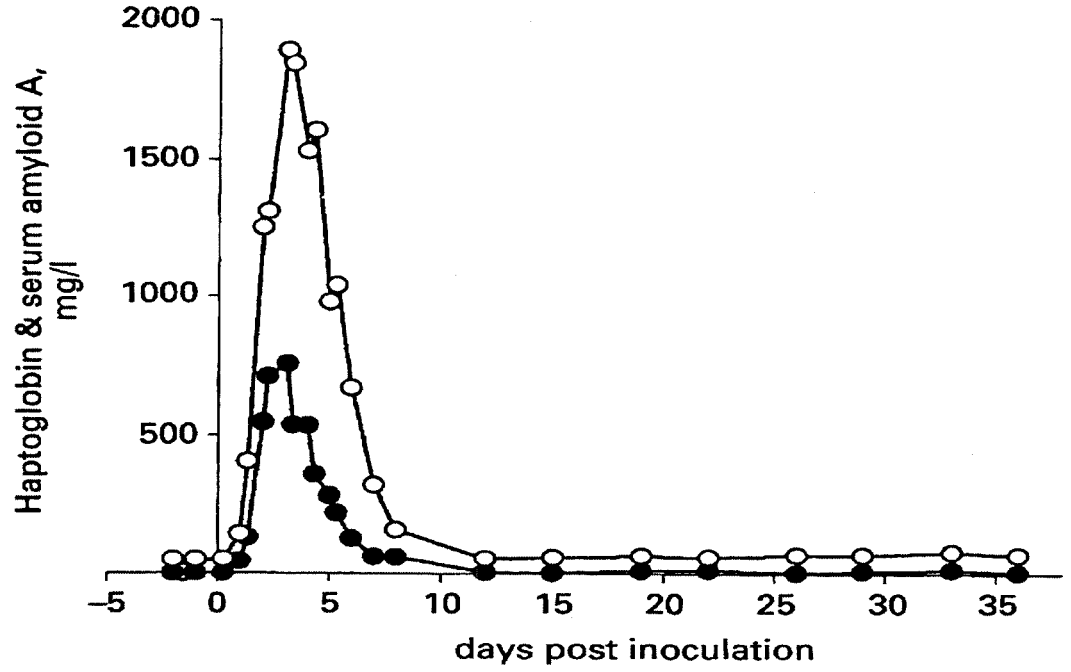


Figure 2-6: Median serum concentrations of haptoglobin (-○-) and serum amyloid A (-●-) At day 0 one udder quarter per cow was inoculated with *S.aureus* (n=6cows). Taken from (Gronlund et al 2003a).

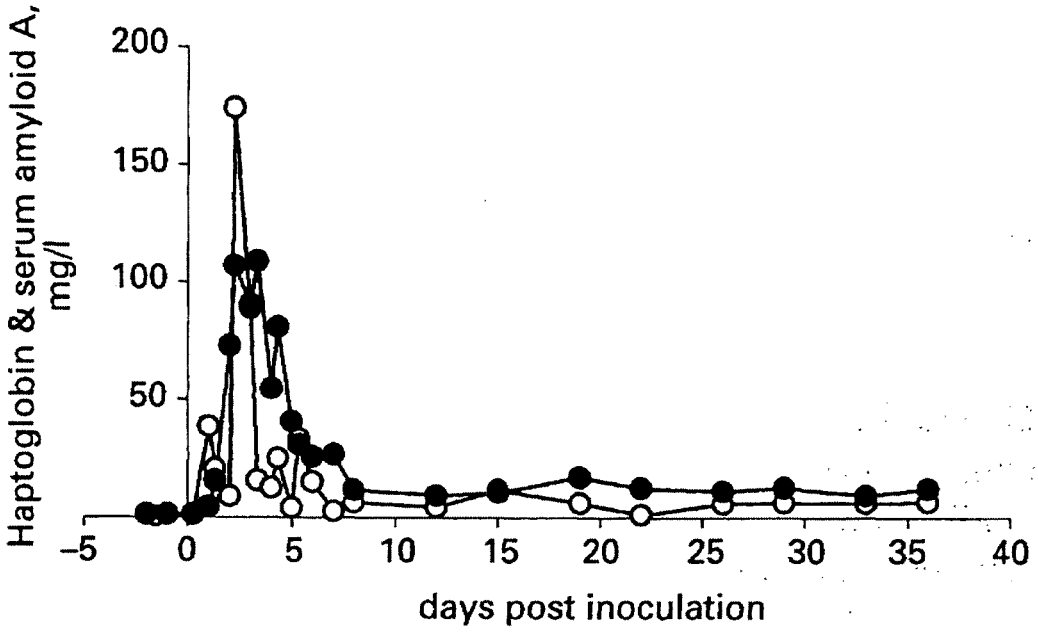


Figure 2-7: Median concentrations of haptoglobin (-○-) and serum amyloid A (-●-) in milk samples from udder quarters inoculated with *S.aureus* at day 0 (n=6cows). Taken from (Gronlund et al 2003a).

2.5.4 Discussion

Haptoglobin is the most widely studied APP in cattle (Gruys et al 1994; Eckersall 1995b; Schroedl et al 2001), and has been found to be increased in a number of disease situations (Makimura and Suzuki 1982; Conner et al 1986; Conner et al 1989; Hirvonen et al 1996; Horadagoda et al 1999). However the majority of studies reporting the potential of Hp in veterinary clinical biochemistry relate to its measurement in serum. Availability of convenient and reliable assays of differing formats, for measurement of serum Hp, have been the backbone of advances in this area. The method of choice for estimation of bovine serum Hp is a sensitive and robust, commercially available assay based on the peroxidase activity of the Hb-Hp complex, but unfortunately it cannot be used to measure Hp in milk due to a lack of sensitivity and interference from other substances in milk (Eckersall et al 1999a; Eckersall et al 2001).

The method reported in this chapter is the first ELISA to measure Hp in bovine milk. The ELISA method, based on the affinity of Hp with Hb, was developed and validated, and was used to evaluate Hp concentrations in milk during experimental mastitis infection. This in itself is a final stage of assay validation in that it is important that a method can be used to detect real pathophysiological changes.

Milk Hp has previously been determined by SRID, however this method is time consuming, lacks sensitivity and can have substantial interassay variation, due to the need for manual measurement of the diameter of the precipitin rings. The speed and practicability of assay procedures are major criteria for the acceptance of an analyte for routine estimation in clinical practice. With regard to this, the ELISA described in this chapter for the detection of bovine milk Hp, holds distinct advantages to previously described methods. First Hp is measured in milk not serum, hence specificity is greatly increased in the diagnosis of milk-related diseases. Second, the method described is sensitive even at low concentrations of Hp, and has been optimised to produce a quick and reliable assay. Third, an ELISA format involves many washing steps and is therefore not affected by the opacity of milk. Finally, milk is a much easier fluid to obtain than serum. Unlike blood vessels, the mammary glands can be accessed with ease, even by unskilled personnel, so that obtaining a milk sample is less traumatic to a cow than obtaining a serum sample and, with a routine diagnostic test in mind, that is extremely important particularly when dealing with lactating dairy cows.

During development of the final assay protocol (section 2.4.4) several stages had to be optimised. Investigations relating to the optimisation of the antibody found that a dilution of 1:400 gave a good result, with a high optical density for maximum values of Hp, but still reasonably low background, giving rise to a steep standard curve (Figure 2.1). Initially serum was considered as a possible standard, so during optimisation both serum and milk were diluted serially in order to determine the effects of these different matrices on the standard curve. Figure 2.2 shows that the slope of the curves for serum and milk demonstrate parallelism, indicating that Hp is being detected in both. It was possible for either serum or milk to be used as the source of standard material in our assay. Milk was chosen in order to eliminate the occurrence of any matrix effect that may be present in serum, although our results show that this is unlikely. In order to optimise assay performance it is essential to prevent non-specific binding. This was achieved by blocking with 5% dried milk powder (Figure 2.3).

Assay validation revealed that the performance of the assay was within acceptable limits for ELISA with an intra-assay CV% of 7.3% and interassay CV% of 9.8% and 26%, the latter was rather high but was at high milk Hp concentration and samples with such high levels were generally diluted to read off a more precise section of the curve. The preferred method for determining the analytical accuracy of an assay is by spiking the test sample with a known amount of a pure homogenous form of the analyte and measuring the recovery. However, as purified bovine Hp was not available, in this experiment the accuracy of the assay was assessed through a dilution experiment. Traditionally new assay studies include a correlation study against an alternative method, quoting the Pearson product moment correlation coefficient, r, as an index of the degree of agreement. The regression analysis (Figure 2.5) gives a correlation coefficient, which is highly significant although there is an obvious bias between the two methods. The significance of the r value can therefore be misleading, in that it does not identify the strength of the relationship between the two parameters, but merely that they are measuring the same substance. It is possible that a strong agreement was not observed between the ELISA and the SRID method because there is already a degree of imprecision associated with the latter. Additionally the slope, intercept and regression coefficient are all heavily influenced by extreme values such as that recorded at 680μg/ml for SRID. Previous studies have used immunoprecipitation in agarose gels to measure bovine Hp but this method gives, at best, semi-quantitative results, is time consuming and requires large volumes of antiserum (Eckersall et al 1989). This, in addition to the high detection limit of SRID, concluded the

ELISA to be a more sensitive and convenient method for the detection of milk Hp in this study.

Clinical validation of the assay showed milk Hp is a prominent marker of acute clinical *S .aureus* mastitis. Moreover, the increase of Hp in milk was specific for the infected udder quarters because levels in the control quarters were unaffected or, on a few occasions only slightly raised. The *S. aureus* mastitis model from which the milk samples were obtained for use in this study was successfully established. All infected quarters developed acute clinical mastitis, and most of them later progressed to chronic subclinical mastitis. The model was therefore suitable for the longitudinal studies of acute and chronic inflammatory responses in the mammary gland. Pre-infection levels of Hp in milk were below the detection limit of the assay, in agreement with the result of previous findings (Eckersall et al 2001). During acute mastitis Hp levels increased rapidly in milk from infected quarters and results from SAA in milk and both Hp and SAA in serum report similar kinetic profiles. The start of the increase coincided with the onset of clinical signs of acute mastitis and the increase in SCC. This study has identified potential for milk Hp as a marker of mastitis. Further studies are needed including investigation of APR during mastitis induced by pathogens other than *S. aureus* and also during naturally occurring mastitis to eliminate any artefacts introduced by the experimental model.

One drawback of the milk Hp ELISA is in the quantification of severely mastitic milk samples with clots in milk causing problems with pipetting, and solubility of the sample. Centrifugation is not advisable, as it is not yet known in what fraction of the milk Hp is present. However, although the method showed some variability, particularly whilst measuring high levels of Hp, the results measured by the developed ELISA still gave an accurate reflection of the clinical situation and were backed up by SCC, SAA and bacteriology, and could aid the diagnosis of clinical mastitis.

The assay for bovine milk Hp described in this study was further developed and commercialised by Tridelta Development Ltd, Dublin, Ireland (http://www.trideltaltd.com/).

The results from this study conclude that both Hp and SAA are sensitive markers of acute clinical mastitis but only SAA is indicative of chronic sub-clinical mastitis. Haptoglobin reached higher concentrations in the milk during the acute phase than SAA, but it is not yet clear if one particular protein is better at detecting mastitis than the other. Further study is required to assess the potential of these APPs as biomarkers of mastitis both together and

individually. Additionally the influence of the experimental model on the kinetics of milk APPs should be addressed by determining the concentration of milk APPs across a time course of naturally occurring mastitis.

Chapter III

Acute Phase Proteins in Milk in Health and Mastitis

3.1 Introduction

3.1.1 Clinical Mastitis

Clinical mastitis is characterised by abnormal milk of varying degrees, depending on the severity of mammary gland inflammation (Figure 3.1a and 3.1b) (Sears et al 1993; Cullor 1996). Signs of inflammation include heat, pain, redness, swelling (Figures 3.2a and 3.2b) and loss of function of the mammary gland (O'Rourke 1992). Clinical mastitis can be further classified as peracute, acute, subacute or chronic (Cullor 1996) depending on the additional signs. Peracute mastitis is categorised by a marked systemic reaction which can include the clinical signs of depression, anorexia, pyrexia and recumbency (Cullor 1996). Clinical signs alone are considered to be insufficient to differentiate between infections caused by Gram positive and Gram negative bacteria with certainty, although recent work indicates that some clinical signs are more likely to be associated with certain types of pathogens than others (Milne et al 2002).

3.1.2 Subclinical Mastitis

Subclinical mastitis occurs when the mammary gland is infected and the number of leucocytes is increased, but where there is no visible abnormality of the milk and there are no clinically detectable signs of inflammation in the mammary gland (Philpot 1984; Sears et al 1993; Cullor et al 1996). Subclinical infections are therefore, difficult to detect and repeated or persistent bouts may lead to fibrosis of the mammary tissue, resulting in a reduction in milk production (Cullor et al 1996).

3.1.3 Mastitis Pathogens

Many different pathogenic bacteria can infect the mammary gland and there are considerable differences in epidemiological and predisposing factors with different types of infection (Dodd 1970). 'Contagious mastitis' and 'environmental mastitis' are labels

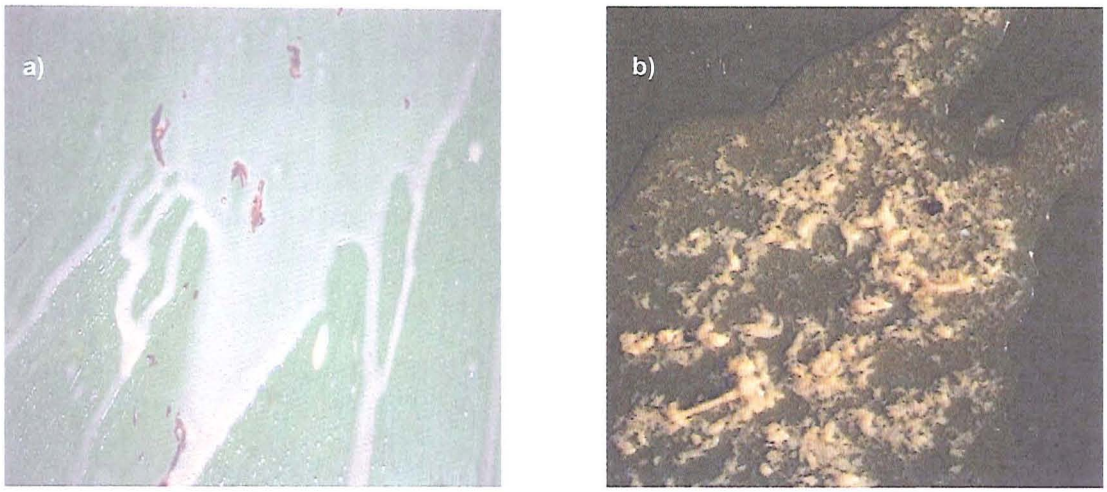


Figure 3-1: Example of the clinical abnormalities of milk resulting from mastitis The milk in picture a) has a slightly pink tinge and also contains true blood clots. The milk shown in picture b) demonstrates the total absence of normal milk, and shows a secretion consisting of thick clots suspended in a clear serous fluid.

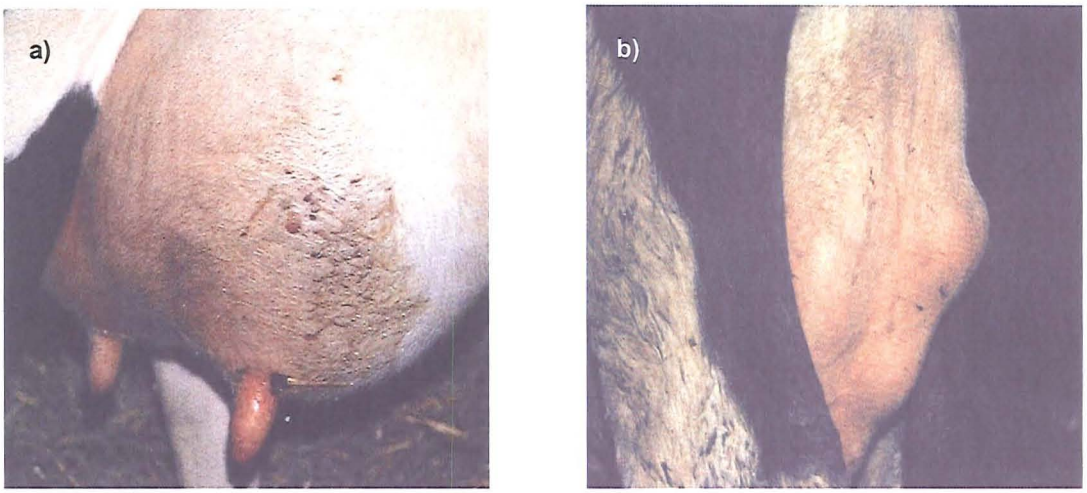


Figure 3-2: Example of the clinical abnormalities of the udder associated with mastitis. a) an enlarged, hard, hot and painful quarter can occur in some cases of clinical mastitis. b) chronic abscessation of mammary tissue

used to classify the epidemiology of pathogens that cause intramammary infections in dairy cows (Bramley and Dodd 1984; Smith and Hogan 1993). Contagious mastitis is transmitted from cow to cow during milking, whereas exposure to environmental pathogens can occur at any time during a cow's life either during the lactating or non-lactating period (Fox and Gay 1993; Smith and Hogan 1993).

3.1.3.1 Contagious Pathogens

Contagious mastitis is caused by organisms that colonise the mammary gland and can be spread by the milking procedure, contaminated machinery, the hands of the milker and nursing calves (Cullor et al 1996). The main contagious pathogens are *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysgalactiae* (Bramley 1984).

Staphylococcus aureus is a gram-positive, aerobic, pathogen which can be found on the skin of the udder, on the teats, and in the intramammary tissue (Cullor et al 1996). Most infections due to *S. aureus* are chronic subclinical infections, although occasional clinical cases of mild to moderate severity do occur (Cullor et al 1996). Such intramammary infections tend to be of long duration (Bramley 1984). *Staphylococcus aureus* is difficult to eliminate, it can survive for extended periods of time on the skin (McDonald 1977) or within neutrophils in the gland, where it is protected from antibiotics (Sandholm et al 1990). *Staphylococcus aureus* is contagious and spreads easily within herds (Fox and Gay 1993). When a number of cows in a herd are infected, bulk milk somatic cell count (BMSCC) increases and legal limits for BMSCC may be violated, or thresholds for premium bonus may not be met (Dekkers et al 1996; Adkinson et al 2001). Hence the control of *S. aureus* mastitis is both necessary and important.

Streptococcus agalactiae is a highly contagious pathogen of the bovine mammary gland (Cullor et al 1996). The primary site of colonisation is in the udder tissue, but teat lesions and teat ducts can also be colonised. *Streptococcus agalactiae* infection can be eradicated from a herd due to the micro-organisms continued sensitivity to treatment with penicillin during lactation (Philpot 1984).

Streptococcus dysgalactiae is not as infectious as *S. agalactiae* (Cullor et al 1996). *Streptococcus dysgalactiae*, found naturally in the udder and skin teat lesions is frequently transmitted during the milking process (Blowey 1990), and often follows teat injury (Cullor et al 1996).

3.1.3.2 Environmental Pathogens

Environmental mastitis is caused by environmental pathogens that do not generally colonise the mammary gland, but can do so when contamination of the cow's environment, teats, udder or milking machine occurs, and the organisms gain access to the teat cistern (Rendos et al 1975). The primary reservoir of environmental pathogens is the dairy cow's environment, but in contrast to contagious mastitis, exposure is not limited to the milking process (Smith and Hogan 1993), and invasion of uninfected quarters by environmental pathogens can occur at any time during the life of a cow (Smith and Hogan 1993). Gram-negative enterobacteriaceae and environmental streptococci, i.e. coliforms and *Streptococcus uberis* are the primary environmental pathogens (Smith et al 1985).

The gram-negative coliform, *Escherichia coli,* is a natural inhabitant of the large intestine and is found surviving in faecal matter (Blowey 1990). *Escherichia coli* can induce severe peracute infection which can occasionally lead to death of the cow (Cullor et al 1996), however, acute mastitis characterised by hard swollen quarters with brief periods of pyrexia and anorexia, is more commonly induced by this micro-organism (Eberhart 1984).

Streptococcus uberis (S. uberis) is a widely occurring causative agent of mastitis in modern dairy herds. It is responsible for the majority of clinical and subclinical cases of mastitis in the UK (Hillerton et al 1993). *Streptococcus uberis* is found naturally on mucous membranes, skin and in faeces (Blowey 1990), and generally causes less severe infection than *E. coli. Streptococcus uberis* is considered to be an opportunistic pathogen that can survive and multiply in extramammary sites (Sandholm et al 1990), including lips, haircoat, tonsils and the rectum of cows (Bramley et al 1979). *Streptococcus uberis* multiplies in most bedding material but especially in straw (Ward et al 2002). *Streptococcus uberis* is difficult to control due to its ubiquitous nature and a lack of knowledge of the pathogenesis of *S. uberis* infections (Leigh 1999). The prevalence of *S. uberis* infections is reported to be high during early and late lactation (Todhunter et al 1995), and there has been little reduction in the incidence of *S. uberis* mastitis over the past 30 years (Leigh 1999).

3.1.4 Detection of Mastitis

Due to the problems of using clinical signs alone for diagnosing mastitis, a number of other methods have been developed and tested for their ability to detect mastitis. Diagnostic tests are used to identify the nature and extent of a mastitis problem in a dairy herd, or to identify an individual animal for segregation, treatment or culling (Bramley 1992).

3.1.4.1 Somatic Cell Count

An increase in somatic cells in the milk gives a good indication as to the degree of inflammation present in the udder (Sears et al 1993)). Milk somatic cells consist of white blood cells and epithelial cells (Nickerson 1985) and the somatic cell count (SCC) is a measure of the number of these cells per ml of milk. Somatic cell counting is a practical and effective method to evaluate the mastitis status of a herd and gives a good indication of the prevalence of infection within a herd, since intramammary infection is the most important factor influencing the number of somatic cells in milk (Reneau 1986). Other factors which influence SCC include breed, age, stage of lactation and physiological stresses such as excitement, high temperatures and trauma (Philpot 1984). Somatic cell count is always elevated shortly after calving. The post-partum period has physiologically elevated foremilk SCC; the duration of which being reported with a range of between 5 and 35 days (Philpot 1984; Blowey and Edmondson 1995; Barkema et al 1999). An elevated SCC in early lactation may also be caused by the fact that a high percentage of udder quarters are infected at calving (Fox and Gay 1993; Todhunter et al 1995).

Somatic cell counting can be done at the quarter level when individual milk samples are collected from each quarter i.e. individual quarter somatic cell count (IQSCC), or at the cow level, when a composite sample of equal volumes of milk is collected from all quarters of the cow i.e. individual cow somatic cell count (ICSCC), or at the bulk tank level i.e. bulk milk somatic cell count (BMSCC). Bulk milk somatic cell count is used as a measure of bulk milk quality as it gives a good indication of the prevalence of infection within a herd (Logue 1997; Booth 1997). Financial penalties for high BMSCC act as an incentive for farmers to control mastitis and are a useful means to assess progress of control programmes (Booth 1981). There is currently no widely accepted definition of a low BMSCC or ICSCC. Published studies have used SCC of less than 250 $x10^3$/ml (de Haas et al 2002) 150 $x10^3$/ml (Barkema et al 1999) and 100 $x10^3$/ml (Peeler et al 2000; Sargeant et al 2001) to define both low cell count herds and individual cows. However previous studies have indicated that ICSCC masks the impact of IQSCC.

3.1.4.2 California Mastitis Test

The California Mastitis Test (CMT) is a qualitative method of estimating SCC. It is based on an anionic detergent containing bromcresol-violet as an indicator reacting with the nuclear content of the somatic cells of milk when mixed to form a viscous gel (Bramley, 1992). The viscosity of the gel allows the SCC of the milk sample to be estimated. Although not very sensitive or specific, the CMT is a cheap, practical and useful on-farm test to identify individual cows with subclinical mastitis (Cullor et al 1996).

3.1.4.3 Bacteriology

Bacteriology can be performed at the bulk tank, individual cow and individual quarter levels. Bulk milk bacteriological examinations have a high specificity but low sensitivity as a test for mastitis, with the result that some infected herds may not be detected on bulk milk samples (Sears et al 1993).

An infected quarter is defined as one producing milk from which bacteria are recovered in pure culture (Newbould 1984). Reliance solely on isolation of bacteriological pathogens to identify mastitis however can give false negative results. This may occur due to variation in the shedding of bacteria, in certain cows and with certain pathogens (Sears et al 1993). Twenty five percent of *S. aureus* infected cows will have a single negative culture result, but with repeated sampling, sensitivity is increased to 94-98% (Sears et al 1993). Accuracy in identification of environmental pathogens by bacteriological culture is also poor. This is thought to be due to the low numbers of environmental pathogens in milk (Neave 1975), and the fact that contamination is likely to be due to the environmental reservoir of infection (Smith 1983).

3.1.4.4 Electrical Conductivity

Increases in the electrical conductivity (EC) of milk are frequently caused by intramammary infection and such changes have formed the basis of various mastitis detection instruments (Woolford et al 1998). Increased EC during mastitis is a result of increased levels of sodium and chloride ions in the milk as a consequence of damage to the epithelium of the udder (Wheelock et al 1966).

In studies using a commercially available instrument, Hillerton & Walton (1991), found subclinical *S. aureus* infections were effectively detected, but EC levels for samples from

subclinical *S. uberis* infections were indistinguishable from uninfected quarters. Using conductivity data for individual quarters, (Nielen et al 1995), found that not all incidences of intramammary infection could be detected before clinical signs occurred.

The conductivity of milk is not considered sufficiently sensitive or reliable for use routinely in automatically detecting both clinical and subclinical mastitis in dairy cows (Mottram 1997).

3.1.4.5 N-acetyl β-D-glucosaminidase (NAGase)

Enzymes are released as a result of tissue damage when the animal's immune responses are reacting to infection. N-acetyl β-D-glucosaminidase (NAGase) is a leukocyte-associated enzyme in milk, and a high concentration indicates a high SCC. The NAGase test can be automated and is therefore suited to high-throughput analysis of samples (Ball and Greer 1991). It is reputed to be the most accurate of the indirect tests, performing similarily to SCC in predicting the infection status of the quarter (Pyorala 1988).

3.1.4.6 Acute Phase Proteins

Two decades ago, scientists recognised the limitations of using standard parameters such as SCC and bacteriology for diagnosis of mastitis (Sandholm et al 1984). It was known that mastitis led to increased permeability of the blood-mammary barrier, so measuring the concentration of blood proteins that leak from the intravascular compartment and into the milk was potentially a new method for detecting mastitis. Bovine serum albumin and α_1-antitrypsin were both considered as potential markers of mastitis, however, limitations in the available methodology for detection at this time led to the measurement of α_1-antitrypsin alone, and this did show a good correlation with SCC (Sandholm et al 1984). C-reactive protein (CRP) has also been reported in the milk of cows with mastitis (Hamann et al 1997).

Concurrent research, and field studies in bovine APPs indicated that measurement of Hp and SAA was likely to be more successful in the detection of mastitis, as these proteins were known to increase in serum over 100 times during the APR, whilst α_1-antitrypsin increases by only two to three fold (Conner et al 1988b), while CRP is not generally recognised as an acute phase reactant in cattle (Eckersall and Conner 1988a).

Recently retrospective analysis of 29 milk samples from cows with naturally occurring clinical mastitis, reported the presence of APPs in milk, including SAA and Hp at concentrations of 0.05 to 0.55 μg/ml and 0.02 to 2 mg/ml respectively (Eckersall et al 2001). In this study the diagnostic values of APPs in differentiating between healthy cows and those with mastitis gave sensitivities and specificities of 86% and 100% respectively for milk Hp and 93% and 100% respectively for milk SAA. Both Hp and SAA also showed correlation with the severity of clinical signs of mastitis. The cases of mastitis were classified as 'mild' if there were clots visible in the milk, and 'moderate' if the clots in the milk were accompanied by changes in the udder including heat, pain, redness, and swelling. In this study, milk SAA was able to distinguish between cows with mild and moderate mastitis, whereas Hp was not. The ability to differentiate the severity of mastitis is very important, as it is likely to influence the therapeutic decisions taken by the farmer or veterinarian. The authors of the study suggested the possibility that a difference in the source of the two proteins could be the reason for this and further speculated on the possibility of local synthesis of SAA within the mammary gland itself. In the same year a mammary-associated form of SAA (M-SAA3)[1], likely to be produced within the mammary gland itself, was described in milk from clinical cases of bovine mastitis and in bovine, equine and ovine colostrum (McDonald et al 2001). Following from the initial reports of SAA and Hp in mastitic milk, the use of APPs in the diagnosis of mastitis was quickly recognised and there have been a number of studies established to investigate further the role of APPs, in both naturally occurring and experimentally induced clinical and subclinical mastitis (Sorensen et al 2002; Winter and Colditz 2002; Pedersen et al 2003; Nguyen et al 2003; Jacobsen et al 2003; Gronlund et al 2003a; Gronlund et al 2003b).

The milk samples used in the clinical validation of the milk Hp ELISA described in chapter 2, were from an experimentally induced *S. aureus* model of mastitis, designed to determine the concentration of M-SAA3 and Hp in the milk and serum of cows with acute clinical and chronic subclinical mastitis (Gronlund et al 2003a). Maximal values of Hp and M-SAA3 in milk were reported 54-80h post infection (p.i.) and 48-120h p.i., respectively. These ranged from 52-323μg/ml for Hp and from 34-286 μg/ml for M-SAA3. Interestingly, maximum concentrations of Hp and M-SAA3, measured during the acute phase in serum, peaked later and for a shorter period of time (72-80 hours pi and 54-104 hours pi, respectively) than in milk. An important part of this study was the controlled use of antibiotics, to transform an acute inflammation into a chronic phase. Maximum

[1] From this point onwards SAA secreted in milk will be referred to as M-SAA3

concentrations of milk Hp and M-SAA3 during the chronic phase were 17μg/ml to 26μg/ml and 10 μg/ml to 43 μg/ml, respectively. In the chronic phase, Hp concentrations in infected quarters were not significantly different from either pre-infection or control quarters, however, M-SAA3 concentrations were significantly different, indicating the potential for milk M-SAA3 analyses in the diagnosis of chronic subclinical *S. aureus* mastitis.

Pedersen et al, (2003), used an experimental model of acute clinical mastitis to elucidate the pathogenesis and the kinetics of Hp and M-SAA3 in milk within the first 12h of intramammary inoculation with *S. uberis*. Mammary associated serum amyloid A in infected quarters began to rise 6h pi, with maximal values recorded 12h after inoculation. The range of M-SAA3 concentrations in milk was 0.47 to 4.6 μg/ml. Haptoglobin ranged from 11 to 92 μg/ml, but no increase was seen until 10h pi, while peak concentrations were found at 12h pi. Haptoglobin was detected only in milk and not in serum, whereas M-SAA3 was detected in both milk and serum. Maximal clinical response, based on measurement of rectal temperature, and heat and oedema of the udder, was reached 11 hours pi. In infected quarters SCC peaked at 8h pi., and a minor initial rise was also observed in the control quarters. The authors concluded that milk M-SAA3 was an appropriate marker for the early detection of mastitis, as the increase in milk M-SAA3 levels at 6h preceded that of SCC (7h) and the development of clinical signs (8-9h).

Gronlund et al (2003b) performed a field study to evaluate the potential for Hp and M-SAA3 as indicators of naturally occurring subclinical mastitis. One hundred and eighty quarter milk samples from cows with subclinical mastitis were assayed for Hp and M-SAA3, using ELISA with detection limits of 0.5 and 0.6 μg/ml, respectively. In 116 samples at least one of the APPs were detected, where SAA ranged from 0.8-151 μg/ml, and Hp concentration ranged from 0.53-358 μg/ml. Five control animals with SCC of <100,000 cells/ml were used in this study, and Hp and SAA were not detected in the milk from any of these animals.

The studies outlined above conclude that the measurement of APPs in bovine milk has good accuracy for the diagnosis of mastitis, thus it was considered appropriate to further characterise the milk APR in mastitis, to establish a time course of APR changes during an infection, and to determine the association between APP in milk and conventional markers of mastitis such as SCC.

3.2 Materials and Methods

3.2.1 Farm Selection

Two commercial dairy farms in the west of Scotland were used in this study; Farm A had a rolling monthly geometric mean BTSCC of 140,000 cells/ml and an annual incidence of clinical mastitis of 17 cases per 100 cows, and farm B had a rolling monthly geometric mean BTSCC of 40,000 cells/ml and an annual incidence of clinical mastitis of 7 cases per 100 cows.

3.2.2 Cow Selection

3.2.2.1 Clinical

Initially the cows were selected on the basis of calving date, with daily sampling beginning at the first milking post-calving, as the incidence of mastitis is higher in early lactation. If the selected cow showed no sign of mastitis within the first four weeks post-calving, then sampling was discontinued and another cow was recruited into the study. Recruited cows that did not complete the study were used as control animals in order to compare parameters with mastitic cows. Forty-seven cows were followed post-calving.

3.2.2.2 Subclinical

An additional 4 cows were recruited to the study mid-lactation, and were identified as having subclinical mastitis by IQSCC of > 60,000 cells/ml and a positive CMT score. Three of the 4 cows were identified as having subclinical mastitis in 2 quarters, accounting for the 7 cases described in the results (Section 3.3.3). Milk samples were taken from the 4 cows for a period of up to 17 days.

3.2.3 Milk Sample Collection

Milk samples were collected into sterile plastic containers daily from all four quarters of each cow, 5 days per week, using an aseptic technique. In order to minimize the risk of contamination of milk samples fastidious attention was paid to teat preparation, as described by Bramley (1992) and Cullor *et al* (1996). The udders and teats were washed, dried and prepared, as normally performed prior to milking, and the teat ends were swabbed with 70% alcohol, prior to sample collection. The furthest away teats were

cleaned first and the nearest teats were cleaned last with the samples being collected in the opposite order, to reduce the chance of contamination by the milk collector. The first 4-6 squirts of milk were discarded to avoid contamination of the milk sample by bacteria from the teat canal. After preparation of the teats, clean disposable gloves were worn by the sampler. For collection of milk the container was held as close to horizontal as possible and the lid of the container was held face down in the same hand in order to minimise contamination. Approximately 20ml of milk sample was taken from each quarter, which was an adequate volume for bacteriological culture, APP assays and somatic cell counting. In between sampling cows, the sampler washed his/her hands with a germicidal solution and, used a new pair of clean disposable gloves for each cow. Samples were collected Monday to Friday.

3.2.4 California Mastitis Test

The California Mastitis Test (CMT) was performed on every cow immediately after the sterile sample was taken. The technique for CMT as described by Cullor *et al* (1996) and Philpot et al (1984) was followed. An equal volume of quarter milk and the CMT reagent were placed in each of the four wells of the CMT paddles. The paddles were rocked gently for a few seconds and observed for any gel formation. The viscosity of the gel was scored from 0-3 (Table 3.1). The CMT score for each quarter was noted on the daily record sheet.

3.2.5 Somatic Cell Count

In this study a threshold of 60,000 cells/ml or below was selected as an IQSCC that can be considered as a healthy quarter and free of mastitis pathogens. This threshold was chosen based on previously published studies described in detail in section 3.1.4.1

Milk samples for somatic cell counting were sent, by post to the Veterinary Science Division, Scottish Agricultural College (SAC), Ayrshire, Scotland, where SCCs were determined using a Fossamatic cell counter. Prior to despatch milk samples were preserved with a Lactab milk preservative tablet (Thomson and Capper Ltd, Runcorn, Cheshire, UK).

3.2.6 Acute Phase Protein Assays

The concentration of M-SAA3 in milk was determined using a commercially available ELISA, (Phase Serum Amyloid A, Tridelta Development Ltd, Ireland) according

CMT Score	Description of visible reaction	Relative Range of Somatic cells (cells/ml)
N (Negative)	Mixture remains liquid, homogeneous, with no evidence of thickening.	< 200,000
T (Trace)	The slight thickening that forms is seen best by tipping the paddle back and forth and observing the mixture as it flows over the bottom of the cup. Trace reactions tend to disappear with continued rotation of the paddle.	150,000 to 500,000
1 (weak positive)	A distinct thickening of the liquid forms, but there is no tendency toward a gel formation. With some milk, the thickening may disappear after prolonged rotation of the paddle	400,000 to 1,500,000
2 (distinct positive)	Mixture thickens immediately, and a gel formation is suggested. As the mixture is swirled, it tends to move in toward the center, exposing the bottom of the outer edge of the cup. When the motion is stopped, the mixture levels out and covers the bottom of the cup.	800,000 to 5,000,000
3 (strong positive)	A gel is formed, which causes the surface of the mixture to become elevated like a partially fried egg. There is usually a central peak that remains projecting above the main mass, even after the rotation of the paddle is stopped	generally > 5,000,000

Table 3-1: Interpretation and scoring of the CMT test and broad estimates of somatic cell levels that may be associated with each score. http://ianrpubs.unl.edu/dairy/g556.htm

to the manufacturer's instructions. The milk samples were diluted 1:50 in assay buffer. The newly developed ELISA based method (see section 2.3.3) was used to determine the concentration of Hp in the milk samples.

3.2.7 Bacteriology

The Department of Veterinary Pathology, at the University of Glasgow Veterinary School, carried out all bacteriological examination using standard laboratory techniques.

3.2.8 Clinical Examination

Examination of the milk and palpation of the udder was carried out if the cow gave a positive CMT, or showed any other signs of being abnormal. Any abnormalities in the milk (clots or blood) or udder (heat or swelling) were noted on the daily record sheet.

3.3 Results

3.3.1 Control Animals

A total of 47 cows (188 quarters) were followed from calving during this study, out of which 35 were classified as control cows based on results of SCC, CMT, bacteriology and the observation of lack of clinical signs. The profiles of SCC and M-SAA3 and SCC and Hp for the normal post-calving cows are shown in figures 3.3 and 3.4. The control cows presented with a raised SCC, M-SAA3 and Hp post-calving (911 $x10^3$ cells/ml, 2.6 μg/ml and 0.8 μg/ml respectively), all three of which fell rapidly between days 1-3 post-calving. By day 7 post-calving, M-SAA3 and Hp were undetectable in all quarters (<0.02μg/ml) and (<0.5μg/ml), respectively (Figures 3-3 and 3-4). The SCCs in figure 3.3 are different to those in 3.4, owing to the fact that these results are taken from a subset (n=9 cows) of the total number of control cows. Somatic cell counts had fallen to <60,000 cells/ml in all control animals by day 10 post-calving. Results for CMT were consistently negative throughout the duration of the sampling period and no bacteria were isolated in milk from any quarters of the control cows.

3.3.2 Cows with Clinical Mastitis

Of the 47 cows followed during this study 6 cows developed clinical mastitis with 5 cows having a single quarter affected and 1 cow having 2 quarters affected, caused through the invasion of the host udder by different pathogens including *S. uberis*, *E. coli* and *S. aureus*. Each of the 7 cases is described individually below, (with reference to figures), including details on clinical signs, levels of SCC, concentration of M-SAA3 and Hp, bacteriological results and duration of infection. There was considerable variation in the concentration of M-SAA3 and Hp measured, so note that it was not possible to maintain a consistent scale in the axes between cases.

Case 1 was followed from calving for a period of 28 days. The first episode of clinical mastitis was diagnosed on day 8 post-calving, by the presence of clots in the milk. Figures 3-5 and 3-6 show the results for SCC and for milk M-SAA3 and Hp. This cow had a high post-calving SCC on day 1 (6036 $x10^3$ cells/ml), which reached a peak at almost 13 $x10^6$ cells/ml on day 8, falling gradually for the next 6 days, and reaching normal levels by day 14. The SCC rose again from day 14 to day 16 with a secondary peak at 7 $x10^6$ cells/ml, then continued to decrease, again reaching normal levels by day 22. Another increase was recorded between days 22 and 23 where a final peak was observed (3165 $x10^3$/ml). Concentrations of M-SAA3, are compared to SCC in figure 3-5. Mammary-associated SAA3 was undetected until day 2 post-calving when the levels rose steadily to a peak concentration of 5.6 μg/ml on day 7, then fell gradually for the next 6 days but rose again on day 14 peaking on day 15 at 3 μg/ml, then continues to decrease between days 15 to 21 before showing a final increase to 1.4 μg/ml on day 22. Milk Hp was raised (108 μg/ml) from day 1 post-calving and peaked at 139 μg/ml on day 6 before falling to undetectable levels by day 13 (Figure 3-6). Haptoglobin then rose between days 13 and 15 and peaked at 87 μg/ml, and continued to decrease between days 15 and 21 before showing a final increase to 70 μg/ml on day 22. Bacteriological culture indicated *S. uberis* as the causative agent of the clinical mastitis.

Case 2 was followed from calving for a period of 56 days. Clinical mastitis was diagnosed on day 15 post-calving by the presence of clots in the milk. Figures 3-7 and 3-8 show the results for SCC and for milk M-SAA3 and Hp. This cow had a high post-calving SCC on day 2 (12184 $x10^3$/ml), which fell steadily until day 8 at which point an initial increase to 6305 $x10^3$/ml on day 9 was followed by a sharp increase to the maximum level for this case of 42750 $x10^3$/ml. Somatic cell count showed a steady decrease from day 14 reaching

normal levels by day 21 and remained at less than 60,000 cells/ml for the duration of the sampling period. Concentrations of M-SAA3, are compared to SCC in figure 3-7. Mammary associated SAA3 was raised (11.4 μg/ml) day 2 post-calving but concentrations fell steadily until day 8 where M-SAA3 begins to rise reaching 6.5 μg/ml on day 9. The concentration of M-SAA3 decreases within the next 24hrs but then rises from day 12 peaking on day 15 at 6.5 μg/ml. Milk amyloid A was undetectable by day 23 and for the remainder of the sampling period apart from a slight rise from days 43 to 50. Concentrations of Hp, are compared to SCC in figure 3-8. Milk Hp was undetectable on day 2 post-calving, however it was present in low concentrations (8-19 μg/ml) on days 5 to 8. Haptoglobin then showed an initial rise on day 8 to 48 μg/ml on day 9 followed by a sharp increase to maximum levels of 198 μg/ml on day 15. The concentration of Hp decreased steadily and was undetectable by day 23 and for the remainder of the sampling period apart from a slight rise at day 34 and from days 43 to 54. Bacteriological culture indicated *E. coli* as the causative agent of the clinical mastitis.

Case 3 was followed from calving for a period of 26 days. Clinical mastitis was diagnosed on day 22 post-calving by the presence of clots in the milk. Figures 3-9 and 3-10 show the results for SCC and for milk M-SAA3 and Hp. This cow had an elevated SCC on day 1 (85 $x10^3$/ml), which fell to normal levels by day 2 and apart from 3 slight rises shown at days 5, 11 and 16, SCC remained <60,000 cells/ml until day 22. Somatic cell count rose steadily from day 19 to a peak concentration of 7643 $x10^3$/ml on day 22, from which point it decreased steadily for the remainder of the sampling period. Concentrations of M-SAA3 are compared to SCC in figure 3-9. Mammary-associated SAA3 was detected (1 μg/ml) on day 1 post-calving, and remained present at very low concentrations (0.07 to 1.5 μg/ml) until day 19 when M-SAA3 concentration rose sharply reaching a peak on day 24 at 49 μg/ml. From day 22, the concentration of M-SAA3 in this cow decreases for the remainder of the sampling period. Concentrations of Hp are compared to SCC in figure 3-10. Haptoglobin was undetected in the milk on day 1 post-calving and remained so until day 22. On day 23, milk Hp concentration peaked at 11 μg/ml and then decreased for the remainder of the sampling period. Bacterialogical culture indicated *S. aureus* as the causative agent of the clinical mastitis.

Case 4 was followed from calving for a period of 26 days. Clinical mastitis was diagnosed on day 3 post-calving by the presence of clots in the milk. Figures 3-11 and 3-12 show the results for SCC and for milk M-SAA3 and Hp. This cow had an elevated SCC on day 1 (6629 $x10^3$/ml), which remained raised and fluctuating from 1500 $x10^3$/ml to 10000 $x10^3$/ml until day 11, at which point the SCC rose sharply and peaked on day 12 at 21554

$x10^3$/ml. Somatic cell count continued to fluctuate at elevated levels for the remainder of the sampling period. Concentrations of M-SAA3 are compared to SCC in figure 3-11. Mammary-associated SAA3 was increased (17.6 μg/ml) on day 1 post-calving and increased sharply in the next 24hrs reaching 45.6 μg/ml by day 2. The concentration of M-SAA3 then decreased slightly for the next 3 days before it rose to a maximum concentration of 82 μg/ml on day 8, after which M-SAA3 levels remained raised and fluctuating (19 μg/ml to 55 μg/ml) for the remainder of the sampling period. Concentrations of Hp are compared to SCC in figure 3-12. Haptoglobin was detected (7.7 μg/ml) on day 1 post-calving, and continued to rise reaching its first peak on day 5, with a concentration of 53 μg/ml. The levels of Hp then fell until day 9 when another rise was seen, which peaked at a concentration of 57 μg/ml on day 15. The concentration decreases, but is again increasing for the remainder of the sampling period with a milk Hp concentration of 83 μg/ml being detected on the final day of sampling. Bacteriological culture indicated *S. aureus* as the causative agent of the clinical mastitis.

Case 5 was followed from calving for a period of 56 days. Clinical mastitis was diagnosed on day 16 post-calving by the presence of clots in the milk. Figures 3-13 and 3-14 show the results for SCC and for milk M-SAA3 and Hp. This cow had an elevated SCC on day 2 (439 $x10^3$/ml), which remained moderately raised and fluctuating (130 $x10^3$/ml to 650 $x10^3$/ml) for the duration of the sampling period with the exception of sharp peaks on days 5 and 16 at 1460 $x10^3$/ml and 1185 $x10^3$/ml respectively. Concentrations of M-SAA3 are compared to SCC in figure 3-13. Mammary-associated SAA3 was detected (1.4 μg/ml) on day 2 post-calving and showed an initial peak at 2.5 μg/ml on day 9, followed by a decrease to day 13, at which point the concentration of M-SAA3 rose to 2.3 μg/ml on day 16, from which point the concentration of M-SAA3 in milk decreased gradually and was undetectable by day 29, but rose again to 2 μg/ml on day 47, but fell to undetectable levels for the final 2 days of the sampling period. Concentrations of Hp are compared to SCC in figure 3-14. Haptoglobin was detected (14 μg/ml) on day 2 post-calving, but fell to undetectable levels by day 7 before rising to give the first of 3 prolonged periods (days 9 to 20; 23 to 34 and 41 to 54) where the concentration of Hp was moderately elevated to levels of 13 to 24 μg/ml; 11 to 23 μg/ml and 12 to 29 μg/ml respectively. Bacteriological culture indicated *S. aureus* as the causative agent of the clinical mastitis.

Case 6 was followed from calving for a period of up to 26 days. Clinical mastitis was diagnosed on day 4 post-calving by the presence of clots in the milk, and a hot and swollen quarter. Figures 3-15 and 3-16 show the results for SCC and for milk M-SAA3 and Hp. This cow had a high SCC on day 3 post-calving (6822 $x10^3$/ml), which increased almost

five fold in the next 24hrs to reach 33699 x10^3/ml by day 4, from which point SCC decreased, but remained elevated above normal levels for the remainder of the sampling period. Concentrations of M-SAA3 are compared to SCC in figure 3-15. Mammary-associated SAA3 was high (42.1 μg/ml) on day 3 post-calving, and rose sharply in the next 24hrs to reach a peak concentration of 255 μg/ml on day 4 post-calving. The concentration of M-SAA3 falls sharply for the next 5 days, but was still detectable in the milk from day 9 for the remainder of the sampling period at concentrations ranging from 0.1 μg/ml to 1.5 μg/ml. Concentrations of Hp are compared to SCC in figure 3-16. Haptoglobin was high (24.4 μg/ml) on day 3 post-calving, but almost a seven-fold increase was recorded over the next 24hrs, and Hp peaked at 161 μg/ml on day 4 post-calving. The concentration of Hp in milk decreased steadily, and was undetected for the remainder of the sampling period from day 15 post-calving. Bacteriological culture indicated *E. coli* as the causative agent of the clinical mastitis.

Case 7 was followed from calving for a period of up to 26 days. Clinical mastitis was diagnosed on day 5 post-calving by the presence of clots in the milk. Figures 3-17 and 3-18 show the results for SCC and for the milk M-SAA3 and Hp. This cow had a high SCC on day 1 post-calving (37174 x10^3/ml), which had fallen sharply by day 2 post-calving (5313 x10^3/ml) but rose again to peak at 12385 x10^3/ml on day 5 post-calving. Somatic cell counts in this cow are elevated for the remainder of the sampling period and range from 365 x10^3/ml to 3921 x10^3/ml. Concentrations of M-SAA3 are compared to SCC in figure 3-17. Mammary-associated SAA3 was high (285μg/ml) on day 1 post-calving, but fell sharply over the next 3 days, before rising to a peak concentration of 68μg/ml on day 5. The concentration of M-SAA3 fell between days 5-10, but remained detected from day 10 onwards at concentrations ranging from 4μg/ml to 32μg/ml. Concentrations of Hp are compared to SCC in figure 3-18. Haptoglobin was high (87μg/ml) on day 1 post-calving, but fell sharply over the next 3 days before rising slightly to 10.4 μg/ml on day 5.With the exception of days 19 and 25, Hp was present in the milk at concentrations of 0.7 μg/ml to 4.3 μg/ml from day 5 onwards. Bacteriological culture indicated *E. coli* as the causative agent of the clinical mastitis.

3.3.3 Cows with subclinical mastitis

Two quarters of one cow from the initial group of 47 cows followed post-calving developed subclinical mastitis, both infections being caused by *S. aureus*. Figures 3-19 and 3-21 show the results for milk M-SAA3 as compared to SCC in both cases. The results for Hp as compared to SCC are shown in figures 3-20 and 3-22. As shown in all four figures

this cow presents with an elevated SCC in both quarters on day 1(89 and 45 $x10^3$/ml) respectively. In both case 1 and 2, the SCC rises sharply and falls again on 3 occasions, reaching peaks on days 4, 8 and 15 with levels of 330, 2044 and 2001 $x10^3$/ml in case 1 and 360, 1617 and 1855 $x10^3$/ml in case 2. Concentrations of M-SAA3 are compared to SCC in figures 3-19 and 3-21. Mammary-associated SAA3 was detectable on day 1 post-calving in both case 1 and 2 (0.2 and 0.1 μg/ml) respectively. In case 1 M-SAA3 was detectable at low concentrations throughout the sampling period with the exception of day 10, and showed peak levels of 0.46, 0.31, 0.24 and 0.43 μg/ml on days 4, 9, 17, and 23 respectively. In case 3 M-SAA3 was detectable throughout the sampling period with the exception of days 8-10, and showed peak levels of 1.1 μg/ml on day 2. Concentrations of Hp are compared to SCC in figures 3-20 and 3-22. In case 4 Hp was detected (1.3μg/ml) on day 1 post-calving, and continued to rise fall throughout the sampling period showing peaks on days 4,7, 10, 13 and 17 with concentrations ranging from 0.85 to 1.86 μg/ml. In case 2 Hp was undetected on day 1 post-calving but rises sharply to give the first peak on day 2 at 0.59μg/ml. Another sharp peak was observed on day 19 at a concentration of 0.48μg/ml. The levels of Hp are rising steadily for the last 5 days of sampling (days 22-26).

The 4 cows in mid-lactation, identified as having subclinical mastitis in 1 or more quarters by a positive CMT, and IQSCC > 60,000 cells/ml. Somatic cell count and M-SAA3 for the 7 cases of subclinical mastitis ranged from 964 to 24735 $x10^3$/ml and 1.84 to 3.45 μg/ml respectively (Figures 3-23 to 3.29). The milk Hp assay was not performed on these samples. Bacteriological culture indicated *S. aureus* was the causative agent for the subclinical mastitis in all 7 cases.

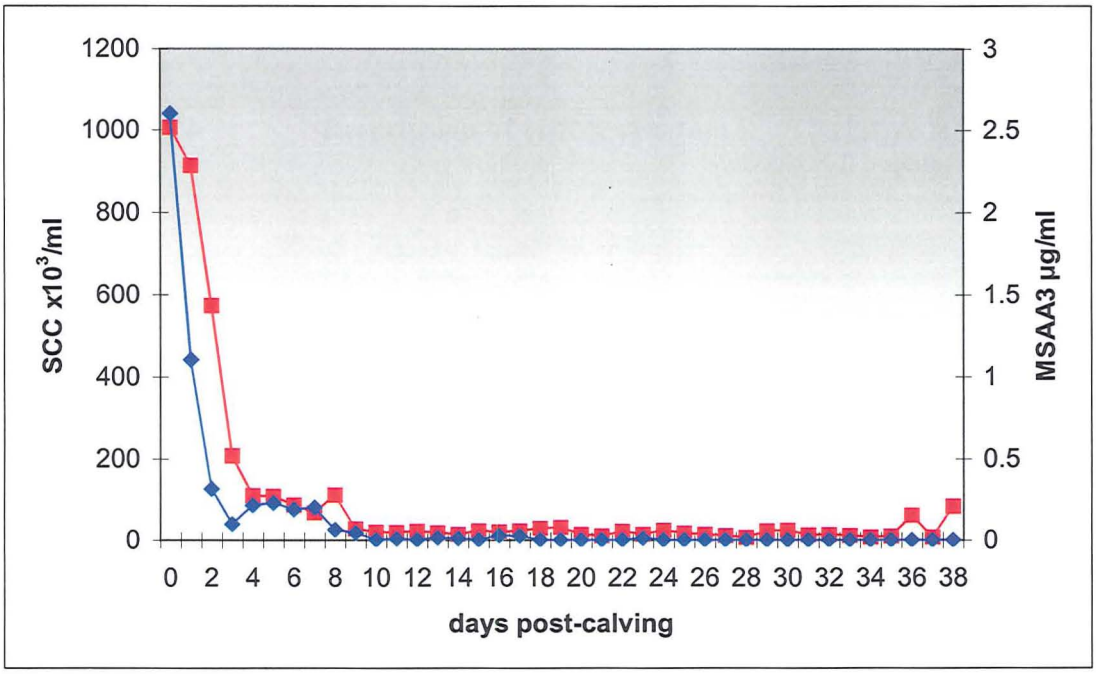


Figure 3-3: The mean somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) for normal post-calving cows (n=35). SCC and M-SAA3 reach normal levels of <60,000 cells/ml and <500ng/ml respectively by day 10 post-calving.

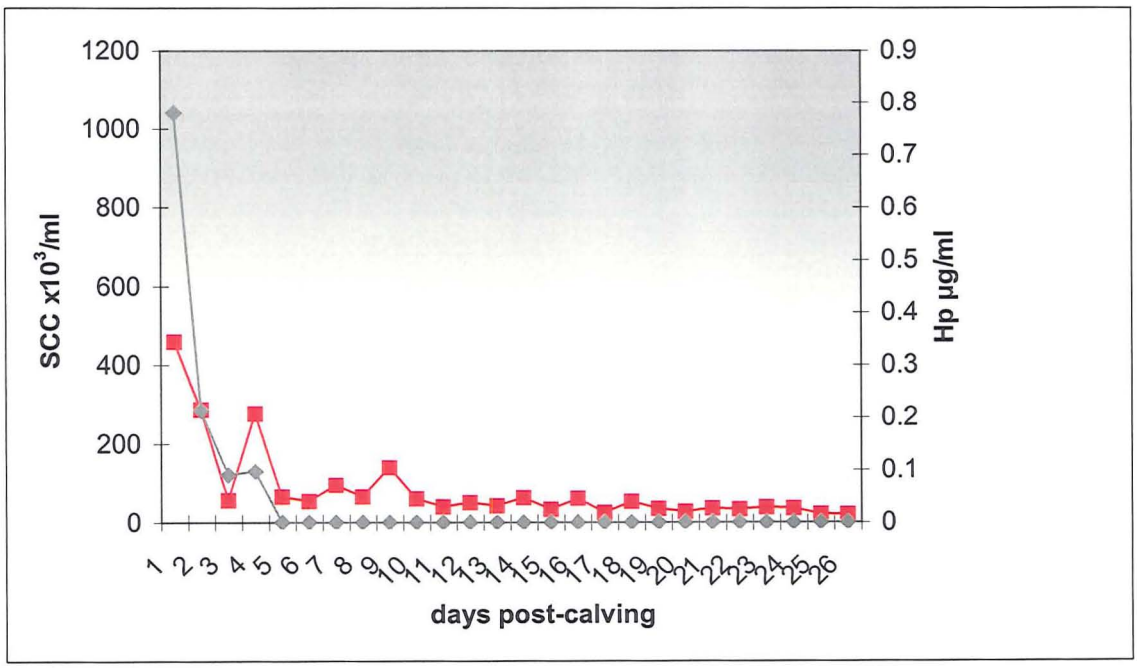


Figure 3-4: The mean somatic cell count (SCC) (—■—) and Haptoglobin (Hp) (—◆—) for a subset of the normal post-calving cows (n=9). SCC and Hp reach normal levels of <60,000 cells/ml and <0.5 µg/ml respectively by day 10 post-calving.

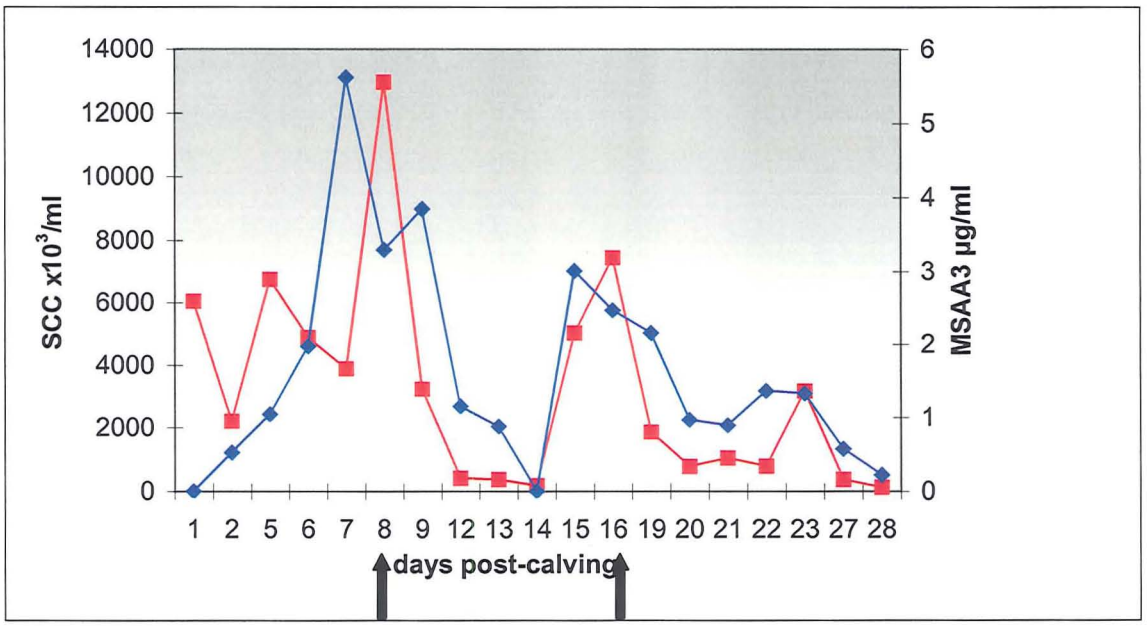


Figure 3-5: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 1. Clinical mastitis developed on day 8 and again on day 16 (as indicated by the arrows). *Streptococcus uberis* was isolated from the milk of this case.

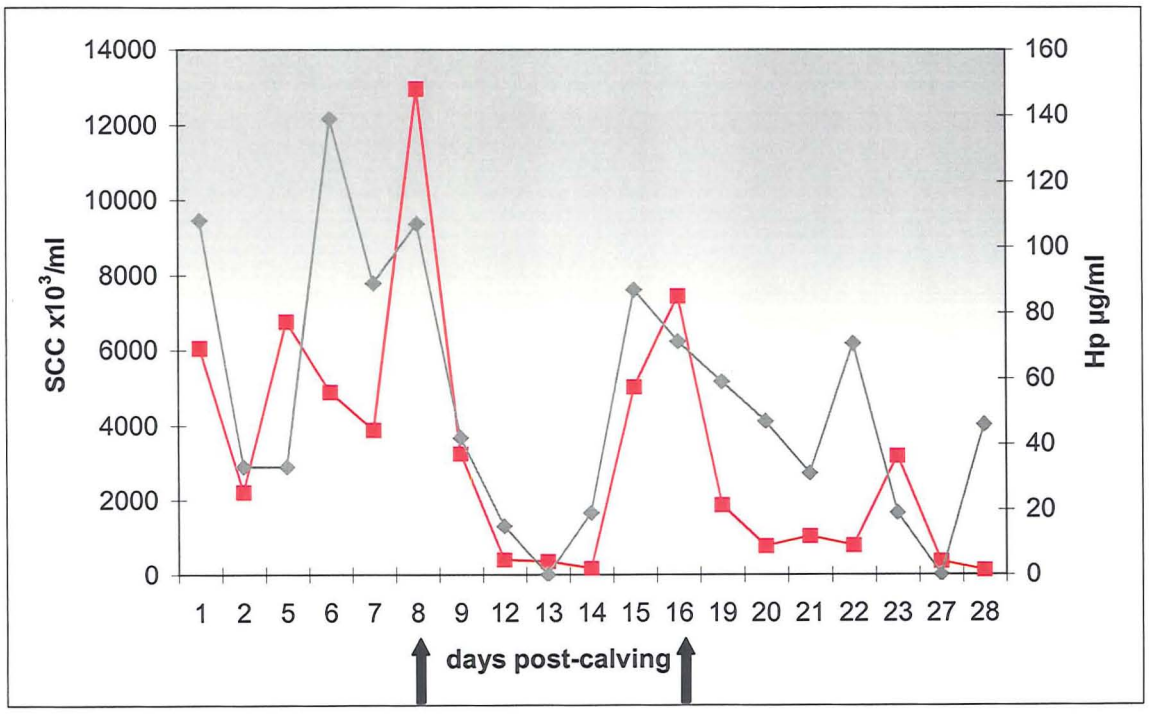


Figure 3-6: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in case 1. Clinical mastitis developed on day 8 and again on day 16 (as indicated by the arrows). *Streptococcus uberis* was isolated from the milk of this case.

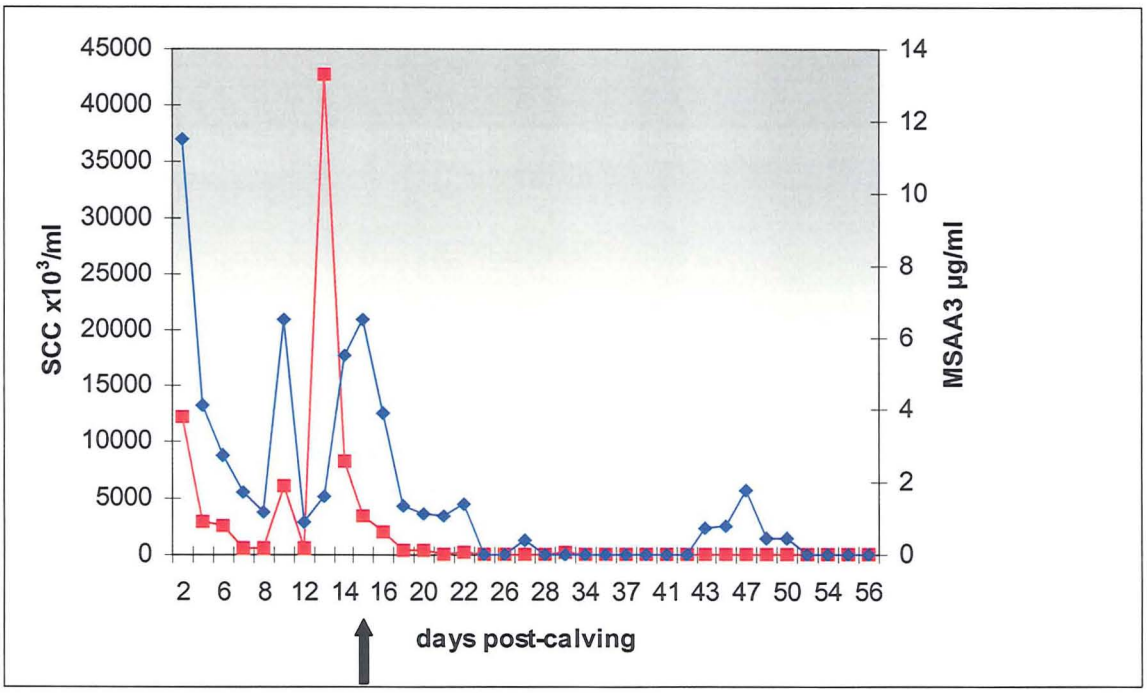


Figure 3-7: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 2. Clinical mastitis developed on day 15 (as indicated by the arrows). *E. coli* was isolated from the milk of this case.

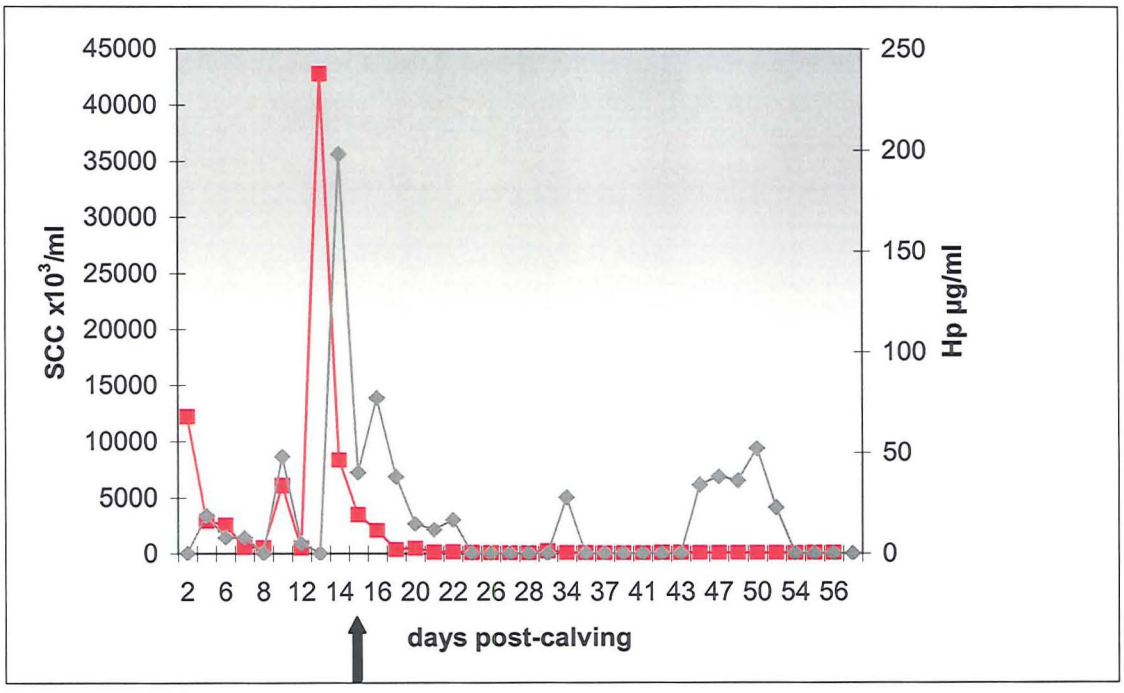


Figure 3-8: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in case 2. Clinical mastitis developed on day 15 (as indicated by the arrows). *E. coli* was isolated from the milk of this case.

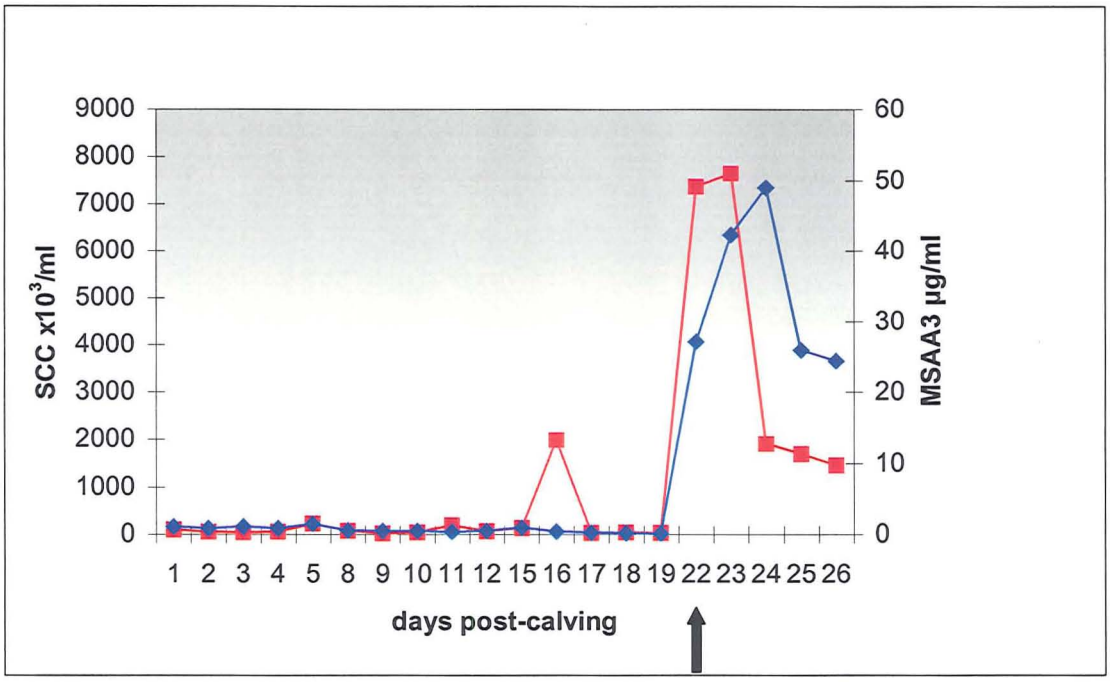


Figure 3-9: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 3, Clinical mastitis developed on day 22 (as indicated by the arrow). *Staphylococcus aureus* was isolated from the milk of this case.

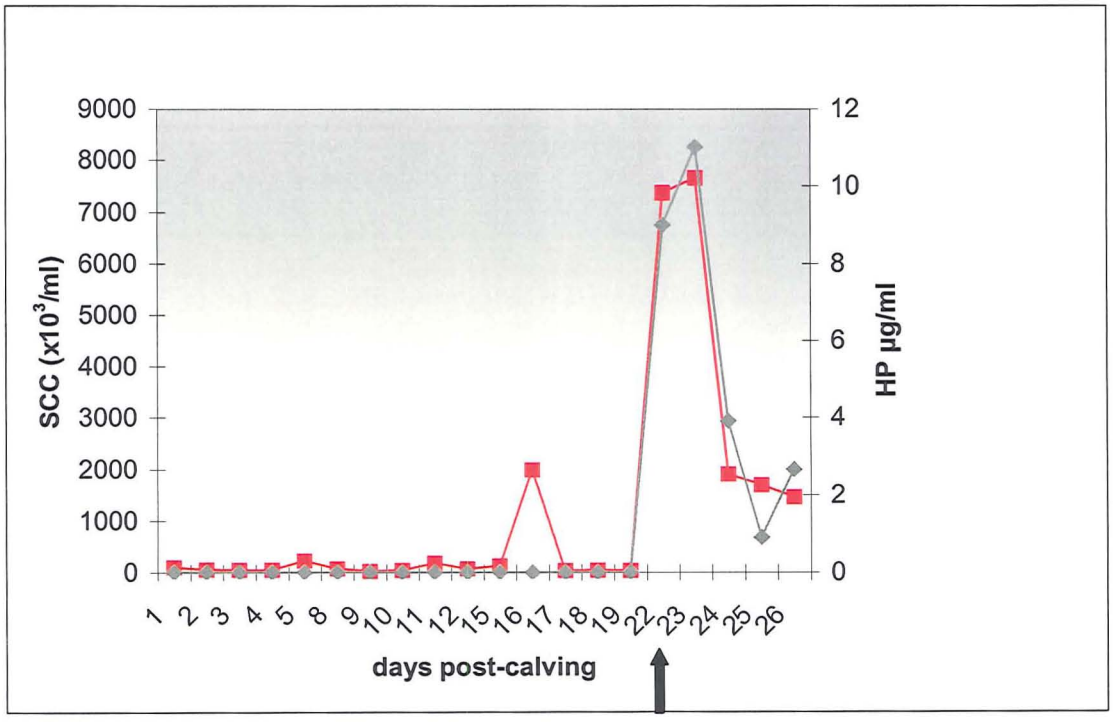


Figure 3-10: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in case 3. Clinical mastitis developed on day 22 (as indicated by the arrow). *Staphylococcus aureus* was isolated from the milk of this case.

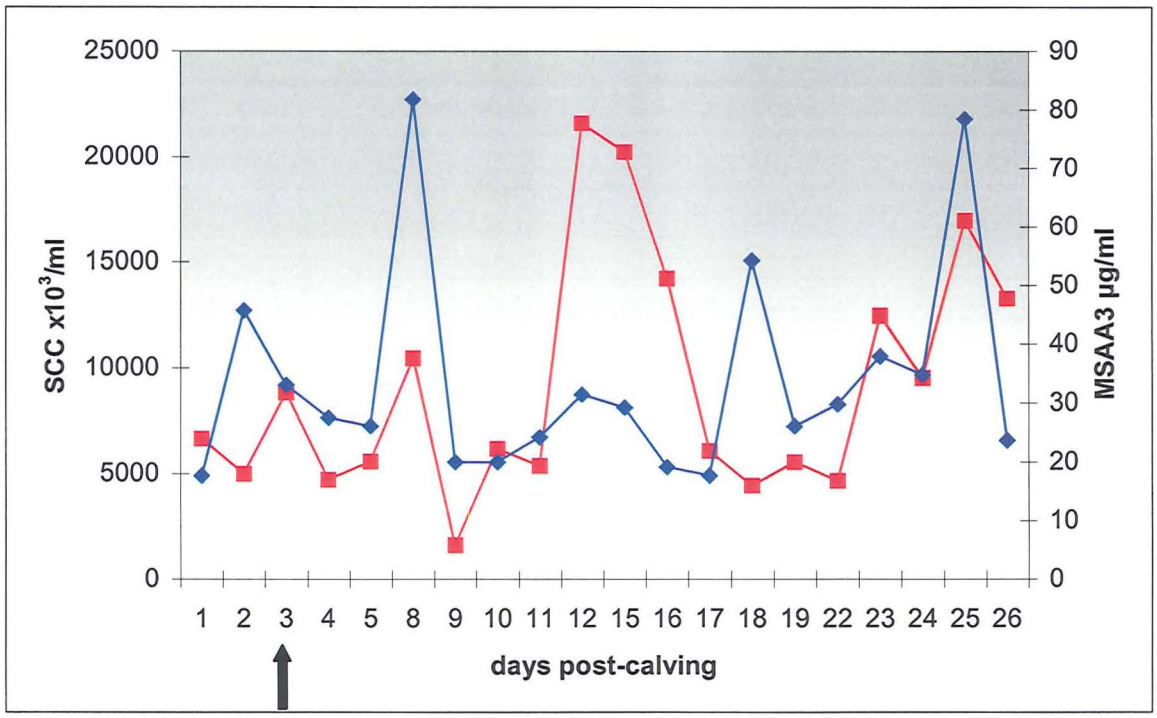


Figure 3-11: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 4. Clinical mastitis developed on day 3 (as indicated by the arrow). *Staphylococcus aureus* was isolated from the milk of this case.

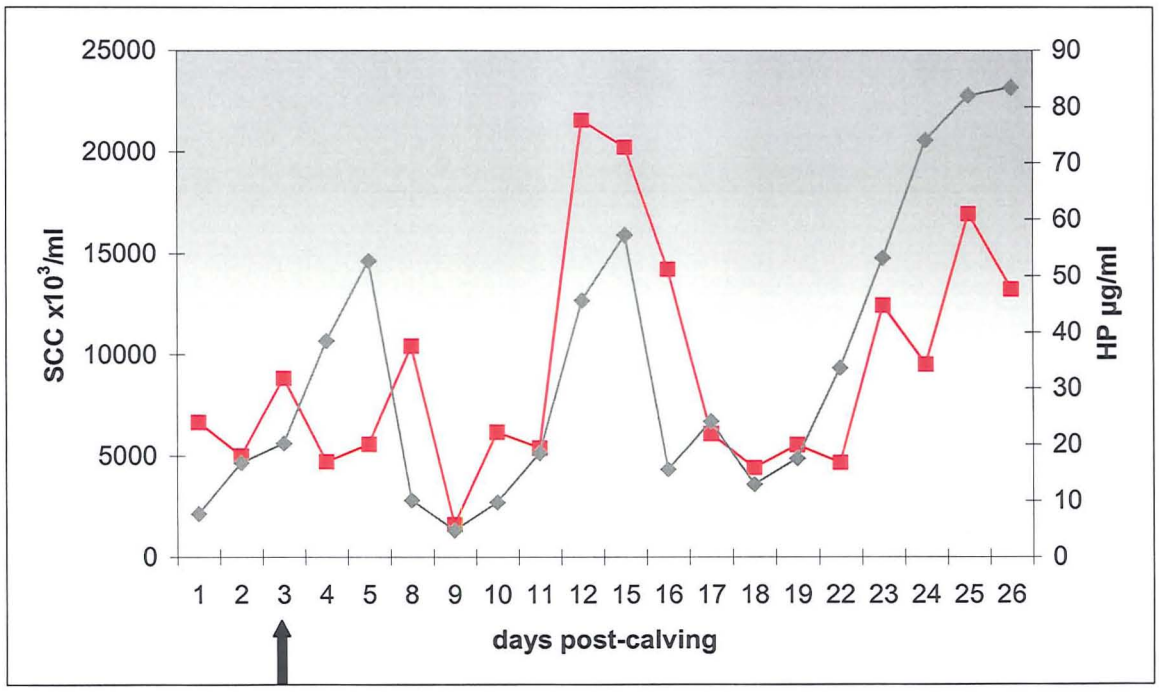


Figure 3-12: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in case 4. Clinical mastitis developed on day 3 (as indicated by the arrow). *Staphylococcus aureus* was isolated from the milk of this case.

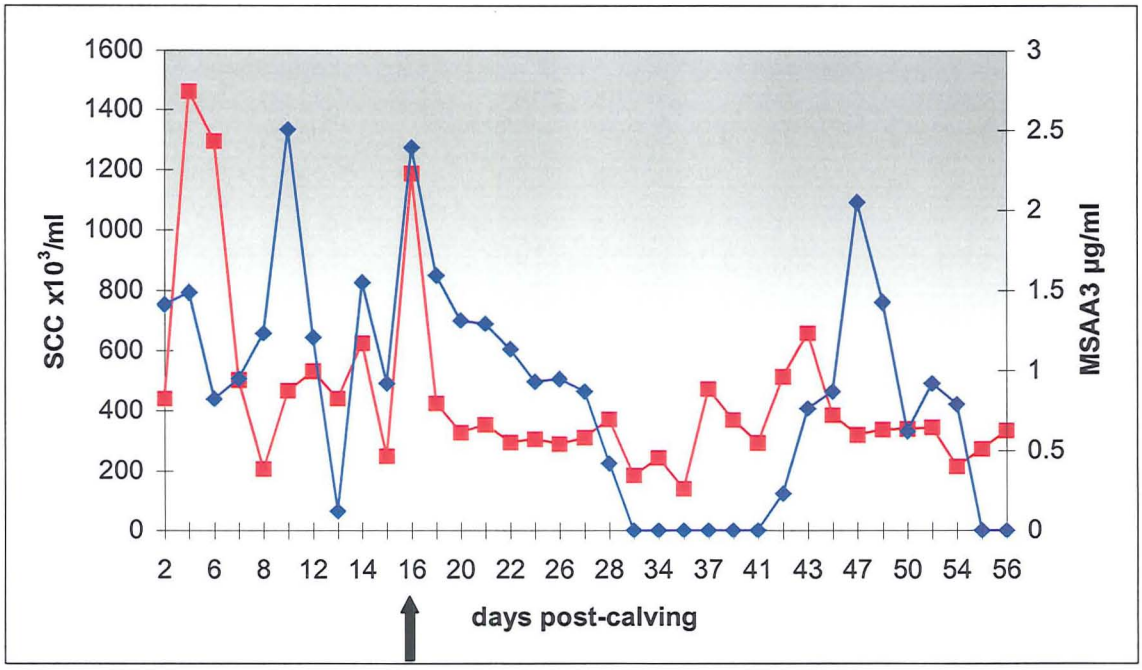


Figure 3-13: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 5. Clinical mastitis developed on day 16 (as indicated by the arrow). *Staphylococcus aureus* was isolated from the milk of this case.

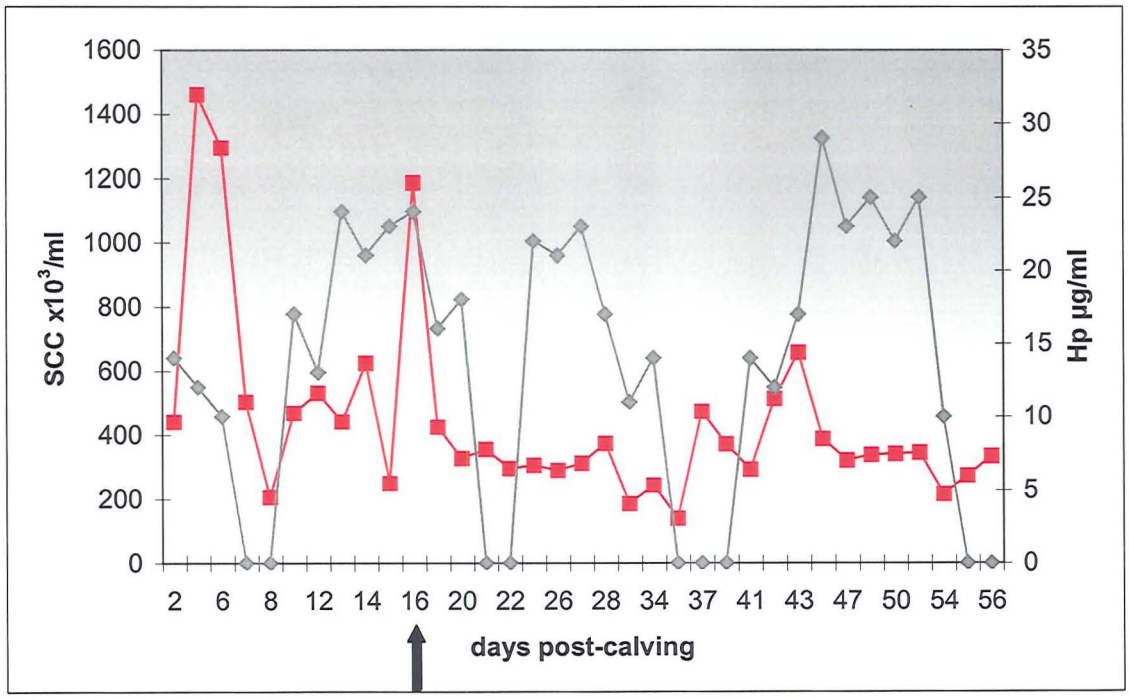


Figure 3-14: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) case 5. Clinical mastitis developed on day 16 (as indicated by the arrow). *Staphylococcus aureus* was isolated from the milk of this case.

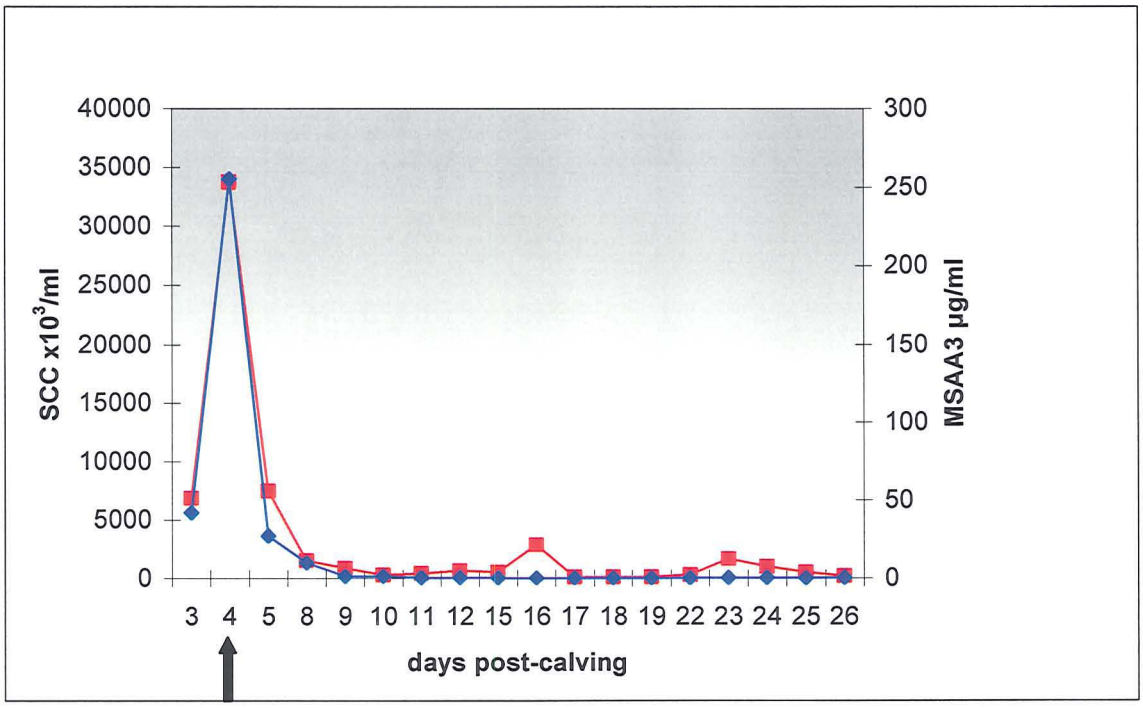


Figure 3-15: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 6. Clinical mastitis developed on day 4 (as indicated by the arrow). *E. Coli* was isolated from the milk of this case.

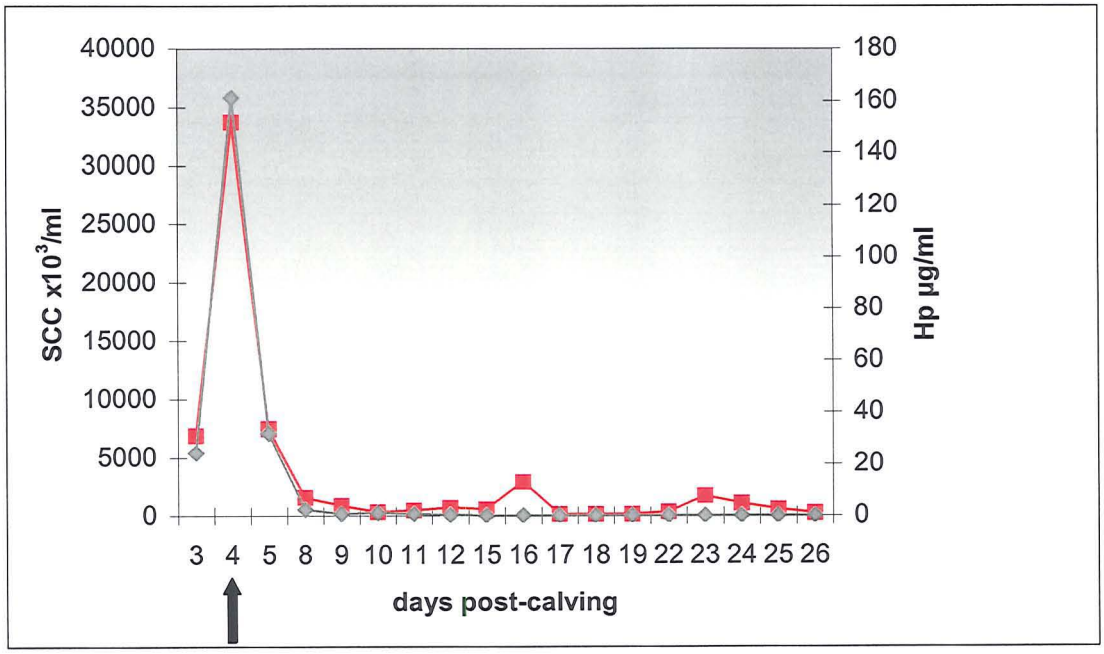


Figure 3-16: The Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in case 6. Clinical mastitis developed on day 4 (as indicated by the arrow). *E.coli* was isolated from the milk of this case.

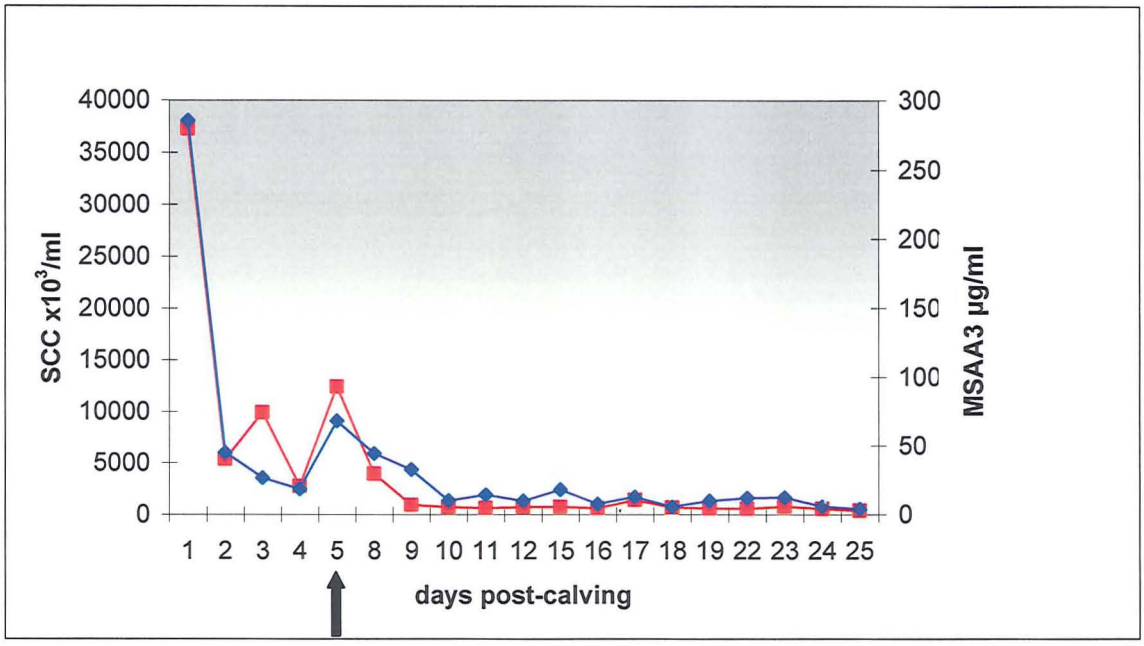


Figure 3-17: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in case 7. Clinical mastitis developed on day 5 (as indicated by the arrow). *E.Coli* was isolated from the milk of this case.

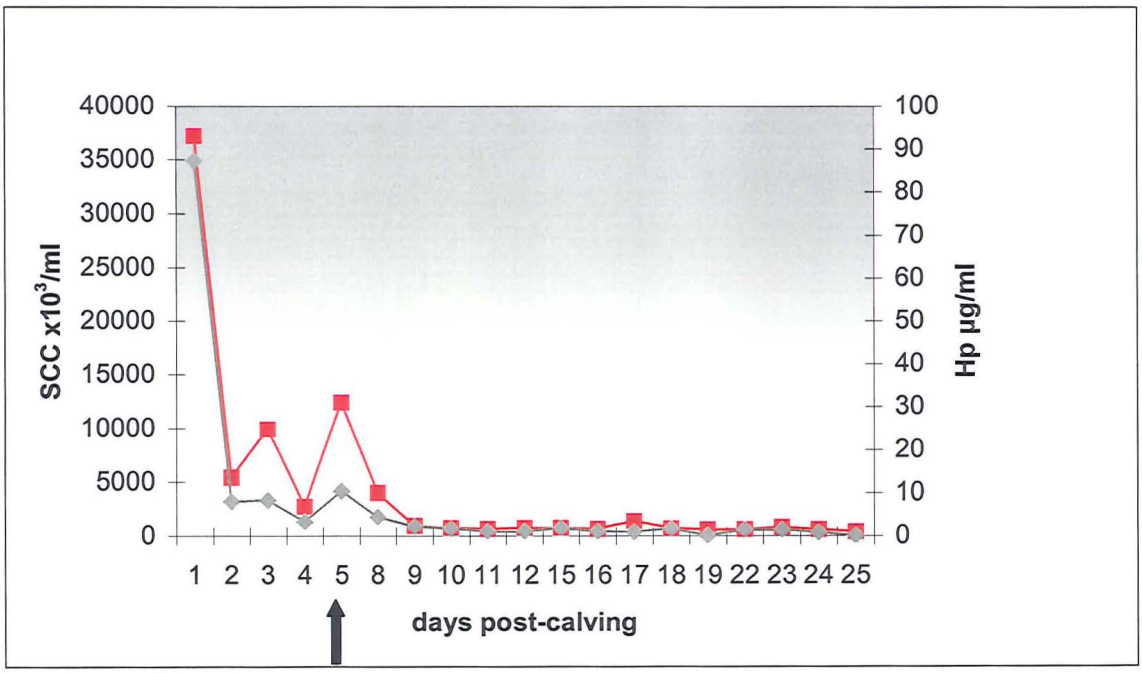


Figure 3-18: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in case 7. Clinical mastitis developed on day 5 (as indicated by the arrow). *E.Coli* was isolated from the milk of this case.

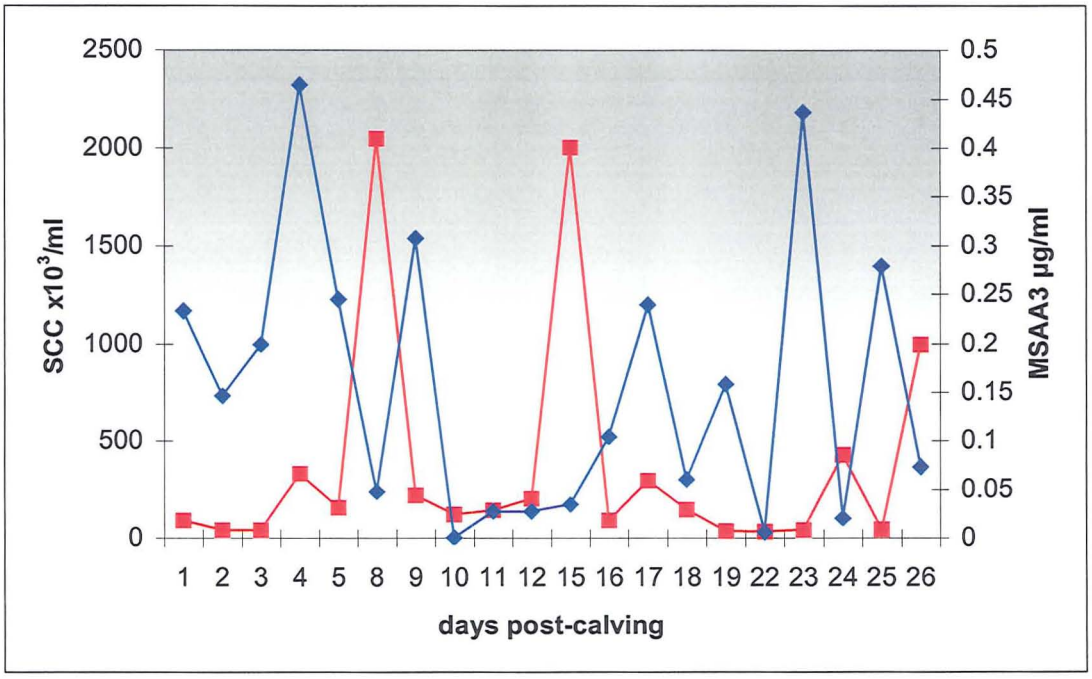


Figure 3-19: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 1, CASE 1 SUBCLINICAL. *S.aureus* was isolated from the milk of this cow.

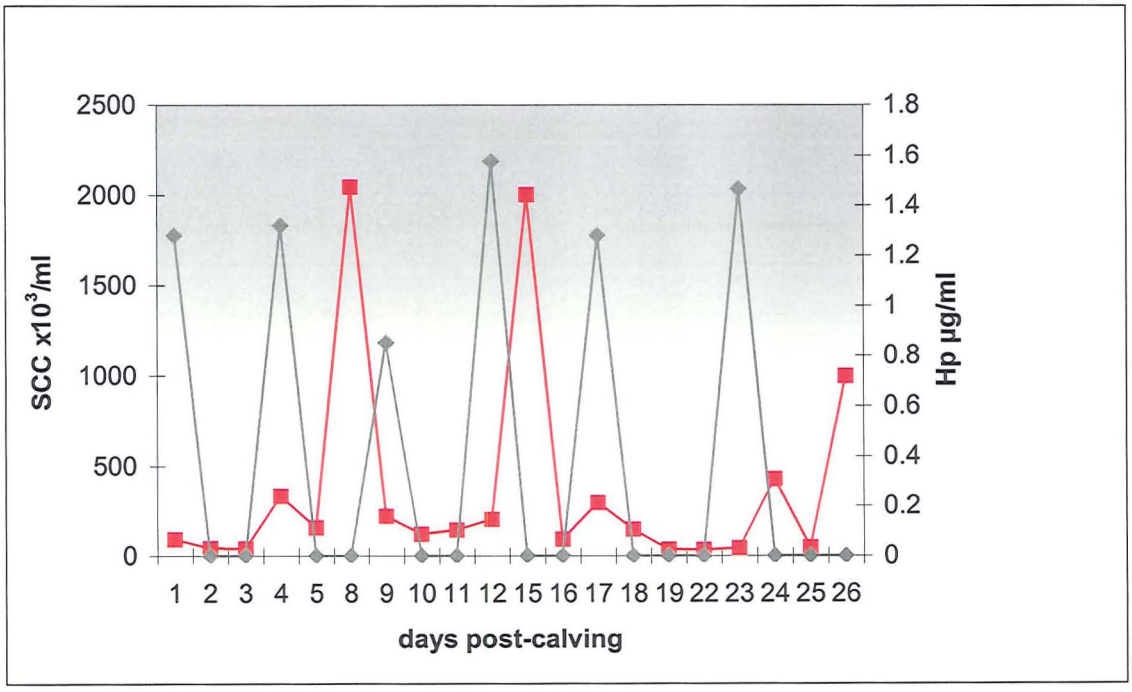


Figure 3-20: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in cow 1, CASE 1 SUBCLINICAL. *S.aureus* was isolated from the milk of this case.

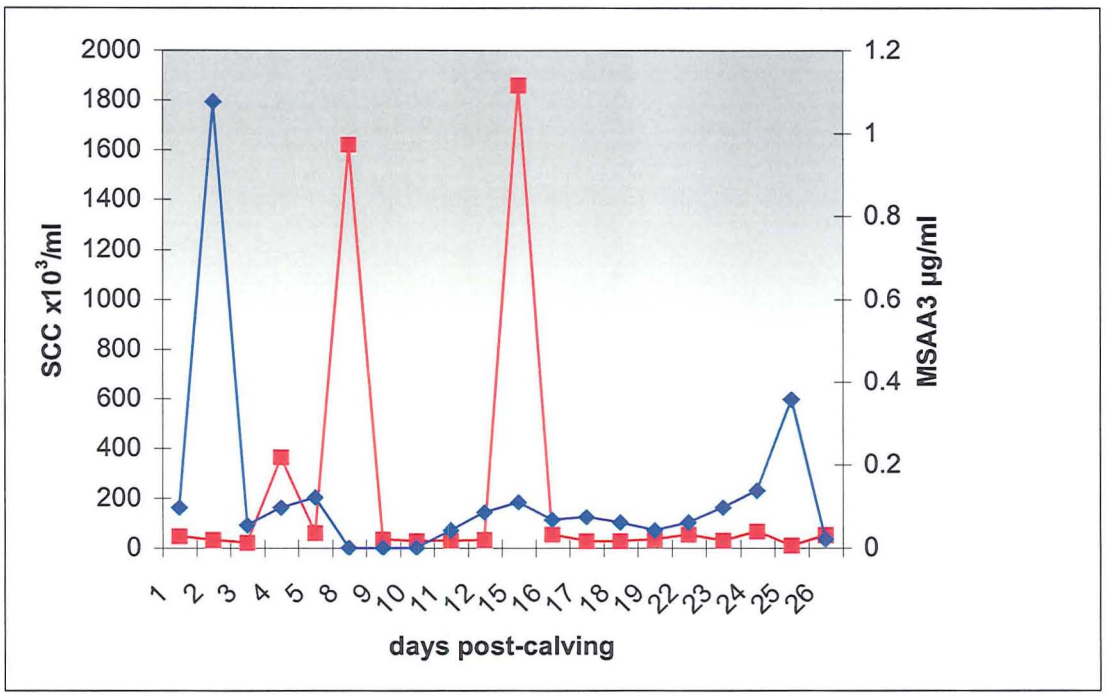


Figure 3-21: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 1, CASE 2 SUBCLINICAL. *S.aureus* was isolated from the milk of this cow.

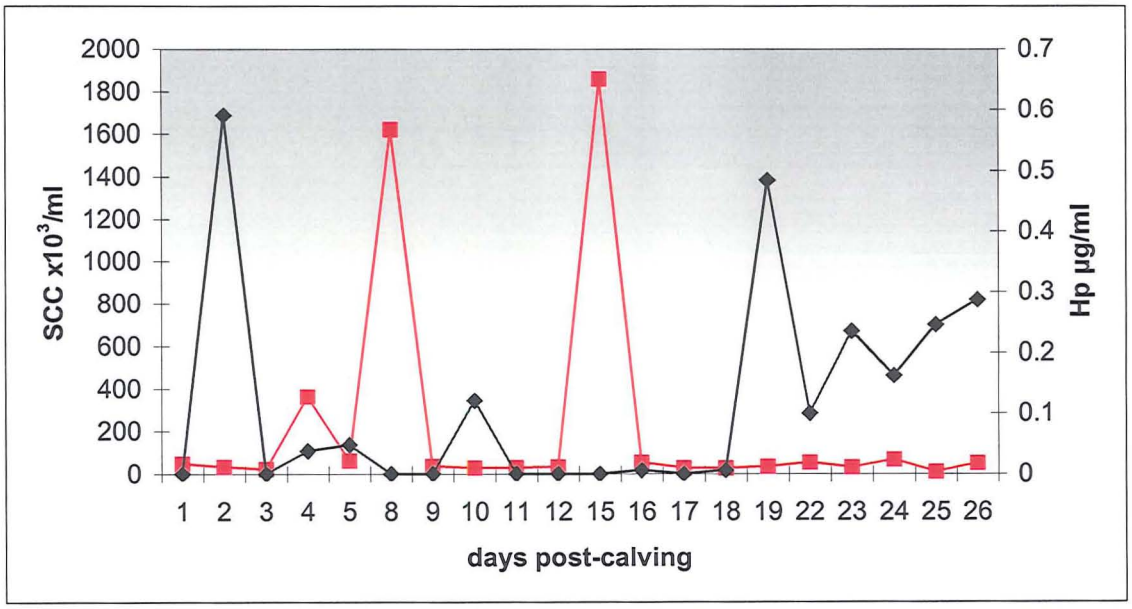


Figure 3-22: Profiles for somatic cell count (SCC) (—■—) and haptoglobin (Hp) (—◆—) in cow 1, CASE 2 SUBCLINICAL. *S.aureus* was isolated from the milk of this case.

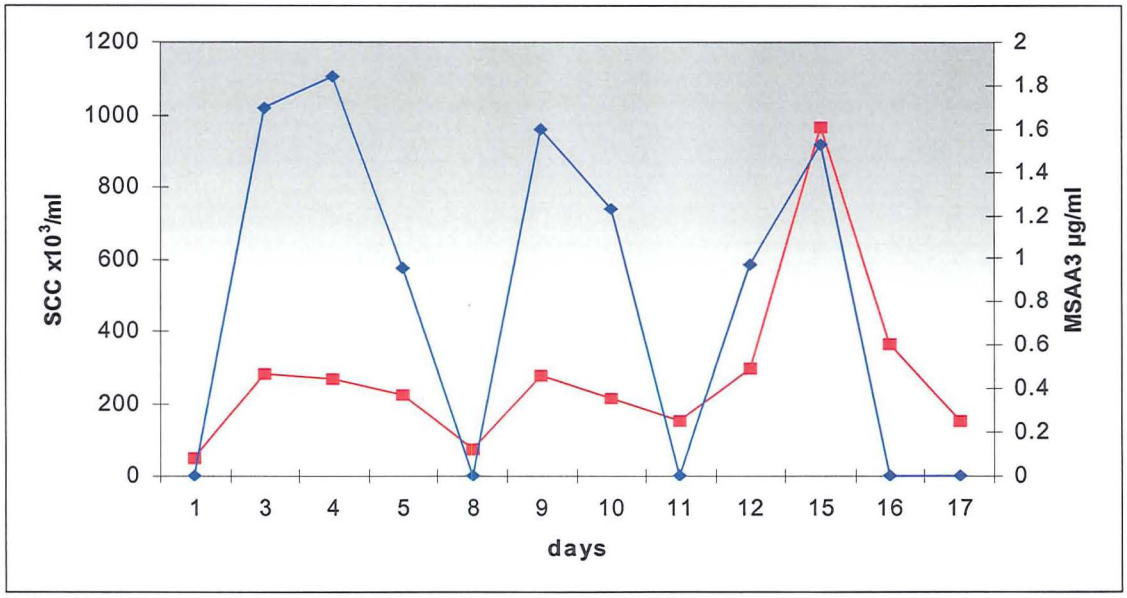


Figure 3-23: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 2, CASE 3 SUBCLINICAL. *S.aureus* was isolated from the milk of this cow.

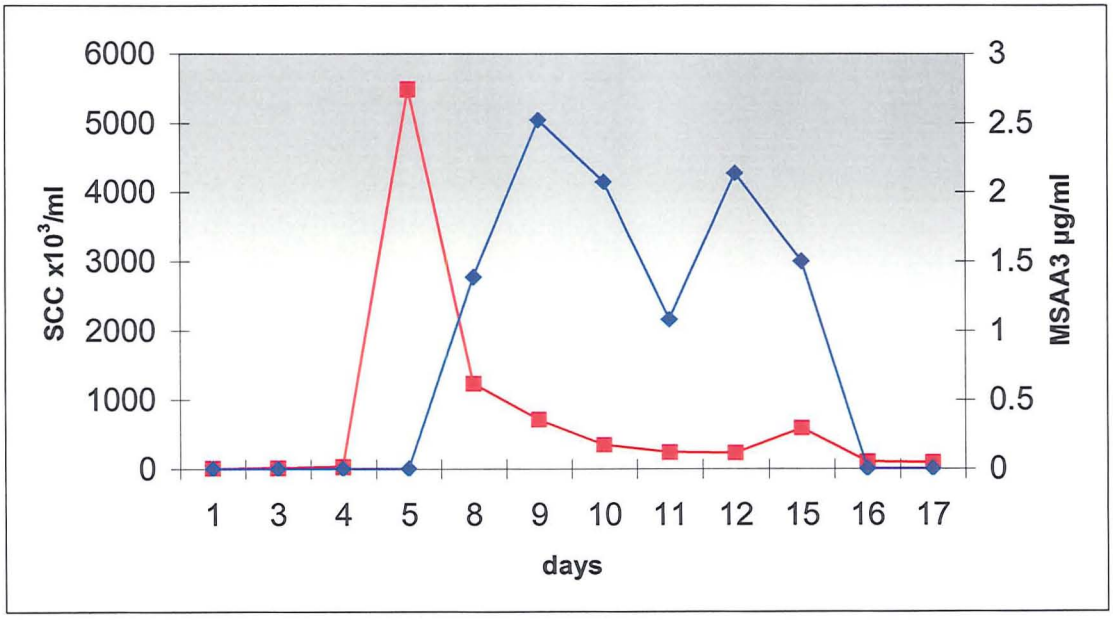


Figure 3-24: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 2, CASE 4 SUBCLINICAL. *S.aureus* was isolated from the milk of this cow.

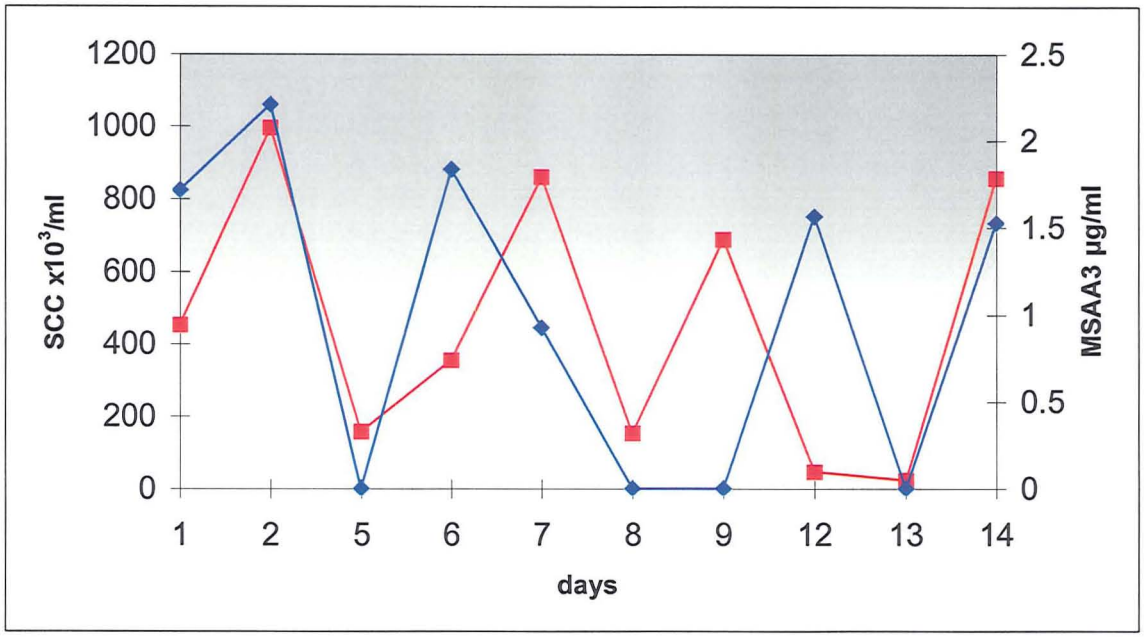


Figure 3-25: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 3, CASE 5 SUBCLINICAL. *S.aureus* was isolated from the milk of cow 3.

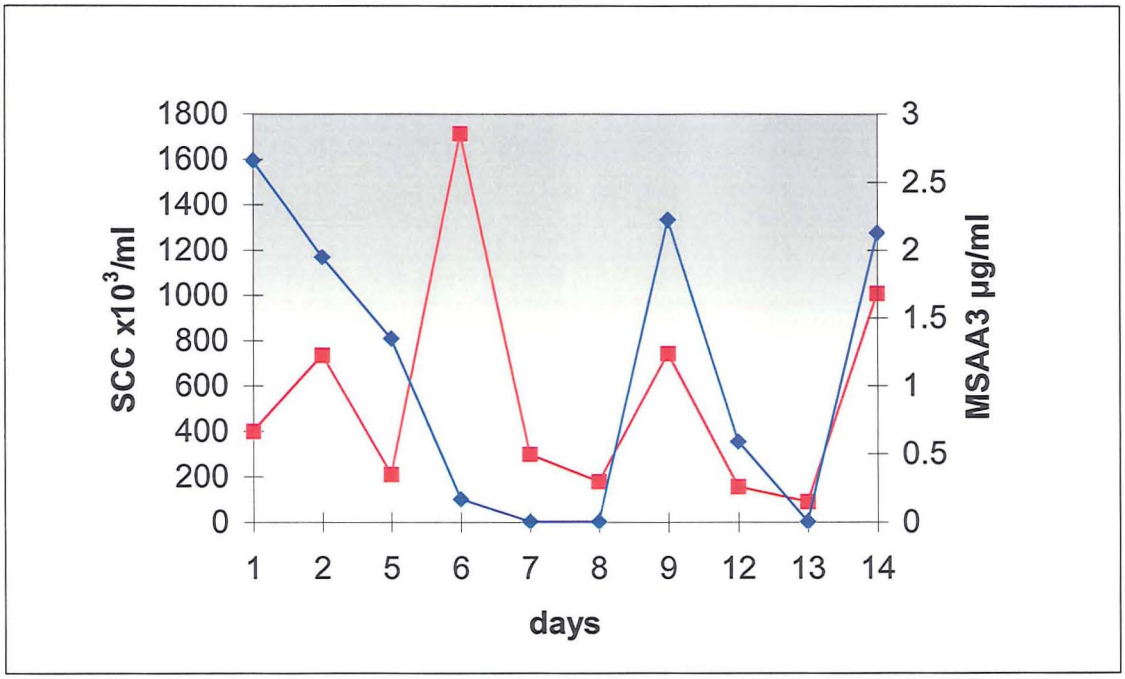


Figure 3-26: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 3, CASE 6 SUBCLINICAL. *S.aureus* was isolated from the milk of cow 3.

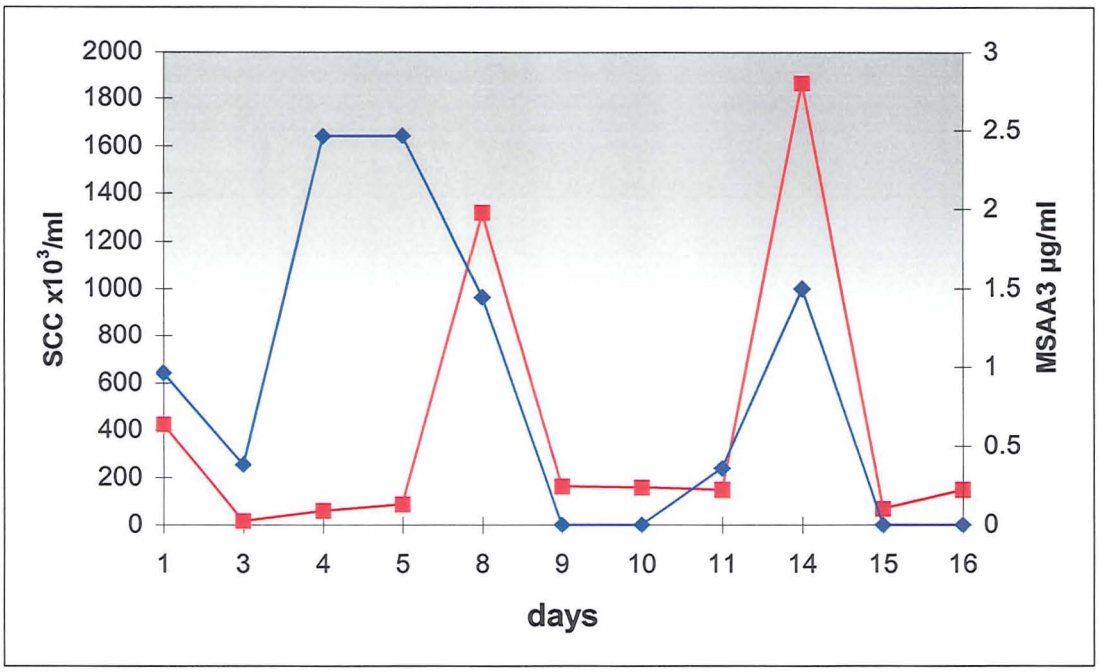


Figure 3-27: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 4, CASE 7 SUBCLINICAL. *S.aureus* was isolated from the milk of cow 4.

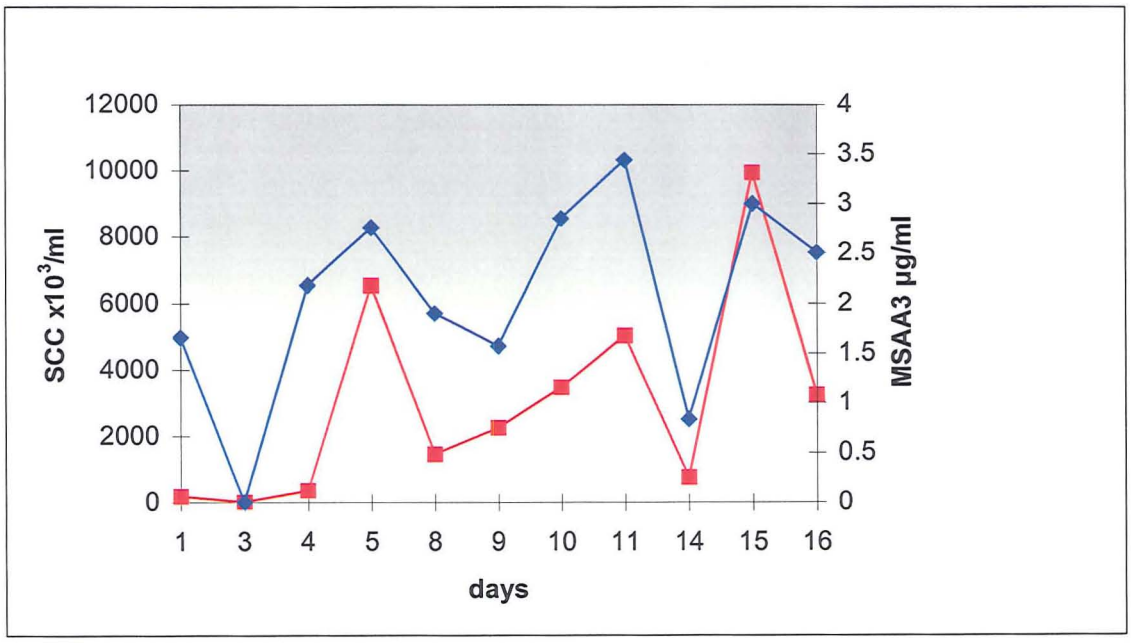


Figure 3-28: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 4, CASE 8 SUBCLINICAL. *S.aureus* was isolated from the milk of case 8 subclinical.

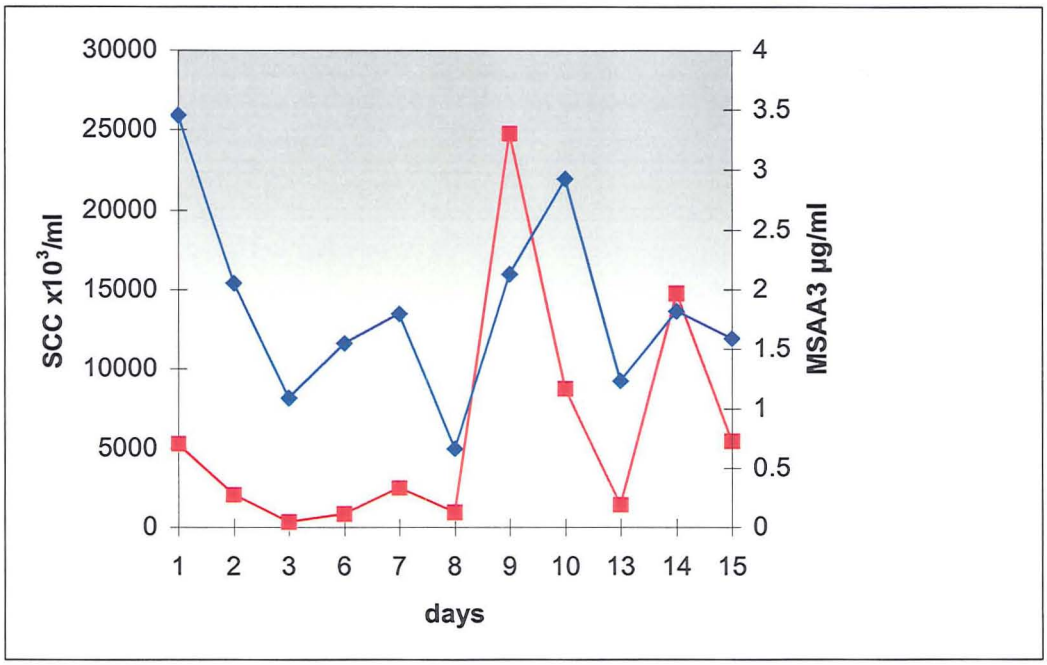


Figure 3-29: Profiles for somatic cell count (SCC) (—■—) and mammary-associated serum amyloid A3 (M-SAA3) (—◆—) in cow 5, CASE 9 SUBCLINICAL. *S.aureus* was isolated from the milk of case 9 subclinical.

3.4 Discussion

Mastitis continues to be the most economically important disease of dairy cattle, and has been identified as the most common cause of death in adult dairy cows (Esslemont and Kossaibati 1997). Earlier diagnosis of mastitis would result in earlier treatment, possibly reducing the adverse effects of mastitis in both economic and welfare terms.

Acute phase proteins have been used as biological markers in a range of inflammatory conditions and in a variety of species, including cattle (Gruys et al 1994). However the recent detection of the major APPs, M-SAA3 and Hp in bovine milk (Eckersall et al 2001; McDonald et al 2001; Pedersen et al 2003; Gronlund et al 2003a) has added further support to their application in a rapid, specific and non-invasive routine diagnostic test for mastitis.

The present study investigated the presence of Hp and SAA in individual quarter milk samples from commercial dairy cows in early lactation, and compared their kinetics to those of conventional markers of inflammation such as SCC. Unlike any previous investigations this project involved taking milk samples from post-calving cows prior to infection in order to establish the earliest time-point of appearance of APP in milk during naturally occurring mastitis. For the purpose of this study it was concluded that early lactation would be the most desirable time to begin sampling as the incidence of clinical mastitis is high during the first month of lactation (Barkema et al 1999).

Somatic cell count is physiologically elevated after calving but then decreases at a rapid rate, particularly during the first 2 weeks of lactation, (Dohoo 1993). In this study, SCC was raised in all cows post-calving, but fell rapidly in the control cows 1-3 days post-calving reaching normal levels of < 60,000 cells/ml by day 10 post-calving. The post-calving levels of SCC in cows that developed clinical mastitis were higher than those of the control cows. The mean SCC on the first milk sample taken post-calving from cows that went on to develop clinical mastitis caused by *S. aureus, S. uberis* and *E. coli* were 2383, 6036 and 18706 $x10^3$ cells/ml, respectively, whereas the maximum post-calving SCC recorded on the first sample in the control cows was 1000 $x10^3$cells/ml. These findings are in agreement with previous studies investigating the association between the incidence of pathogen-specific clinical mastitis and BMSCC, which concluded that different pathogens are associated with different baseline levels for SCC (de Haas et al 2002).

Milk Hp and M-SAA3 were elevated in all control cows post-calving but fell rapidly between days 1-3 post-calving and were undetectable in all quarters by day 6 and 10 for Hp and M-SAA3 respectively. In the 7 cases of clinical mastitis, both M-SAA3 and Hp were detected together in 4 cases and individually in another 2 cases on the first day of sampling post-calving. The concentrations of M-SAA3 and Hp detected at the first sampling post-calving ranged from 1μg/ml to 285μg/ml and 11μg/ml to 108μg/ml, respectively. The highest post-calving concentration of M-SAA3 was recorded in a clinical case that developed *E. coli* mastitis (case 7, Figure 3.11a), whereas the highest concentration of Hp was recorded in a cow that developed *S. uberis* mastitis (case 1, Figure 3.5b). The lowest post-calving concentrations of APPs in milk were found in the cows that subsequently developed clinical mastitis caused by *S. aureus* mastitis. With the exception of the highest post-calving Hp levels being found in the *S. uberis* mastitis case, the results for milk APP at calving in relation to pathogen type were in agreement with the SCC results, namely *S. aureus* resulted in the lowest levels of SCC and *E. coli* the highest. Somatic cell count decreased more rapidly in uninfected quarters than in infected quarters in early lactation, indicating that using SCC early postpartum gives a reliable indication of intra-mammary infection status (Barkema et al 1999). Accordingly, determination of APP levels in milk at calving may be used to give an indication of the infection status of the mammary gland, and possibly APPs may give an indication of the probable day of onset of clinical mastitis in early lactation, and may indicate the pathogen responsible, although further work is required to substantiate this.

A key part of this study was to determine the relationship between milk APPs and conventional markers of mastitis. Somatic cell counts were measured in milk and used essentially to validate the use of milk APPs as a new diagnostic tool for mastitis. On analysis of the profiles of APP in relation to SCC, before and after clinical mastitis both M-SAA3 and Hp showed similar kinetics and are similar to SCC. Peak concentrations of M-SAA3 and Hp occurred in conjunction with SCC and on the same day as clinical signs were detected, in 3 of the 7 cases of clinical mastitis. Examination, of the profiles for case 1 (Figures 3.5a and 3.5b), showed that maximum values of M-SAA3 and Hp, clearly preceded that of SCC and clinical signs on both episodes of mastitis recorded. The situation for the remaining 3 cases is slightly more complex, and although the APP did not reach maximum values before the detection of clinical signs, they rose in concentration either prior to (case 4 and 5) or in conjunction with (case 2 and 3) SCC.

Of the 7 cases of clinical mastitis 3 were caused by *E. coli*, 3 by *S. aureus* and 1 by *S. uberis*. On examination of the APP concentrations within the pathogen specific groups (*E.*

coli and *S. aureus)*, it appears that the pathogen responsible for the mastitis could influence the APP concentration. The concentrations of both M-SAA3 and Hp in *E. coli* infection are much higher than those seen in *S. aureus* infections. The pathogen-specific effects on the APP curves showed clearly differential effects for *S. aureus* and *E. coli*. *Staphylococcus aureus,* is known to cause chronic mastitis with periodic episodes and is often present subclinically for some time before clinical symptoms are observed (Harmon 1994). This is considered the likely situation in clinical cases 5 and 6 where the overall patterns of SCC and APPs showed daily fluctuations and were elevated for a prolonged period of time, before and after the onset of clinical signs. In contrast are the 3 clinical cases 2, 6 and 7 where *E. coli* was the pathogen responsible, all 3 parameters (SCC, M-SAA3 and Hp) increased sharply over the 24h period prior to the onset of clinical signs and decreased rapidly post-infection in each of the 3 cases. The presence of a low SCC prior to the onset of *E.coli* clinical mastitis and the fact that such infections are rarely persistent has been studied previously (Peeler et al 2003). However SCC reached baseline levels before both APPs fell to undetectable levels. A rapid decline in SCC post-infection has previously been reported in *E.coli* mastitis (de Haas et al 2002). Over the years pressure to reduce BMSCC has led to recent suggestions that cows with a very low SCC might be more susceptible to clinical mastitis because their ability to respond to intra-mammary infection would be reduced, and hence when a pathogen does successfully colonise the udder and establish an infection it is likely to be more severe. More than 50% of the clinical cases of mastitis isolated in this study were caused by environmental pathogens, which is likely to be due to the fact that samples were collected during early lactation, when the prevalence of such pathogens is at its highest (Dingwell et al 2003).

From the results both M-SAA3 and Hp can be considered as sensitive and reliable markers of clinical mastitis, and also show potential as an early bio-marker of the disease prior to the onset of clinical signs. There is much speculation on the mechanisms involved which lead to the presence of M-SAA3 and Hp in milk. However the recent discovery of a mammary- associated SAA3 expressed and secreted in colostrum (McDonald et al 2001), supports the theory that APP may be locally produced within the udder rather than entering the mammary gland by traversing the blood-mammary barrier. The production of Hp and M-SAA3 may be associated with different cytokine profiles, which in turn may be dependent on the duration and type of inflammatory stimuli in the mammary gland. In four of the seven clinical cases, the concentrations of Hp were higher than that of M-SAA3. Although comparisons to the few other studies reporting the presence of APP in milk are useful they must be treated with caution due to very different experimental designs and techniques applied. The experimental infection with *S. aureus* by Gronlund et

al (2003a), for acute clinical mastitis gave a range of 1.2 to 323 μg/ml and 1.4 to 286 μg/ml for Hp and M-SAA3 respectively. The maximum values are higher than the maximum values of M-SAA3 and Hp recorded for *S. aureus* clinical cases in this study: 49 and 83 μg/ml, respectively. The methods used to determine both APPs in the study by Gronlund et al (2003a) were exactly the same as in the present study, however in an experimental infection the first line of defence is circumvented which may explain why the values of milk APP were substantially higher in experimental infection than those obtained from infection caused by natural exposure of the teat end. The second published study involving an experimental infection, although in this case with *S. uberis* (Pedersen et al 2003), found a low range of results for M-SAA3 (0.47 to 4.62 μg/ml), however this is likely due to the fact that the cows were culled only 12h after inoculation at which time the APPs in milk were still rising. The APP results from the initial findings of M-SAA3 and Hp in milk during naturally occurring mastitis (Eckersall et al 2001), reported high concentrations of Hp in milk as compared to M-SAA3. The Hp was determined in the previous study by SRID, and may have resulted in the high values due to methodological differences. Further research is needed to clarify the difference between the characteristics of these two APPs and to unravel the biological relationship between them, before it could be concluded that either one is a more desirable biomarker of mastitis than the other.

The results from this study suggest the concentration of APP varies with the occurrence of different pathogens, and could therefore be used to predict the pathogen involved in a case of clinical mastitis. Mastitis control measures are aimed at the management of specific pathogen types (Sargeant et al 2001), and the earlier they are identified the sooner appropriate treatment may be administered. At present, bacteriological culture of milk samples is the standard method for identifying pathogen type, which takes 2-3 days, this delay alongside financial considerations involved with bacteriological sampling have precluded this technique from being widely adopted as a screening method for mastitis by the dairy industry.

Two cases of subclinical mastitis were diagnosed from the original group of 47 cows that were sampled in early lactation. Mammary-associated SAA3 and Hp were detecting in a fluctuating cycle similar to the pattern observed in the cases of clinical mastitis induced by *S. aureus*. The concentrations were however, much lower than those on the clinical cases: 0.1 to 0.46μg/ml and 0.6 to 1.86μg/ml for M-SAA3 and Hp respectively. Although the focus of this study was clinical mastitis, the two cases of subclinical mastitis did provide interesting results, and therefore additional cows were recruited to the study mid-lactation, in order to further characterise the APP response in subclinical mastitis. These 7 cases

were only tested for M-SAA3 as determination of its concentration was easier than that of Hp due to the ready availability of the commercial assay for the former. The levels of M-SAA3 in these cases showed very similar kinetics to the early lactation subclinical cows, with daily fluctuations at low levels, and the maximum value of M-SAA3 recorded being 3.4μg/ml. *Staphylococcus aureus* was isolated as the major pathogen causing subclinical mastitis in each of the nine cases. This finding is in agreement with the most recent annual Veterinary Investigation Diagnosis Analysis Database (VIDA) statistics compiled by the Veterinary Laboratories Agency which give an insight into the current profile of mastitis pathogens, and confirmed *S. aureus* as the most common cause of subclinical mastitis in the UK (Bradley 2002). Quarters within cows are not independent; infection within one quarter increases the probability that additional quarters in the same cow will be positive (Barkema et al 1997), hence the finding that 9 cases of subclinical mastitis identified in this study were isolated from different quarters of only 5 cows is not unexpected. This may be due to increased susceptibility of certain cows or to within-cow transmission of pathogens (Barkema et al 1997). Intracellular survival and resistance to host immune responses results in a poor cure rate of *S. aureus* infections (Fox and Gay 1993). At present, an increased SCC is often the only means of detecting chronic subclinical mastitis. Monthly screening of most dairy herds using SCC at the cow level is routine. However this can lead to false negative results when pooling of the milk from all 4 quarters may mask a high IQSCC. Therefore validation of an effective means for identifying intra-mammary infection at the quarter level would be beneficial to the welfare and productivity of the lactating dairy cow as this could potentially result in faster and more targeted therapy increasing cure rates and decreasing premature culling.

The continued development of robotic milking systems in which the daily contact between the cow and stock worker is lost means that manual diagnosis of mastitis through examination is no longer practical, so a need for new monitoring systems has been created.

The results of this study show that the major APPs, M-SAA3 and Hp can be detected in the milk of cows with clinical and subclinical mastitis. Based on results presented, the concentration of APP present in milk at calving is indicative of the risk of the cow developing mastitis during early lactation. Additionally, should further work support the findings from this study, measurement of APP in milk could provide information on the pathogen type. This has very important implications on treatment of the cow leading to improved targeting of therapy and earlier treatment as the time-consuming and costly procedure of bacterial culturing could be phased out. The concentrations of APP in milk may be used to differentiate between clinical and subclinical mastitis, and may also be a

more sensitive marker of intra-mammary infection than SCC, due to the fact levels take longer to return to baseline levels after the occurrence of an infection, possibly indicating that the pathogen has not yet been eradicated from the udder. Based on the results of our study milk APP, have considerable potential for the use as biomarkers of mastitis. The fact that they can be measured in the milk, eliminates the need for invasive intervention, which usually involves skilled personnel, and presents the basis for a quick, reliable and sensitive diagnostic test for mastitis which could greatly improve the health and welfare of dairy cows.

Chapter IV

Investigation of the Bovine Milk Proteome in Health and Mastitis

4.1 Introduction

Control of mastitis remains a major problem to the dairy industry worldwide. Early diagnosis is paramount if economic loses and adverse animal welfare resulting from mastitis, are to be reduced. Recent studies have identified the presence of APPs such as Hp and M-SAA3 in the milk from cows with mastitis (Eckersall et al 2001; McDonald et al 2001; Pedersen et al 2003; Gronlund et al 2003a) and have prompted further investigation of the effect of this disease on the bovine milk proteome. Using milk as the analyte in a diagnostic test is highly advantageous because of the relative ease of collection by non-invasive methods. Therefore identification and characterization of milk proteins is important in the development of new, clinically useful biomarkers as well as for studying the biochemistry and pathogenesis of disease processes in the mammary gland.

4.1.1 Mastitis Induced Changes in Bovine Milk

Changes in milk composition during mastitis have been widely studied (Kitchen 1981; Watanabe et al 2000). Somatic cell count (SCC) in bovine milk is used as a measurement of mammary-gland health and of milk quality (Munro et al 1984). During infection of the mammary gland SCCs increase as an integral part of the cow's immune defence mechanisms. This occurs alongside alterations in the protein constituents of milk, which includes an influx of serum proteins, such as bovine serum albumin (BSA), due to the increased capillary permeability of the infected gland, resulting in changes which are among the principal features of mastitic milk (Kehrli, Jr. and Shuster 1994; Lindmark-Mansson et al 2000).

Bovine milk from a healthy gland contains 3.9% fat, 3.3% protein, 5% lactose, and 0.7% minerals (Wong et al 1996). Milk proteins include α_{s1}, α_{s2} and κ-caseins, β-lactoglobulin (β-LG), α-lactalbumin (α-LA), BSA, lactoferrin and immunoglobulins (Wong et al 1996). Caseins are the principal proteins in milk comprising approximately 80% of the total protein content. They are grouped together in micelles: spherical structures containing α, β,

and κ casein, and calcium phosphate. It is the casein fraction that gives milk its white-blue colour. It can be removed from milk through acid precipitation at pH 4.6, and is essential to the manufacture of cheese. After precipitation of casein the supernatant is known as 'whey' and β-LG and α-LA are the major whey proteins found in bovine milk accounting for between 7 to 12% and 2-5% respectively of the skim milk total protein. The whey proteins are soluble globular proteins, transparent in solution. β-Lactoglobulin is the most abundant whey protein, followed by α-LA. β-Lactoglobulin is a member of the lipocalin family, and although no biological role for this protein has yet been confirmed, its family origin and its identified similarity to retinol-binding protein have led to suggestions of it having a role in transport (Sawyer et al 1998). Present on the outer surface of this protein is a cavity lined with hydrophobic residues. This hydrophobic cleft is able to accommodate fatty acids like palmitate and stearate, a property, which would be exploited for the removal of this protein from solution. α-Lactalbumin is a small (Mr 14 200), acidic (pI 4-5), Ca^{2+} binding protein, which is essential for lactose biosynthesis. It is one of two components of lactose synthase, which catalyses the final step in lactose biosynthesis in the lactating mammary gland (Permyakov and Berliner 2000).

The reviews of Kitchen (1981) and Munro *et al* (1984) both noted that there was a decrease in casein concentrations during mastitis, largely due to post-secretory degradation of casein by proteinases originating from bacteria, leucocytes or the blood. The activity of the major oxidizing lipolytic and proteolytic enzymes is noticeably higher in mastitic milk than in normal milk. β-casein is hydrolysed into smaller fragments by plasmin, resulting not only in a decrease of total casein, but also an abnormally high ratio of κ-casein to β-casein. However, coupled with the increase in proteins of blood origin entering in milk with high SCC, the overall change in total milk protein is often negligible (Auldist and Hubble 1998). The concentrations of β-Lg and α-LA can drop by more than 70% of their normal level during mastitis. Unlike caseins however the major whey proteins are relatively resistant to proteolytic attack. Their reduction could be due in part to impaired cellular synthetic and secretory function, but is more likely due to leakage of these proteins out of the milk and into the extracellular fluid (Auldist and Hubble 1998). This theory is supported in a study which reported elevated concentrations of α-LA in the blood of cows with increased SCC (Mcfadden et al 1988). Reduction in milk yield is one of the clearest clinical signs of mastitis. The decrease in α-LA leads to a proportional decrease in lactose: the osmotic regulator of milk volume. Inflammatory reactions change the composition of milk in terms of quality and quantity. As the degree of inflammation increases the chemical composition of milk approaches that of blood. This is largely due to the disruption of the integrity of the mammary epithelia by bacterial toxins coupled with the

customary increase in vascular permeability at inflammatory sites and inflammation induced changes in the regulation of milk protein secretion (Watanabe et al 2000).

4.1.2 Proteomics

The term proteome describes the 'total protein complement of a genome'(Wasinger et al 1995) and was coined in 1994 as the linguistic equivalent to the concept of the genome by Wilkins and Williams of Macquarie University, Sydney, Australia. This definition implies a static nature of the proteome similar to that of the genome. In fact the proteome is a highly dynamic system, with the subset of expressed proteins changing in abundance, state of modification, subcellular location etc, in accordance to the physiological state of the cell or tissue (Aebersold and Goodlett 2001). This dynamic range of protein expression makes the identification of the entire proteome a far bigger and more complex challenge than sequencing of the genome (Naaby-Hansen et al 2001). Proteomic studies can be used to obtain a global, integrated view of disease processes leading to the identification of disease specific proteins and discovery of novel drug targets (Page et al 1999; Banks et al 2000). Currently the best established applications of proteomics are in the clinical and biomedical fields where expression differences in the proteomes of tissues or body fluids such as serum can be measured directly and correlated with a particular disease (Blackstock and Weir 1999). Proteomics is a technology that integrates the significant advances in two-dimensional electrophoretic separation of proteins, mass spectrometry and bioinformatics (Page et al 1999), (Figure 4-1).

4.1.2.1 Two-dimensional electrophoresis

Study of the proteome dates back to the 1970s when it was demonstrated that it was possible to separate proteins based on their isoelectric points (pI) and molecular weights (Mr) by electrophoresis on polyacrylamide gels (O'Farrell 1975). Although the techniques have been available for over 2 decades, recent advancements in the technology including the availability of immobilized pH gradients (IPG) in the first dimension have led to the production of reproducible two-dimensional gels, suitable for large scale analysis of protein expression and termed two-dimensional electrophoresis (2-DE) (Gorg et al 2000; Chich 2001). Immobilised pH gradient gels are prepared by covalently linking a small number of chemically defined molecules to the polyacrylamide backbone, resulting on a series of buffers distributed throughout the pH range of the strip. These allow attainment of true equilibrium throughout the focusing stage, which greatly increases the reproducibility

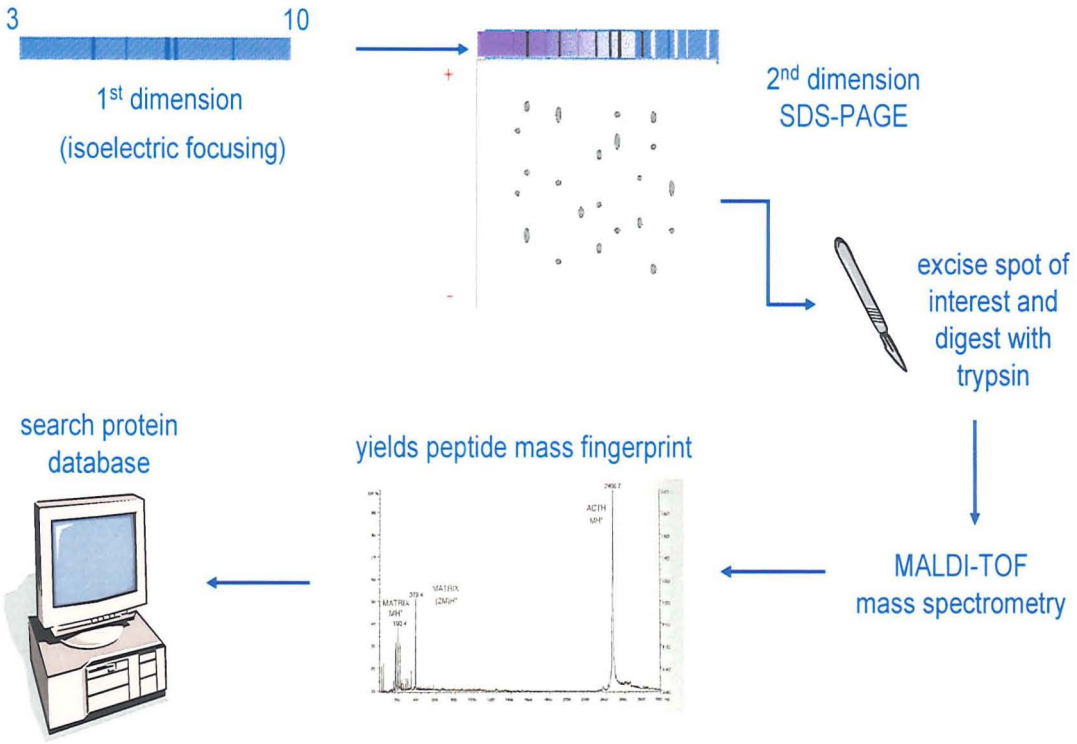


Figure 4-1: Two- dimensional Electrophoresis coupled with mass spectrometry and database searching. The proteins are separated in the first dimension by isoelectric focusing on a plastic backed polyacrylamide strip containing an immobilized pH gradient. On application of a current the polypeptide subunits migrate in an electric field until they reach their isoelectric point resulting in an IPG strip with protein bands across its length. The strip is then applied to a SDS-PAGE gel and the proteins are separated in the second dimension by molecular weight. The 2-DE gel is stained with colloidal Coomassie and the spots of interest are excised from the gel, digested with trypsin and analysed by MALDI-MS. The resultant peak list from the peptide mass fingerprint is subjected to computer database searching.

of 2-DE (Gorg et al 2000; Pennington and Dunn 2001). Separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension is based on the original method (Laemmli 1970). Reproducibility and resolution have been increased with the introduction of ready-made pre-cast gels, including gradient gels, however financial considerations and relatively short shelf-life frequently drive researchers to cast their own gels (Lefkovits 2003). Two-dimensional electrophoresis can potentially resolve up to 10,000 protein spots, dependent on sample preparation and detection strategy (Jenkins and Pennington 2001). The protocol for 2-DE is very well developed and has recently been reviewed (Gorg et al 2000). However, no single method of sample preparation can be applied universally due to the diverse nature of samples that are analysed by 2-DE. In general pre-fractionation procedures for 2-DE should be kept as simple as possible, target different molecular properties, and employ solutions that are compatible with downstream separation methods in order to minimize sample loss and the chance of degradation or introduction of artefactual protein modifications (Naaby-Hansen et al 2001). It is now generally accepted that a combination of chaotropes (ie. urea and thiourea) and zwitterionic detergents (i.e. CHAPS) are essential in the extraction buffer to solubilise most proteins from a wide range of organisms (Rabilloud et al 1997). Two-dimensional electrophoresis (2-DE) is a powerful tool for analysing complex protein mixtures and remains the core technology of choice in the majority of proteome projects, however there are limitations with the usage of this technology, including the analysis of hydrophobic and/or membrane proteins, low abundance proteins and very basic proteins (Blackstock and Weir 1999). Additionally, 2-DE includes many steps of manual handling (sample preparation, gel casting and gel staining). Furthermore the complete analysis of 1 gel including mass spectrometry and data analysis can take up to 3 days (Hille et al 2001). Automated proteome analysis is necessary to improve speed but also to reduce human contamination of samples (e.g. by keratin) (Rabilloud 2002).

4.1.2.2 Mass Spectrometry

Mass spectrometry (MS) is an essential tool in the field of proteomics. Identification of protein spots separated by 2-DE remained laborious until the 1990s when biological MS had developed into a sufficiently sensitive and robust technique (Mann et al 2001). The introduction in the late 1980s of two new 'soft' ionization methods: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) fundamentally changed the analysis of proteins (Blackstock and Weir 1999; Aebersold and Goodlett 2001; Mann et al 2001; Pennington and Dunn 2001; Aebersold and Mann 2003).

Mass spectrometers have 3 basic components 1) an ionization source, 2) a mass analyzer, and 3) a detector. They measure the mass to charge ratios (m/z) of ions under vacuum, and various combinations of ionization sources and mass analyzers are available. The time-of-flight (TOF) mass analyzer most commonly used with MALDI is robust, simple and sensitive and has a large mass range (Aebersold and Goodlett 2001). Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry is the method of choice for obtaining a peptide mass fingerprint (PMF), which in conjunction with a protein database is a powerful method for the identification of protein spots (Blackstock and Weir 1999). The set of masses generated by mass spectrometric analysis of a digestion of a protein with a specific protease (i.e trypsin) can act as a fingerprint, and this property is unique to that protein (Yates et al 1993; Pappin et al 1993). Trypsin, specifically cleaves the peptide bond at the C-terminus of arginine and lysine residues, except if they are followed by a proline and is the enzyme commonly used for the digestion of proteins. To generate gas phase, protonated molecules, the analyte is dissolved in a large excess of matrix, typically a small organic energy-absorbing molecule, most commonly α-cyano-4-hydroxycinnamic acid (CHCA). The mixture is then irradiated, by a pulse laser to generate a short burst of ions. The ions are accelerated to a fixed amount of kinetic energy and travel down a flight tube, therefore the arrival time of the ions at the detector is inversely proportional to their m/z ratio, producing the TOF spectrum (Mann et al 2001; Pennington and Dunn 2001).

4.1.2.3 Database searching

In order to identify the proteins, the set of peptide masses (PMF) obtained from MALDI-MS are extracted and subjected to database searches (Yates 1998). A number of search engines have been developed to enable the rapid searching of protein and nucleotide sequence databases with PMF data. They all work on the common principles of comparing the masses of the measured proteolytic peptides to the theoretically expected tryptic peptide masses for each entry in the database. A score is then calculated which reflects the accuracy of the peptide matching (Fenyo 2000; Mann et al 2001). There are a number of search engines available on the internet (Table 4.1), each of which is continually evolving in size and technology. All are based on the principles outlined above, the recent addition of more sophisticated scoring algorithms, has increased the sensitivity and specificity of protein identification (Perkins et al 1999; Mann et al 2001). The MOWSE scoring system implemented in the MS-FIT, MASCOT® search engine takes into account the relative abundances of the peptides of a given mass in the database when calculating the score, so that the chance of getting a random match to a larger peptide is lower and therefore it will

Name	Internet Location
PepSea	http://www.pepsea.protana.com/PA_PepSeaForm.html
PeptIdent/Multident	http://www.expasy.ch/tools/peptident.html
MS-Fit	http://prospector.ucsf.edu/ucsfhtml3.2/msfit.html
ProFound	http://www.proteometrics.com/prowl-cgi/ProFound.exe
MASCOT®	http://www.matrixscience.com/cgi/searchform.pI?SEARCH=PMF

Table 4-1: List of search engines available for protein identification from peptide mass fingerprint data

contribute to a higher degree to the score (Pappin et al 1993). The second feature of the MASCOT® is that it uses probability based scoring. The probability that the resultant match is a random event is calculated for each protein sequence in the database and the proteins are then ranked with decreasing probability of being a random match to the experimentally obtained data (Fenyo 2000). This is particularly effective in the reduction of false positive results (Perkins et al 1999).

Protein identification by PMF analysis has a high degree of success (50-90%) in organisms with fully sequenced genomes (Mann et al 2001). However, limitation of proteomic based studies becomes apparent with the poor success rate in identification of proteins from species whose genomes are not yet fully sequenced (Lester and Hubbard 2002). At present the success rate for identifying proteins from microorganisms such as *E.coli* with fully sequenced genomes (OBrien 1997) remains higher than that from mammalian studies (Fenyo 2000).

4.1.2.4 Proteomic studies of milk

The milk proteome has previously been investigated with the first reports dating back to as early as the 1980s. Early studies utilized the basic 2-DE system proposed by O'Farrell (1975) to identify the major components of bovine and human milk (Anderson et al 1982; Holt and Zeece 1988). As this was before the use of mass-spectrometry in conjunction with 2-DE, identification of the proteins was based on the comparison of the Mr and pI values obtained for each spot to existing literature on such properties of milk proteins. Hence due to the limitations in the technology at this stage, there was no scope for the identification of novel milk proteins. However N-terminal sequence tagging has been used in conjunction with data-base searching to identify whey proteins of wallaby milk (Molloy et al 1997). The coupling of MS with 2-DE in the 1990s has revolutionized proteomic studies and as a result most of the publications in this field are in the last 7 years. Casein is the major protein component of bovine (Goldfarb 2001) and human (Goldfarb 1999) milk. The overpowering effect of casein on the milk proteome is evident in all 2-D images of whole milk that have previously been shown (Galvani et al 2000; Galvani et al 2001). This poses a problem in identification of additional components of the bovine milk proteome, in particular low abundance proteins. Several studies have exploited pre-fractionation techniques to remove casein from the milk allowing examination of the remaining 'whey' proteins of lower abundance. One such study reported the identification of several minor proteins such as fatty acid binding protein, prealbumin, clusterin and serotransferrin by N-terminal sequence analysis following the removal of casein, α-LA, lactoferrin and

secretory IgA (Murakami et al 1998). A similar approach was taken in an investigation of the bovine whey fraction, but IgG was also removed leading to a further depletion of the major proteins (Yamada et al 2002). Here albumin, serotransferrin and lactoferrin were found in the low abundance fraction of mature milk, though several more proteins were found in bovine colostrums including β-fibrinogen, chitinase and α-antitrypsin. Although there have been previous investigations of the bovine milk proteome, the focus has been either on the caseins of the milk, which are of interest to the manufacture of cheese, or in the differences between the proteins expressed in whole milk from those expressed in colostrums. To date there is only one study using modern proteomic methods to investigate the changes induced in bovine milk through mastitis (Baeker et al 2002). After removal of casein by acid precipitation the milk from 7 quarters with sub-clinical mastitis was compared to milk from healthy quarters and the appearance of lipocalin-type prostaglandin D synthase in mastitic milk was observed and proposed as a possible biomarker of mastitis.

It is evident from this review of the literature devoted to the field of milk proteomics that few studies have been performed, most of which have been in the recent past. This is probably due to improvements in the necessary technology that have occurred in the last few years and it is expected that further developments will lead to an increase in research activity on the analysis of the milk proteome.

The importance of proteomic analysis is its ability to study post-transcriptional control as well as post-translational modifications (PTMs) such as phosphorylation, glycosylation, acylation and methylation. Since the major milk proteins are known to be extensively post-translationally modified (Anderson et al 1982), a feature that is likely to influence the function of the protein, targeting research at the protein rather than the gene level will result in a better understanding of the biological phenotype in connection with a particular disease state.

The aim of this study was to use proteomic methods to identify the differential protein expression in bovine milk during clinical mastitis. The specific objectives were: 1) to optimise the best solubilisation method for proteins from bovine milk 2) to maximise sensitivity and reproducibility of proteomic analysis 3) to determine a pre-fractionation method compatible with 2-DE methods and resulting in effective removal of highly abundant milk proteins 4) to identify changes in the protein profile of bovine milk associated with clinical mastitis, and finally to identify novel protein markers of clinical mastitis.

4.2 Materials and Methods

4.2.1 General Reagents

General chemicals and other materials were obtained from obtained from Sigma-Aldrich Co Ltd, Dorset, UK, unless otherwise stated.

4.2.2 Milk Samples

Milk samples were collected from a commercial dairy farm in the West of Scotland. Mastitis was identified on the basis of clinical signs (heat, pain, redness and swelling of the udder, or clots in the milk).

4.2.3 Sample Preparation for 2-DE

Sample preparation is the first step in any proteome analysis study. The solubilisation protocol should be optimised in order to give reproducible and clearly resolved 2-D gels. Whole milk was separated by centrifugation at 4000g for 20 min at 4°C, and the lipid- rich cream supernatant and the cell debris pellet were discarded. In the first instance the caseins were precipitated by acidification of skim milk to pH4.6 with 1M HCL (Molloy et al 1997) and removed by centrifugation (4000 x g, 4°C, 20min). The pH of milk decreases with mastitis so the volume of HCL required, to precipitate the casein varied between samples. This variable was eliminated through replacing the acid precipitation method used in previous studies (Molloy et al 1997; Baeker et al 2002) with ammonium sulphate fractionation. In the final protocol the caseins, which comprise approximately 80% of the total protein content in milk were precipitated by ammonium sulphate fractionation by addition of saturated ice-cold $(NH_4)_2SO_4$ to the milk samples in an ice bath to a final concentration of 35% (v/v) of $(NH_4)_2SO_4$. After centrifugation at 4000g for 20 min at 4°C, the supernatant (milk whey) was removed and dialysed against deionised water at 4°C overnight. The protein content of the milk whey sample was determined (bicinchoninic acid), using bovine serum albumin as a standard. The whey samples ranged in protein content from 2.5 mg/ml to 10 mg/ml. Each sample was diluted in rehydration buffer (8M urea; 4% w/v, CHAPS; 0.5% v/v ampholyte and 100mM DTT), to give a final sample volume of 300µl containing 500μg total protein.

4.2.4 Two-dimensional Gel Electrophoresis

Samples were applied by rehydration on non-linear immobilized IPG strips (17cm, pH 3-10 or 4-7 BIO-RAD, Hemel Hempstead, UK), and focussed in a BIO-RAD Protean IEF Cell for 74750 volt hours (Vhrs) using the following voltage rampage programme: 50V, 500Vhrs; 500V, 250Vhrs; 2000V, 1000Vhrs; 5000V, 5000Vhrs; 8000V, 8000Vhrs; 10000V, 60000Vhrs.

IPG strips were equilibrated in SDS equilibration buffer (50mM Tris-Cl pH 8.8, 6M urea, 30% v/v glycerol, 2% w/v SDS, .001% w/v bromophenol blue) containing 1% (w/v) dithiothrietol for 15mins in the first step followed by 2.5% (w/v) iodoacetamide in equilibrium buffer for 15mins in the second step. The IPG strips were then run in the second dimension in 12% polyacrylamide gels using 1.5M Tris-HCl, pH 8.8, as separating gel buffer and Tris-glycine-SDS, pH 8.3, as electrode buffer, in a BIO-RAD Dodeca Cell. Gels were run at 200V for 7 hours.

4.2.5 Gel Staining and Image Acquisition

Polyacrylamide gels were stained using colloidal Coomassie blue G dye according to manufacturers instructions (Sigma-Aldrich, Dorset, UK), and destained in 10% (w/v) acetic acid in 25% (v/v) methanol for 60 seconds and then for a further 24 hours in 25% (v/v) methanol. Gel images were acquired using a UMAX PowerLookIII scanner (UMAX Technologies Inc, CA, USA) under the following settings: transmissive mode, true colour RGB and a resolution of 600dpi.

4.2.6 In-gel Trypsin Digestion and Mass Spectrometry

Spots of interest were excised with a scalpel blade from the gel, and washed with 300µl of deionised water for 15mins to remove residual destain solution. Coomaasie blue stain was removed from each excised gel piece by washing with 50% (v/v) acetonitrile in 100mM ammonium bicarbonate and drying in a Speedvac evaporator (Jencons-PLS Leighton Buzzard, UK). Each gel piece was rehydrated in 12µl of 20mM ammonium bicarbonate containing 12.5µg/ml of modified porcine trypsin (Promega, Southhampton, UK). After 30 minutes, more 20mM ammonium bicarbonate was added to any gel pieces that required further rehydration. The gel pieces were subsequently incubated at 37°C overnight with shaking. The supernatants from the tryptic digests were desalted using C18- ZipTips^{tm}, according to the manufacturers instructions (Millipore, Watford, UK). Two micolitres of

the supernatant were then crystallized in a matrix consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) prepared in 50% (v/v) acetonitrile containing 0.1% (v/v) aqueous trifluoroacetic acid (TFA), and applied directly to the MALDI plate. Samples were left to dry at room temperature before being analysed by MALDI mass spectrometry. MALDI-MS was performed on a Voyager DE Pro (Perseptive Biosystems, CA, USA). Sufficient transients were collected to give an acceptable sound to noise ratio.

4.2.7 Protein Identification Search Engines

The peptide mass fingerprint, obtained by MS was subjected to search by pasting the peptide masses into a protein database (MASCOT, Matrix Science), which uses a probability-based scoring system to define a significant match. The spectra were first baseline corrected, smoothed, calibrated against internal trypsin autolysis peptides and de-isotoped. The threshold was set to allow approximately 15 peaks/spectra to be saved. Search parameters were consistent for all proteins spots that were analyzed throughout the study (Table 4.2). The data entry form for MASCOT® which allows selection of these parameters is given as an example (Figure 4-2 and Table 4.3).

4.2.7.1 The NCBInr database

The NCBInr database contains the translated protein sequences from the entire collection of annotated DNA sequences kept at GenBank, and also protein sequences in the Protein data Bank (PDB). SWISS-PROT and Protein Information Resource (PIR) databases, covering most of the publicly available data.

4.3 Results

Milk whey proteins from normal cows and those with clinical mastitis, were separated by 2-DE using two different pH range IPG strips (pH 3-10 and pH 4-7) in the first dimension, and 12% (w/v) polyacrylamide gels in the second dimension. After staining of the gels with colloidal Coomassie blue, the patterns of separated proteins were revealed. Separation of the milk proteome using a broad range IPG strip (pH 3-10) in the first dimension showed clear differences (by visual inspection) between the proteins expressed by normal and mastitic milk (Figure 4-3). However with complex samples, such as milk employed on this study, 2-DE using a broad-range pH gradient reveals only a small percentage of the

Parameter	Selected Option
Database	NCBInr
Enzyme	Trypsin
Protein mass range	0 to 3000KDa
Tolerance	50ppm
Missed cleavage	1
Fixed modification	Carboxymethylation
Variable modification	Oxidation of methionine
Charge state	MH^+

Table 4-2: Parameters selected for database searches using MS data. These parameters were used with the MASCOT® search engine

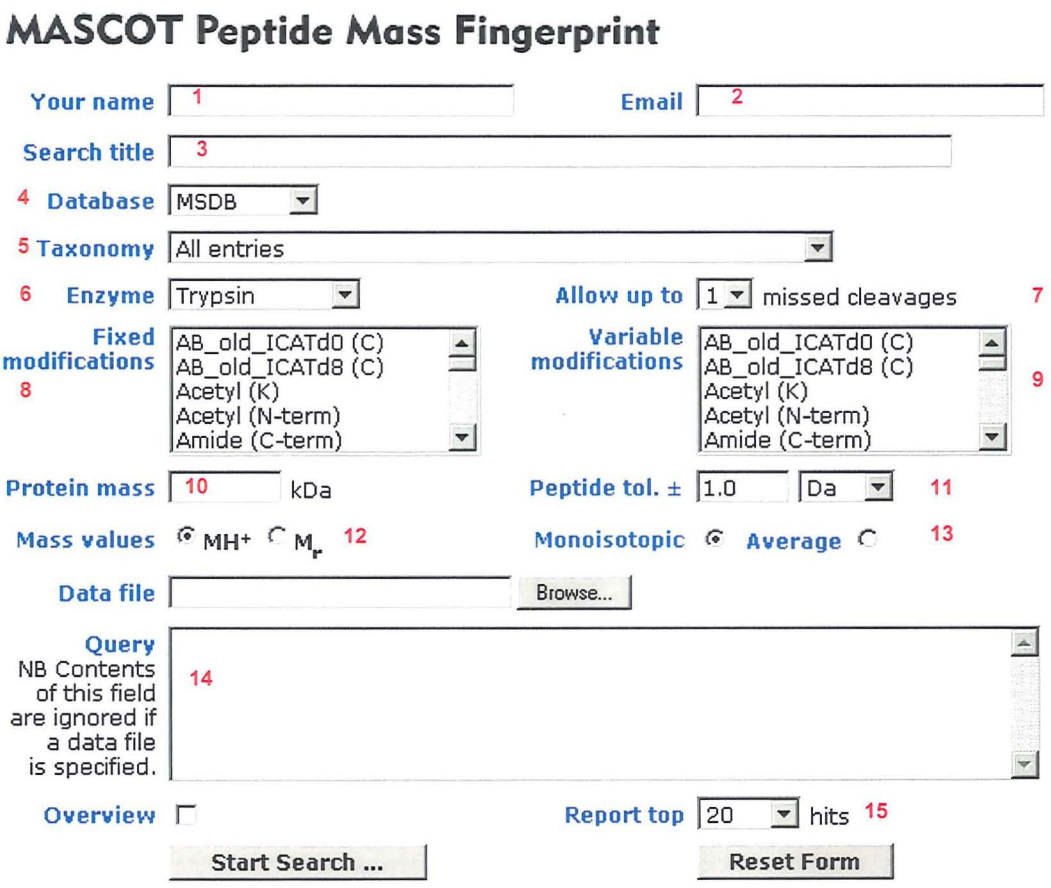


Figure 4-2: Peptide Mass Fingerprint search form from the MASCOT® search engine. Each parameter is labelled with a number and described in table 4.2. Reproduced from http://www.matrixscience.com

parameter number	parameter description
1	Enter your name and e-mail address. The search results will be e-mailed to you if the connection is lost during the search.
2	
3	Identify the sample (e.g. spot number) to keep track of mass lists
4	Select database, NCBI was first choice in this study
5	Select taxonomy. Higher scores will be achieved with narrower search parameters. Start with closest related taxa and broaden the search as needed
6	The enzyme used to digest the protein should be entered
7	One missed cleavage is often observed with in-gel trypsin digestion
8	Enter modifications that will occur on all the proteins (e.g. acetyl N-term) due to equilibration with iodoacetamide
9	Oxidation of methionine may occur with in-gel trypsinization
10	Enter the probable mass of the protein that generated the peptide. Preferably through comparison with molecular weight markers on the 2-D gel image.
11	With internal calibration of trypsin the peptide mass accuracy is within 50ppm (0.1 Da)
12	For MALDI data the peptides in the mass list are protonated (MH^+)
13	A monoisotopic mass list is generated
14	The peptide mass list is pasted here
15	Enter the number of hits that you would like the search engine to report.

Table 4-3: List of parameters for selction prior to database searching.

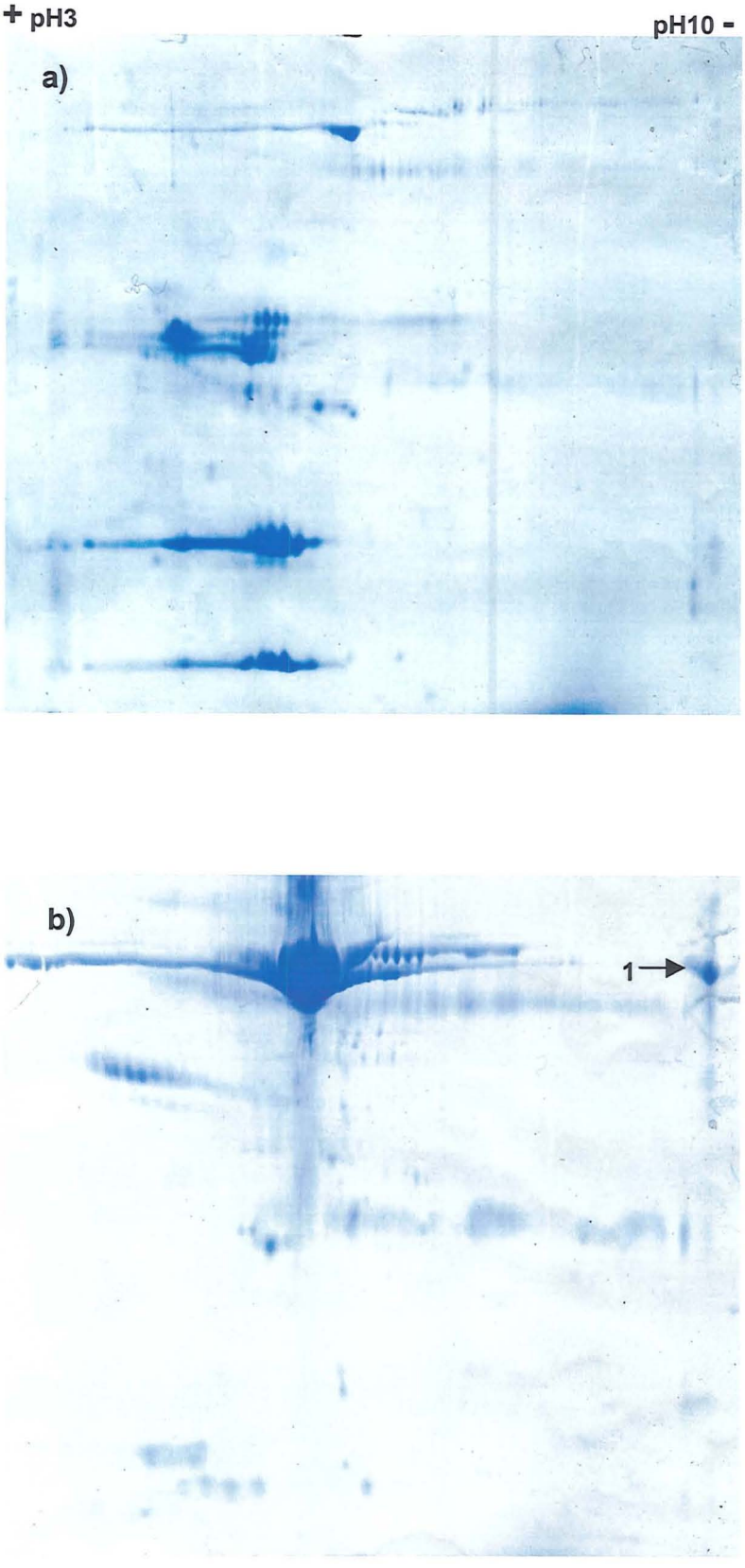


Figure 4-3: 2-D gels of normal whey a) and mastitic whey b), pH 3-10. Broad range IPG strips (17cm) (pH 3-10) were used for isoelectric focusing in the first dimension; the gels were stained with colloidal coomassie. Protein spot 1 indicated by an arrow (image b) was differentially expressed between the two milk samples. As this spot, lies out with the 4-7 pI range of the images shown in Figure 4-4, it was annotated in this figure instead, and described in table 4.3.

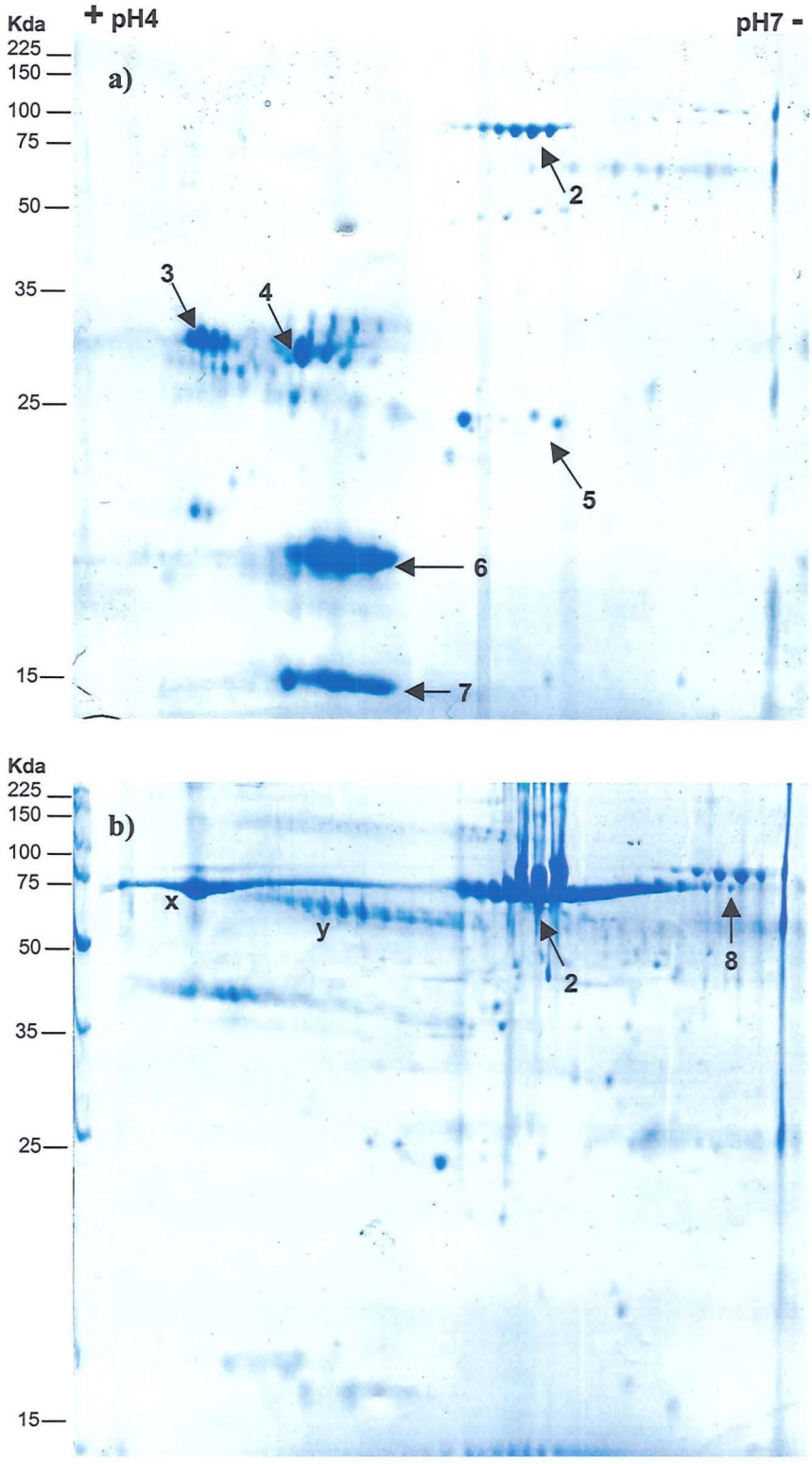


Figure 4-4: 2-D gels of normal a) and mastitic b), pH 4-7 IPG strips (17cm) were used for isoelectric focusing in the first dimension, the gels were stained with colloidal coomassie. The numbered protein spots were digested and the resultant tryptic peptides were analysed by MALDI-TOF MS. The identities of the spots are summarised in table 4.4.

whole proteome because of insufficient separation. The first proteome maps can be considered useful to give a general picture of the protein distributions over pH 3-10, but the number of protein spots resolved was limited. Improved resolution was obtained by using a narrower range IPG strip (pH 4-7) in the first dimension, as shown for one milk sample in Figure 4-4. As a result almost all of the protein identification using MALDI-MS was performed on the gels separated using pH 4-7 in the first dimension. One differentially expressed protein spot however was visible at the basic end of the mastitic milk when separated over a pH gradient of 3-10 (Figure 4-3b) and is indicated with an arrow (spot 1). This spot was the only spot excised from this gel and analysed by MALDI-MS. Mass spectra were acquired by MALDI-MS for spots 2 to 8 on the pH 4-7 gels (Figure 4-4), producing a PMF, an example of which is shown in Figure 4-5. The monoisotopic mass list obtained from the PMF was used to search the computer database producing a histogram (Figure 4-6) from which a significant score can be distinguished from an insignificant score. The results obtained from searching the NCBInr database from spot 1 in Figure 4-3b and those from figures 4.4a and b are summarised in table 4.4.

4.3.1.1 Identification of Major Proteins in Milk

Figure 4-4 gel a) shows the 2-DE pattern of normal bovine whey. The major whey proteins (β-lactoglobulin and α-lactalbumin), caseins and albumin were identified in normal whey (Figure 4-4a). Unnumbered spots were subjected to MALDI-MS but were not successfully identified probably because the sequence data for these protein spots is not currently available in the databases, or there was insufficient concentration of protein in the sample. Figure 4-4b displays the changes taking place from mastitic milk. The high molecular mass region of the gel shows an increase in the proteins mainly of blood serum origin, including albumin and serotransferrin. Albumin and serotransferrin labelled as spots 2 and 8 respectively, in both a) and b), was much increased in mastitic milk. Serotransferrin was not identified by MALDI-MS in the normal milk displayed in Figure 4-4 a), however it does appear to be present but in very low abundance in this milk sample, which explains why its identity was not confirmed by mass spectrometry. The large intense spot (x), at the acidic end of Figure 4-4, gel b, and at a similar molecular mass to albumin, and also the less intense chain of spots (y) seen in this area but at a slightly lower molecular mass to albumin, were not identified by MALDI-MS.

Proteins in the middle molecular mass region of gel a, displaying the normal milk proteome, were shown, to be caseins despite the precipitation of the majority of such protein by ammonium sulphate during sample preparation. However examination of the

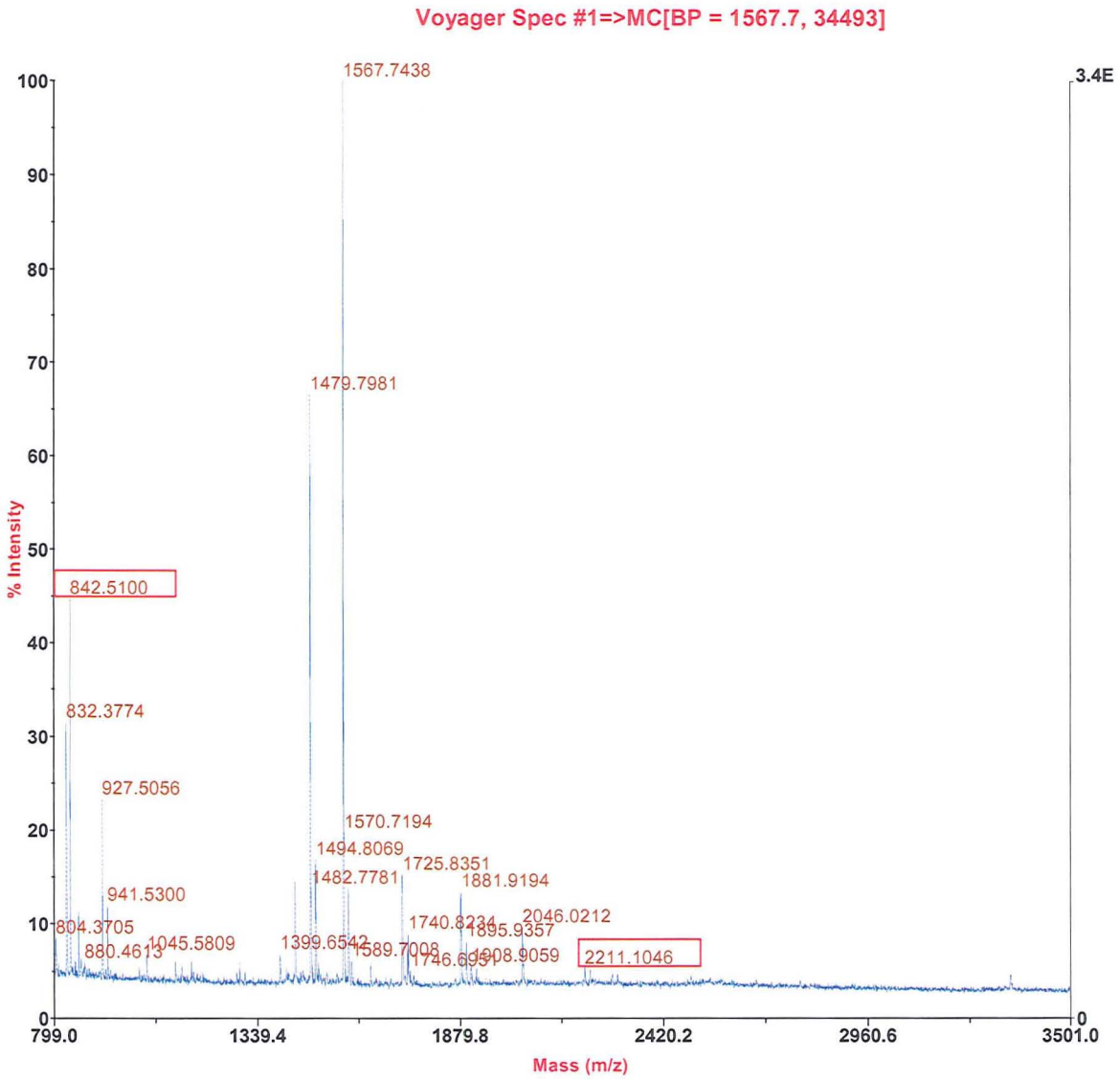


Figure 4-5: Example of a MALDI mass spectrum. The peptide mass fingerprint was derived from the in-gel digestion of protein spot 2. The typical spectrum was obtained by MALDI-MS in reflectron mode using α-cyano-4-hydroxycinnamic acid as the matrix. Two hundred laser shots were acquired and the resultant peptide mass fingerprint (PMF) shown above was internally calibrated using the porcine trypsin autolysis peptide peaks as highlighted at 842.510 and 2211.1046 Da $[M+H]^+$

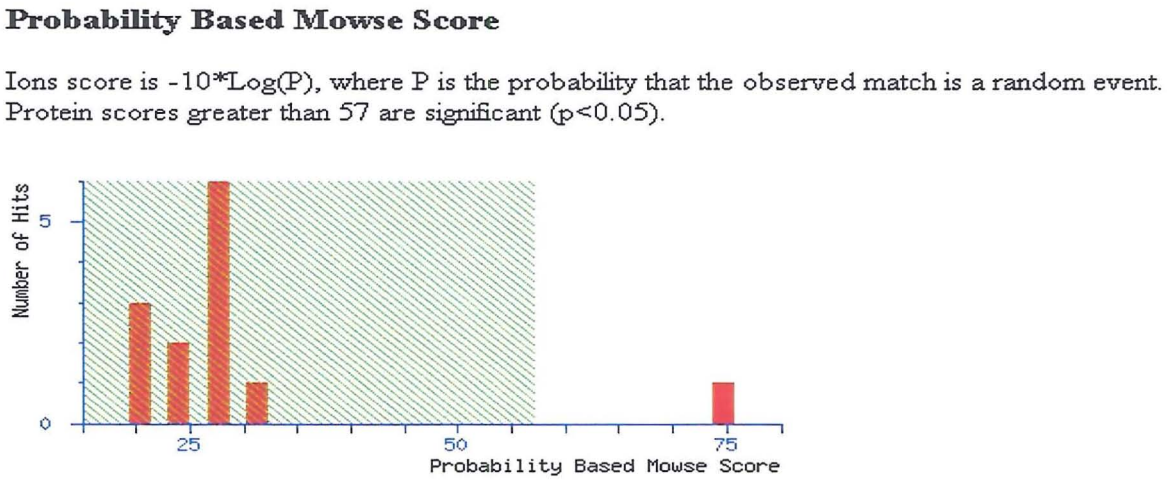


Figure 4-6: Score histogram for a typical peptide mass fingerprint. Mascot incorporates a probability based implementation of the Mowse algorithm (Perkins et al 1999). Only scores outwith the green region are significant. Reproduced from http://www.matrixscience.com/

Spot ID	Name	pI/Mr	Score
1	Microsomal triglyceride protein *(Bos taurus)*	8.74/98970	53
2	Bovine serum albumin	5.82/69278	155
3	αS_1 Casein *(Bos taurus)*	4.98/24523	207
4	β-casein A_3 - bovine	5.26/25082	52
5	κ-casein	6.29/21269	124
6	β-lactoglobulin	4.93/19908	105
7	α-lactalbumin	4.9/16246	115
8	Serotransferrin precursor *(Bos taurus)*	6.8/77703	116
9	Lactadherin precursor (milk fat globule-EGF factor 8)	6.81/47411	78
10	Annexin 1	6.44/39200	55
11	Annexin 4	5.55/34938	38
12	Apolipoprotein A-1 precursor (Apo-A1) (Bovine)	5.71/30258	98
13	Bactenecin	7.57/17931	104

Table 4-4: Molecular mass (Mr), isoelectric point (pI) and database score for protein spots successfully identified by MALDI-MS

similar area of the mastitic milk (Figure 4-4b) shows that mastitis induces obvious changes in the caseins of milk. Two large intense spots at the lower molecular mass region of the gel of normal milk (Figure 4-4a) were identified by MALDI-MS as the major whey proteins β-LG (spot 6) and α-LA (spot 7). In whey from cows with severe clinical mastitis, the concentration of both these proteins was greatly reduced (Figure 4-4b)

4.3.2 Milk Isoforms

The 2-DE gel images showed the presence of several proteins as multiple spots (Figure 4-7). The appearance of such beads of spots, with similar molecular mass but different pI values is indicative of post-translational modifications such as glycosylation or different protein isoforms.

4.3.3 Casein Pellet

The 2-DE gel of the casein pellet after ammonium sulphate fractionation of the milk (Figure 4.8) shows caseins to be the major proteins, and the obscuring effects that these abundant proteins would have on other spots within the milk proteome.

4.3.4 Identification of Lower Abundance Milk Proteins

The bulk of the identified proteins are shown as intense spots, the majority of which have pI values within the relatively narrow range of 4-9 to 5-2. Throughout this study the major protein spots in the milk of both normal and infected samples were reproducible on 2-DE gels. However given the nature of the milk samples it was expected that a greater number of protein spots would have been observed. Some of the 2-D gels of milk did contain a greater number of protein spots, primarily of lower abundance, and higher pI values than the major spots observed in the milk proteome, although greater variation between samples was observed with such spots. Figure 4-9 examines closely the changes observed in the lower abundance proteins of milk during mastitis as compared to normal milk. Comparison to previous works show that many of these proteins are likely to be casein fragments (Yamada et al 2002) and bovine serum albumin fragments (Baeker et al 2002). Protein identification of the spots in figures 4-9 and 4-10 using MALDI-MS was only successful in identifying a few of the spots. The proteins identified by MALDI-MS in this region of the gel included annexin-I, annexin-IV, apolipoprotein A-1 precursor, lactadherin precursor and bactenecin, all of which were only identified in the disease state, and are indicated by an arrow. Spot 9 in Figure 4-10 was identified as lactadherin precursor protein

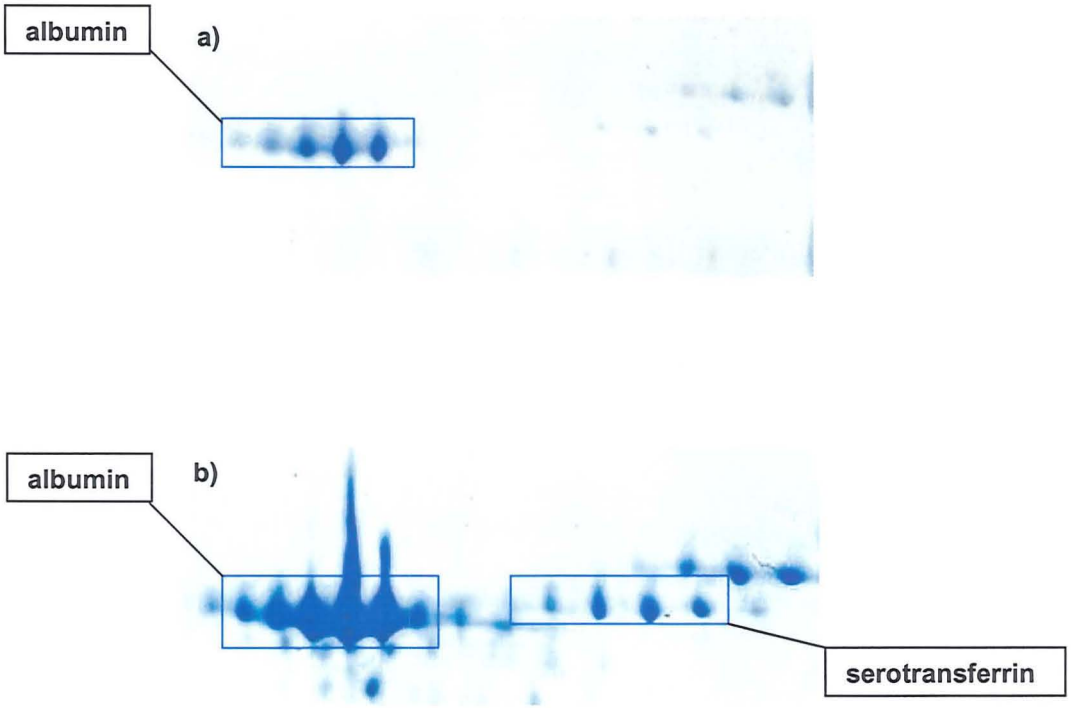


Figure 4-7: Zoom view of 2-DE gels from normal milk (a) and mastitic milk (b) at pH 5.5 to 7 and molecular weight 100kd to 65kd. The close-ups show the characteristic pattern of protein isoforms with similar molecular weight but slight variations in pI. The increased concentration of albumin and serotransferrin is again observed in the infected milk sample.

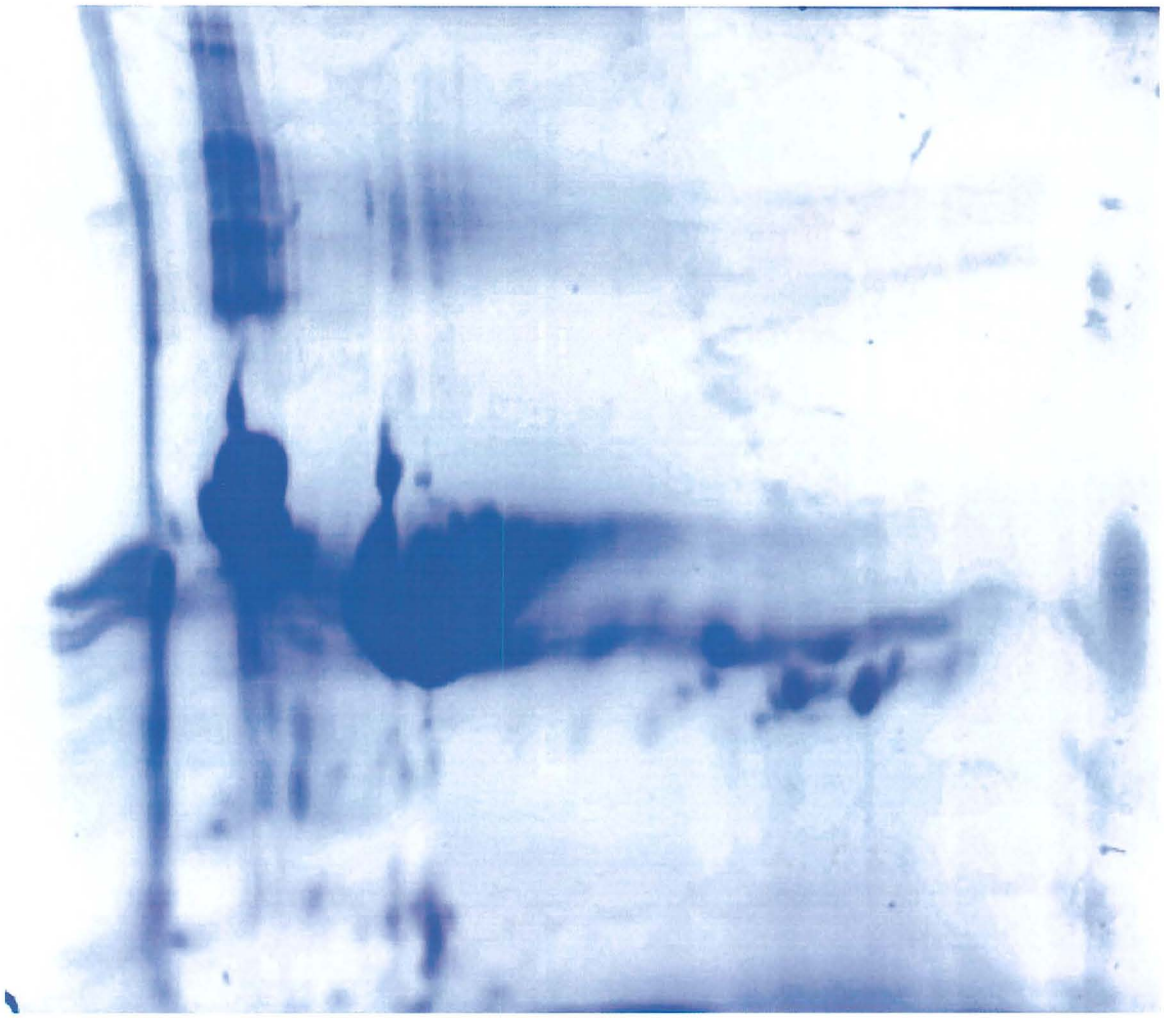


Figure 4-8: 2-DE gel of casein pellet of milk after ammonium sulphate precipitation

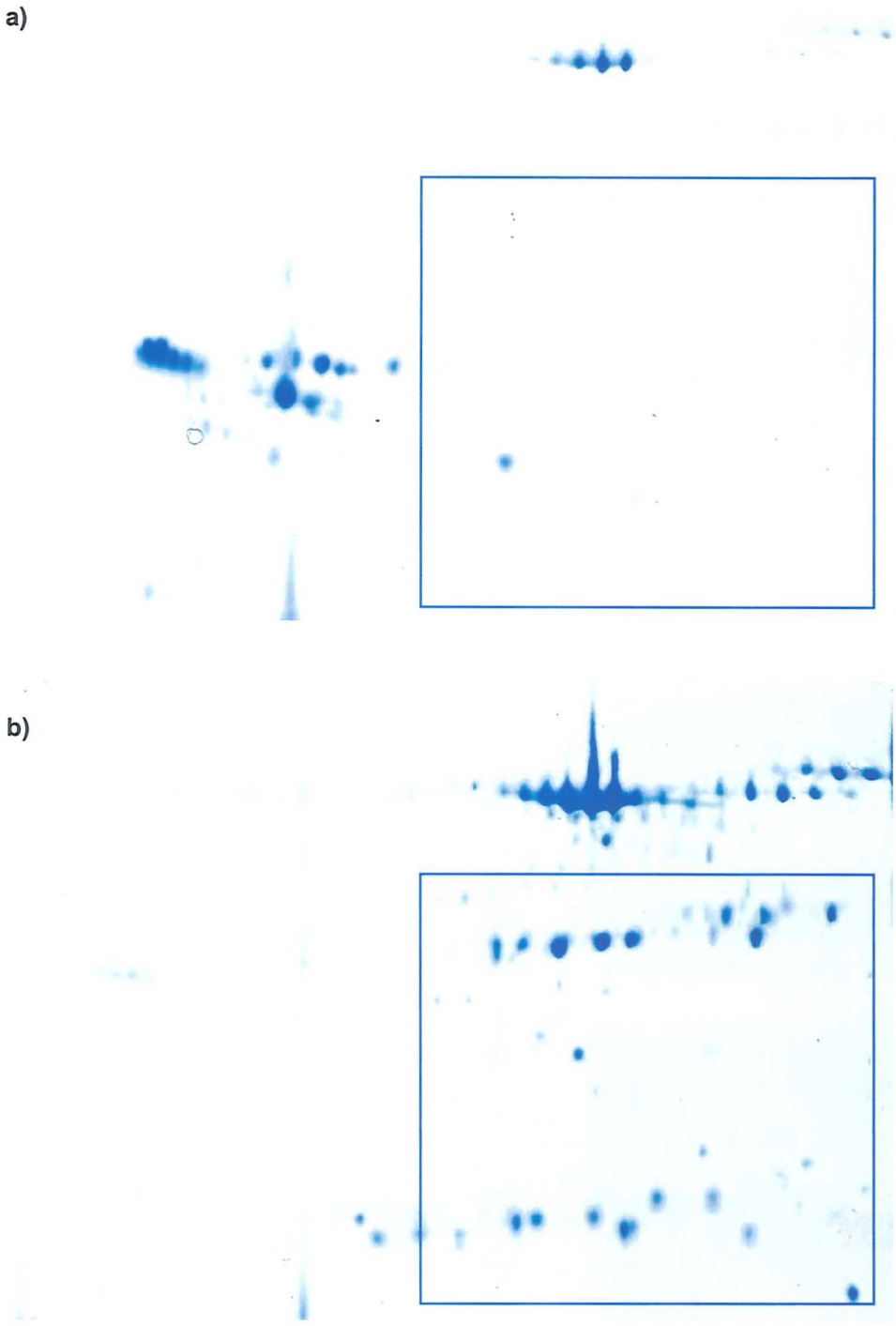


Figure 4-9: 2-DE gels from normal milk (a) and mastitic milk (b), highlighting areas of interest which are described in detail in Figure 4-10

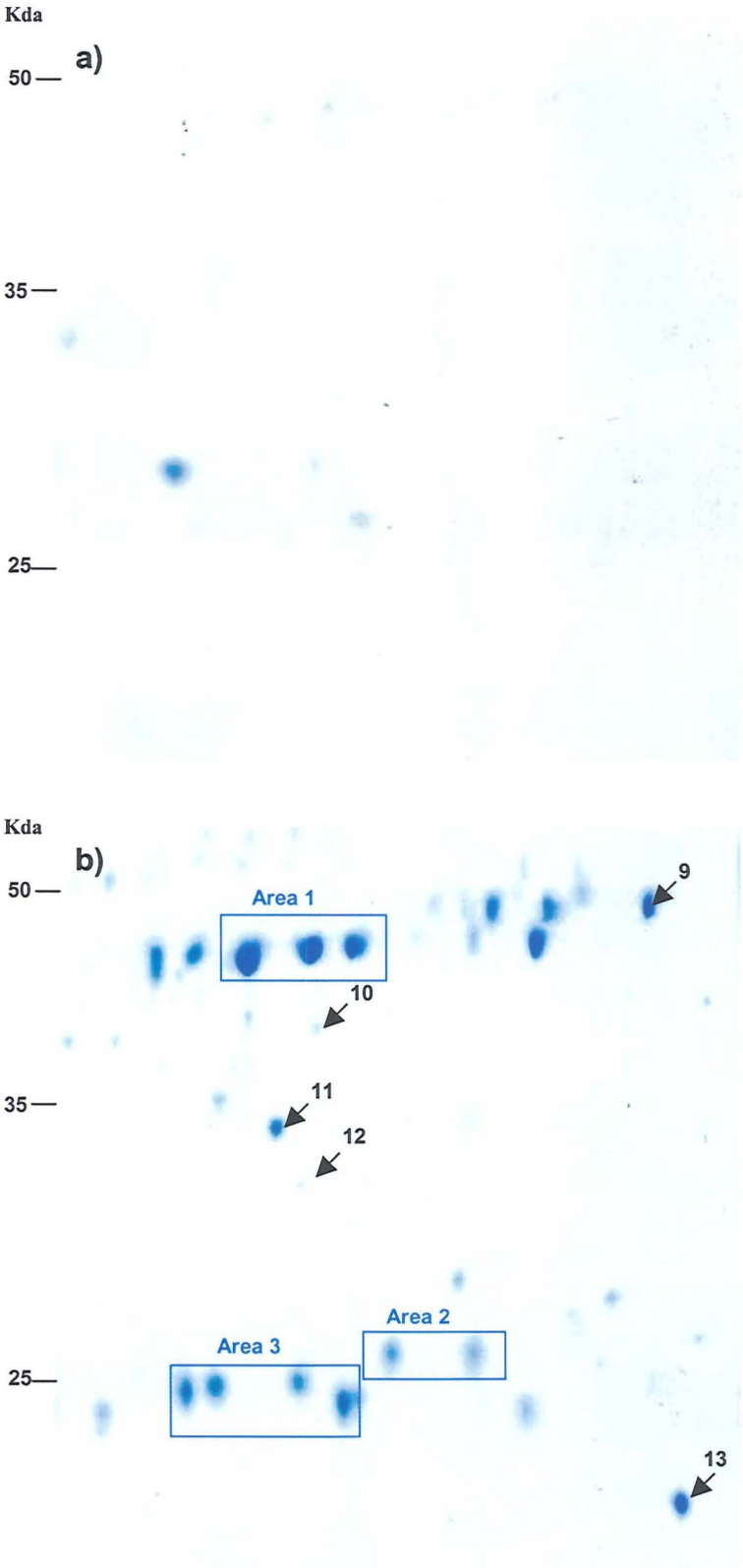


Figure 4-10: Zoom view of 2-DE gels from normal milk (a) and mastitic milk (b) at pH 5.5 to 7 and molecular weight 100kd to 65kd. Examination of the lower abundance proteins in milk reveals that there are several such proteins present in mastitic milk but not in normal milk. Protein spots identified by MALDI-MS are numbered and spots in the boxes are assigned speculative identities through comparison with previous works.

(pI 6.81, Mr 47411). Spots 10 and 11 were identified as bovine annexins I and IV with a pI/Mr of 6.44/39200 and 5.55/34938 respectively. Both of these spots were only observed in infected milk, and annexin IV is in greater abundance than annexin I. Apolipoprotein A-1 (ApoA-I) (pI 5.71 and Mr 30258), was identified as the very faint spot 12. It is possible that ApoA-I is also present in normal milk, as there was a very faint spot lying at the correct pI and Mr in the 2-DE gel of normal milk (Figure 4-10a), however mass spectrometry for this spot did not produce a significant identity. Bactenecin (pI 7.57 and Mr 17931), an antimicrobial peptide was identified strongly as spot 13, and is also absent from normal milk.

The remainder of the spots were given a speculative identity through comparison to previously published 2-DE gels of milk. Proteins in areas 1, 2 and 3 of Figure 4-10 gel b were identified tentatively through comparison to previously resolved 2-DE gels of milk protein (Yamada et al 2002; Baeker et al 2002). The spots that were identified by mass spectrometry were used as reference markers to ensure a realistic pI and Mr was being associated with the protein spots in the highlighted areas. The strongly stained spots in area 1 when compared to previously published 2-D gel images of milk (Murakami et al 1998; Yamada et al 2002), correspond well to both the pI and Mr of IgG heavy chain. The location and intensity of the spots in area 2 corresponded to IgG light chain identified previously in milk and colostrum by N-terminal sequencing (Murakami et al 1998; Yamada et al 2002). The results from MALDI-MS from the other spots in this region of the gel give support to assigning an identity to the unidentified spots, through considering their location in relation to such previously characterised spots. Additionally the spots in both areas 1 and 2 display similar patterns of isoform distribution to IgG heavy and light chains previously identified.

A recent study of mastitic milk identified 4 spots differentially expressed at 26Kd in the inflamed quarter as isoforms of bovine prostaglandin D synthase (PGD-S) (Baeker et al 2002). The 4 spots presented in area 3 of Figure 4-10 could possibly be PGD-S.

4.4 Discussion

The important contribution of bovine milk to human nutrition and its availability in large amounts have made it the subject of biochemical studies for many years. There has been considerable interest in milk proteomics in the recent past due to two main factors. First, identification of bioactive milk-derived peptides with possible pharmaceutical applications has generated a commercial interest (Clare and Swaisgood 2000). Second from a health

perspective there have been a number of reports suggesting correlations between particular genetic variants of milk proteins and some disease states including diabetes (Elliott et al 1999). For these reasons characterisation of the bovine milk proteome is timely.

Inflammation of the mammary gland, induced by bacterial infection, results in changes in the composition of bovine milk. The approach of this study was to analyse the entire protein complement of bovine milk during mastitis and in healthy cows using 2-DE coupled with mass spectrometry. A method of sample preparation aiming to precipitate the caseins allowing the investigation of milk whey proteins was developed and resulted in clearly resolved 2-DE gels allowing examination of the protein changes taking place in milk during clinical mastitis. This has been a commonly used approach to investigating milk proteins in whey (Molloy et al 1997; Galvani et al 2000).

The most prevalent protein in whey is β-lactoglobulin, comprising 10% of the total milk protein, and approximately 58% of whey protein and was identified in 2-DE gels in this study by MALDI-MS as a series of spots with a Mr of 19.9kD and pI of 4.93. The biological functions of β-LG have been a matter of speculation however, in the early 1970s, β-LG was found to bind retinol, and it may therefore have a role in the transport of vitamin A to neonates (Wong et al 1996). Alpha-lactalbumin, a calcium binding protein, accounts for about 13% of the total whey protein and was also identified by MALDI-MS in a series of protein spots with Mr of 16.2kD and pI of 4.9. Under physiological conditions in the mammary gland, α-LA functions as a specifier protein, in that it modifies the substrate specificity of galactosyltransferase, and in complex with this enxyme catalyzes the final step in the biosynthesis of lactose. Both β-LG and α-LA appear as a string of spots in the 2-DE gels demonstrating that they are present as isoforms of differing charge, presumably as a result of post-translational modifications, which are known to occur to these proteins prior to secretion (Anderson et al 1982).

The attempt to remove from the samples, the caseins, the most abundant protein in milk, by pre-treatment with ammonium sulphate precipitation, was only partially successful, as several spots were identified as either αS_1 casein, β-casein or κ-casein, indicating that the process had not removed all the casein proteins present. A 2-DE gel of the casein precipitate was run which showed by MALDI-MS caseins to be the only abundant spots, indicating that this process removed no other major proteins. Caseins are a series of proteins unique to milk that comprise approximately 80% of the total protein content. They are phosphorylated proteins that undergo substantial post-translational modification. Of these, α-casein is the major protein in bovine milk, so that the absence of identified protein

spots following mass spectrometry indicates that this casein isoform had been removed by the pre-treatment (Wong et al 1996).

Bovine serum albumin, with Mr of 69.2 kD and pI of 5.82 which is known to be a component of normal milk at low concentration (Auldist and Hubble 1998) was the only other protein identified in normal milk by MALDI-MS. Defence mechanisms of the mammary gland against invasion of infectious agents are complex. Stimulation of neutrophil migration from the blood to the mammary gland is achieved through the production of inflammatory mediators at the local site of infection, including: cytokines, chemokines, complement fragments and arachidonic acid metabolites. Mastitis-induced disruption of normal secretory functions of the mammary gland leads to a break down of the blood-mammary barrier and the appearance of various serum compounds such as albumin, serotransferrin, complement proteins and Hp have previously been reported (Baeker et al 2002). Proteomic examination of the major proteins in whey from dairy cows with clinical mastitis in this study (Figure 4-4) confirmed that, in this condition, there is a major increase in serum albumin, presumably due to the increased leakage from the circulating blood, as described above. Serotransferrin (Mr 77.7 kD, pI 6.8) was also identified in mastitic whey again probably due to passive leakage from the serum, across the blood- mammary barrier and into the milk as the infection progressed.

In addition to revealing that proteins such as albumin and serotransferrin are increased in concentration in milk whey during clinical mastitis, it was clear from a comparison of the 2-DE gels that the major normal milk proteins were virtually absent from the whey of clinically mastitic udders. Thus there were minimal protein spots corresponding to β-LG, α-LA or the casein remnants. Previous work has shown that there is a decrease in casein and whey protein concentrations during mastitis (Munro et al 1984). The phenomenon is possibly due to post-secretory degradation of the proteins by proteinases originating from bacteria or, leukocytes (Moussaoui et al 2002). The main proteolytic enzyme in milk is plasmin, which originates in the blood. However somatic cell proteases including cathepsin G, elastase and collagenase all display increased activity in milk during inflammation of the mammary gland (Moussaoui et al 2003). However, if this were the case, protein and peptide fragments, identifiable as such by MALDI-MS would be expected on the 2-DE gel and these were not found to be present. An alternative explanation is that the reduction in β-LG, α-LA and casein remnant concentrations in mastitic whey is due to a reduction in the synthesis and secretion of the proteins (Mcfadden et al 1988), either as a result of the physical damage to the mammary epithelial cells by bacterial toxins or as a

pathophysiological response of the mammary tissue to the presence of intramammary infection. The host response to toxins or to secondary mediators such as cytokines, nitric oxide or prostaglandins may play a role. Indeed β-LG, the protein in milk with an ability to bind retinol, could be responding in the same way as the major serum protein with the same activity (retinol binding protein, RBP), which is known to be downregulated, during the cytokine-mediated acute phase response to infection (Ingenbleek and Bernstein 1999).

It has been confirmed that the major proteins of milk do not vary widely in pI (Holt and Zeece 1988), as the 2-DE gels of normal milk whey clearly show β-LG, α-LA and the casein remnants focus in a relatively narrow range of pH 4.5-5.3. This suggests an alternative approach to removing these abundant proteins, which could be applied in future investigation of less concentrated proteins in milk. Immobilised pH gradients strips with a narrow pH range of pH 5.0-7.0, pH 6.0-8.0 or pH 7-10 could be used in the first dimension of 2-DE to examine milk proteins with low abundance and pI > 5.0, as with these gradients the major milk proteins would not focus but would instead migrate towards the anode, allowing examination of lower abundance proteins without interference from the major milk proteins.

Identification of lower abundance proteins differentially expressed in mastitic milk was achieved for a small number of protein spots by MALDI-MS. Differences in the 2-DE patterns of the low-abundance proteins between normal and mastitic milk (Figure 4-9) were found in a number of mastitic samples. Such spots however were not detected in close proximity to any of the major spots, as highlighted by the zoom images shown (Figure 4-10). These differences in the low abundance milk proteins showed more variation between animals than was observed with the major protein spots. Further investigation of the functions of the low-abundance protein spots, (spots 1, 9-13, Table 4.4) which were upregulated in infected milk revealed a possible association of the proteins in inflammatory disease processes.

The annexins (spots 10 and 11, Table 4-4) consist of a family of at least 20 structurally related proteins. They all share a core structure containing phosolipid and calcium binding sites, and have been linked with intracellular signalling processes through protein kinase C (PKC) receptors (Andersen et al 2002). Macrophages and neutrophils are rich in these proteins. Annexins have previously been detected in the mammary glands of lactating cows. These proteins are thought to play a role in the suppression of inflammation, and

have previously been implicated as having a role in fatty liver, pneumonia and mastitis in cattle (Katoh 1995).

The bovine glycoprotein lactadherin (spot 9, Table 4-4) is an abundant peripheral protein associated with the milk fat globular membrane but is also found in a wide range of tissues and is present in several body fluids (Andersen et al 2000). Bovine lactadherin precursor protein was identified in this study as a low-abundance protein in mastitic milk with a pI of 6.81 and an Mr of 47411 Da. Structurally, the protein consists of two epidermal growth factor (EGF) - like domains, one of which (domain 2) contains the presence of an arginine - glycine - aspartic acid (RGD) sequence motif which was identified on examination of the amino acid sequence, and this motif is known to bind to integrin receptors, hence inferring a role for this protein in cell adhesion and motility.

Apolipoprotein A-1 (ApoA-I) (spot 12, Table 4-4) is the major protein of plasma high density lipoprotein and plays an important role in lipid transport and metabolism the protein interacts specifically with lipoprotein and endothelial cell protein annexin II.(Kanno and Katoh 2001). This protein was identified as a very faint spot as indicated by an arrow in Figure 4-7. Interestingly, the APP, Hp is synthesized extrahepatically by adipocytes (Friedrichs et al 1995), and has recently been shown as being distributed in the lipoprotein fractions (Katoh and Nagakawa 1999), and may have a role in the regulation of lipid metabolism (Porta et al 1999). Haptoglobin has previously been analyzed in serum by 2-DE, followed by subsequent detection by either immunoblot or silverstaining (Kanno and Katoh 2001). From the outset it was expected that Hp would have been identified by 2-DE methods, in mastitic milk samples as previous chapters have used immunoassay to measure the increased concentrations of this protein in milk during infection (chapter 3) (Eckersall et al 2001). With a known Mr of 20Kda (α-subunit) and 35Kda (β-subunit) (Morimatsu et al 1991) and pI of 5.27 (α-subunit) and 5.8 (β-subunit), if the Hp subunits were present they should have focused within the pH range of the IPG strips used in the experiment. However Kanno et al (2001) used 2-DE to detect Hp in serum where concentrations are much higher than in milk and used the more sensitive silver stain, and Hp was still only shown as a minor component in comparison to other protein spots including APO-A1. Therefore it is likely that milk Hp may need more sensitive methods for detection.

By comparison to previously published data showing 2-DE patterns of bovine milk, the number of protein spots detected on the 2-DE gels for milk presented here is low, with previous studies reporting the presence of up to several hundred proteins (Galvani et al

2000; Galvani et al 2001). It has been noted however that although the protein numbers are high, only a few genes are responsible for them and so the majority probably represent proteolytic products (Holland et al 2004). All the recommended steps were included in the sample preparation protocol to obtain a high level of protein extraction and therefore the detection of a maximum number of protein spots by 2-DE (Rabilloud 1998; Molloy 2000; Lanne et al 2001). These included the use of chaotropes for the disruption of hydrogen bonds, prevention of hydrophobic interactions by surfactants and destruction of intramolecular and intermolecular disulphide bonds by reducing agents. Use of staining methods of greater sensitivity than colloidal Coomassie, such as silver stain or sypro-ruby would probably have led to an increase in the number of proteins being detected, however there are drawbacks associated with the use of such stains. Silver staining gives poor reproducibility, a limited dynamic range, and selective staining of protein spots with some staining negatively or not at all. In addition, low detection limits often occur in mass spectrometry because the stain modifies cysteine residues and the gluteraldehyde and formaldehyde used in the procedure alkylate protein amino groups making the protein spots unsuitable for mass spectrometric analysis (Patton 2002). The fluorescent stain Sypro ruby can detect in the range of 1-2ng of protein per spot, and the linear range is over three orders of magnitude (Gorg et al 2000). However, being a fluorescent stain, Sypro ruby renders the manual excision of protein spots from the 2-DE gel and requires special equipment such as the Amersham Ettan robotic workstation, which was not available at the time of this study.

The known association of bovine mastitis with the accumulation and activation of inflammatory mediators and immune cells within the milk and udder (Holland et al 2004) would suggest the likelihood of detecting secretory products such as IL-8, tumour necrosis factor-α and Hp and M-SAA3 would be high (Barber et al 1999). However in this study no such products were identified in either normal or mastitic milk, probably because they are present in low concentrations. It can be difficult to elucidate low abundance proteins of interest in the presence of relatively abundant proteins using 2-DE (Corthals et al 2000). Such proteins could possibly be identified by 2-DE and MALDI-MS after enrichment of the crude sample, for example by using high affinity antibodies to the proteins of interest (Oda et al 2001). Alternatively immunoblotting could be used to confirm the presence of low abundance proteins on 2-DE gels.

A number of proteins giving clear spots or strings of spots in the 2DE gel of mastitic milk were not identified. The bioinformatic approach of matching proteins from peptide mass fingerprinting to published protein sequence databases is reliant upon the 'unknown'

protein being partially, or ideally, fully represented in a current database. However there are still many species whose genomes are not sequenced, and are unlikely to be sequenced in the near future. This poses a problem in current proteomic studies, but for such organisms whose genomes are not yet fully sequenced it may be possible to use cross-species identification (Lester and Hubbard 2002). Due to the limited number of *Bos taurus* database entries, identification of the unknown protein spots may have to be performed by cross-species matching. Identification of marsupial milk proteins using a mammalian database has previously been employed successfully to identify wallaby casein, β-LG and α-LA (Molloy et al 1997). Entry number and content varies largely between databases, so database selection within the search engine (Molloy et al 1997), can also strongly influence the success of the identification procedure, in combination with human judgement and selection criteria selected within the database.

Comparison to previously published 2DE gels of bovine milk gives support to both IgG heavy and light chains being present in mastitic milk. Immunoglobulin G is present in the database, but was not identified by MALDI-MS and database searching in this study. There are a number of reasons as to why this can happen. It is possible that the spots analyzed were a mixture of proteins, either from the gel or with keratin contaminations. In that situation protein mixtures score poorly on Mascot® due to the high number of proteins that do not match, and hence a mixture often fails to be identified correctly. It is also possible that an insufficient number of peptides were identified to get a significant match on Mascot®, and there are a variety of reasons that can lead to this happening. Presence of a high number of trypsin cleavage sites results in the peptides produced being too small, but if the number of trypsin cleavage sites is too low then the peptides produced are to large. Additionally many peptides do not ionize well, and many do not bind strongly to the carbon resin present within the ZipTips^{tm}, leading to a low concentration of peptides being extracted from the gel. Finally it is possible that there was a problem with the tryptic digest itself, for example proteins with a complex tertiary structure are often resistant to proteolysis.

Two-dimensional electrophoresis coupled with MALDI-MS represents a powerful method to identify the changes induced by mastitis on the bovine milk proteome. However it remains that milk is a very complex fluid and the number of proteins within, that have not experienced post-translational modifications or proteolysis is rather limited (Galvani et al 2001). Post-translational modifications in proteins are of immense importance for protein folding and function. Such proteins remain the most difficult to characterise, and further steps should be taken in future studies to tackle this problem. Traditionally this is achieved

using methods that can identify the mass increment or mass deficit relative to the known molecular mass of the protein followed by amino acid sequencing of the modified protein by tandem mass spectrometry (MS/MS) (Jensen 2004). However, determination of PTMs by mass spectrometry may require additional preparative steps such as enrichment of the protein of interest using immunoprecipitation (Mann et al 2001). The complete characterization of the primary structure of proteins will remain a laborious and challenging task for proteomics for many years to come (Mann and Jensen 2003).

Chapter V

A Proteomic Approach to Study the Bovine Milk Proteome during an Experimental *Staphylococcus Aureus* Model of Mastitis

5.1 Introduction

In the previous chapter a proteomics approach was developed and used, to successfully investigate differentially expressed proteins in bovine milk during naturally occurring bovine mastitis. However, by definition samples were from cases of clinical mastitis in which major changes were found. In addition, as all samples were from established disease, the alteration to the milk proteome during the early or sub-clinical stages was not examined. The objective therefore, of the study described in this chapter was to monitor the proteome in an experimental infection designed to mimic the condition frequently encountered on farm, of subclinical or early infection periods.

This was achieved by collaboration with an ongoing project with the University of Glasgow Veterinary School and the Hannah Research Institute (Ayr) in which an experimental model of *S. aureus* mastitis was established. The objective of the model was to examine the alteration to the milk concentrations of Hp and M-SAA3 during subclinical and early mastitis. However, milk collected from the *in vivo* experimental model which would allow examination of the milk protein during the development of mastitis was available. Specialist software for identification of protein altering from sample to sample had the potential to identify changes in major and minor protein during the disease process.

Having made minor modification to the previously described method (section 4.2.4) the optimal conditions for analysis of the milk proteome were established and samples were analysed from selected time points from the infected and control quarters of the experimental infection with *S. aureus*. Milk samples from 7 selected time points were analysed with the objective of identifying consistent differences as the disease progressed.

5.2 Materials and Methods

5.2.1 Cows

Twenty Holstein dairy cows from the herd at the Hannah Research Institute, with low SCC (<100,000 cells/ml) in previous lactations were selected for the experiment. The cows were grouped as either control cows (n=10) or infected cows (n=10). The infected cows were challenged by intramammary infusion of *S. aureus* mastitis. The control cows were infused with sterile phosphate buffered saline (PBS).

5.2.2 Infection

The 10 cows in the experimental infection group were infused with a low virulence strain of *S. aureus* isolated from a case of subclinical mastitis (Young et al 2001) at a dose of 5 x 10^6 organisms in 10ml of sterile PBS. Each of the 10 cows was challenged on 2 occasions. First, a single quarter in each cow was infected. Second, on day 28 after the first infusion, two quarters of the same cow were infused, 1 of which was the previously infused quarter and which will therefore be undergoing re-challenge. The cows were then euthanased 3 days after the second infection (day 31 after initial treatment).

5.2.3 Milk Samples

Milk samples (20ml) from each quarter of the 20 cows were collected weekly, for the 4 weeks preceding infection. On the day of infusion (day 1), and for the following 3 days milk samples (20ml) were collected from each quarter of all cows at both daily milkings. Thereafter, milk samples were collected daily on days 4-7 post-infection (p.i.), and then on days 9, 12, 15, 19, 23 and 26 p.i. On day 28 (day of re-challenge), 29, 30 and 31 milk samples were collected at both daily milkings. Milk samples were stored at -20°C until analysis.

Milk samples from 1 quarter of 3 control cows and 1 quarter (the quarter of double challenge) from 3 experimentally infected cows showing the highest acute phase response, were analysed using 2-DE at the following time points days –7, 1, 2, 3, 7, 28, and 29.

5.2.3.1 Somatic cell count

Milk SCCs were determined as described in section 3.2.5.

5.2.3.2 Acute phase protein analysis

Milk samples were assayed for Hp and SAA as described in section 3.2.6

5.2.3.3 Sample preparation

The milk samples were prepared prior to 2-DE as described in chapter 4, section 4.2.3

5.2.4 Two-dimensional Gel Electrophoresis

Samples (120µg in total volume of 125µl of rehydration buffer) were applied by rehydration on non-linear immobilized IPG strips (7cm, pH 4-7) (BIO-RAD, Hemel Hempstead, UK), and focussed in a BIO-RAD Protean IEF Cell for 26000 volt hours (Vhrs) using the following voltage rampage programme: 50V, 600Vhrs; 2000V, 2000Vhrs; 4000V, 24000Vhrs. The IPG strips were then equilibrated as described in chapter 4, section 4.2.4 and then run in the second dimension in 12% polyacrylamide gels using 1.5M Tris-HCl pH 8.8 as separating gel buffer and Tris-glycine-SDS pH 8.3 as electrode buffer, in a Mini-PROTEAN 3 Dodeca cell (BIO-RAD, Hemel Hempstead, UK). Gels were run at 200V for 1h. The mini-PROTEAN 3 Dodeca cell has the capacity to run up to 12 mini-gels simultaneously.

The gels were then stained and scanned as described in chapter 4 section 4.2.5.

5.2.5 Image Analysis

Phoretix software (Nonlinear Dynamics, Newcastle, UK) was used to create a 'difference map' based on the pre-infection samples, to highlight any spots that exhibited different expression levels across the course of the infection. The creation of the difference map required a process of optimisation of spot detection, editing and filtering, background subtraction and creation of a reference gel.

The computer analysis of the gels was carried out on a time series of 2-DE gels for 3 of the infected cows. The 7 time-points (days -7, 1, 2, 3, 7, 28, and day 29) from each cow were analysed together in 1 experiment using the tools outlined below.

5.2.5.1 Spot detection

Spot detection is the first step in the computer analysis of a 2-D gel. It is designed to automatically identify the maximum number of spots on the gel, however some degree of manual editing is necessary to achieve accurate detection.

The spot detection parameters were optimised visually using the detection Wizard on a representative part of the gel image. The spot detection wizard works by performing several detections on the same area of the image using a combination of sensitivity and operator size parameters. It then displayed 9 different combinations of these two parameters. The aim in this operation was to achieve a similar appearance in all 9 panels. Stringency of spot detection was altered by adjusting the sensitivity, noise, operator size and background parameters. Once the spot detection was optimised, the selected parameters were applied to the entire gel.

5.2.5.2 Spot editing and filtering

The removal of spots that were not required for reasons of size, shape or relevance can be achieved by a combination of manual editing and by use of the automated 'Spot Filtering Facility'. Spots below a certain area, peak height, volume and/or spots displaying poor circularity could be filtered out. The circularity filter is a very useful way of removing streaks detected on the image. A threshold volume was set in each experiment, to enable the removal of low volume spots, which are usually background noise.

5.2.5.3 Background subtraction

Background subtraction is necessary to ensure that low intensity spots can be distinguished from background, and also so that a true pixel intensity value can be calculated for a given spot. "Mode of non-Spot", was used to subtract the background from the gels. This works by calculating the pixel intensity between the background and a selection of the least intense spots.

5.2.5.4 Creating a reference gel

Before any expression changes can be explored using the software, a reference gel must first be created from 1 of the gels in the experiment. In this project, the day -7 gel was chosen as the reference gel for each cow, as it is the within-cow control.

5.2.5.5 Creating a difference map

In order to explore either an increase or a decrease in the expression levels of the milk proteins, across the time series of infection, a difference map is created. Spots showing more than a two-fold increase or decrease in spot volume than the equivalent spot on the reference gel are highlighted in green. Differentially expressed spots can be further explored by creating a table that will display the volume for the spot in each gel being analysed in the experiment.

5.3 Results

An experimental model of *S. aureus* mastitis was successfully established in dairy cows infused with *S.aureus*, judged by conventional criteria of raised SCC. The results for SCC and APP in the 3 selected infected animals are shown (Figures 5-1 to 5-6), to demonstrate that the cows developed sub-clinical mastitis. The milk from 1 quarter of three infected cows with the greatest increase in APP and 1 quarter of three control cows was subjected to proteomic analysis at 7 time points: days -7, 1, 2, 3, 7, 28, 29.

5.3.1 Somatic Cell Count

The SCC increased rapidly following infection, from ~30 $x10^3$ cells/ml pre-infection, to ~5 $x10^6$ cells /ml within 15h of infection. In the infected udder SCC remained elevated until the time of the second infusion with *S. aureus* on day 28.

5.3.2 Acute Phase Proteins

In milk from the infused quarter of infected cows, increases in Hp and M-SAA3 were present within 15h of infusion reaching an initial peak at 48h post infusion. Milk Hp rose from 0.22 ± 0.07 μg/ml to 7.2 ± 13.7 μg/ml and M-SAA3 increased from 0.19 ± 0.37 μg/ml to 8.8 ± 2.3 μg/ml. Elevated levels of these APPs were present until the time of second infusion. Thereafter both Hp and M-SAA3 rose again to highest level (Hp: 0.8 ± 49.0 μg/ml, M-SAA3: 0.35 ± 73.5 μg/ml). It was not possible to determine whether there was a difference between the onset of the APP response in milk and the rise in SCC as both analytes were elevated in the first samples of milk taken after the first infusion (at 15h).

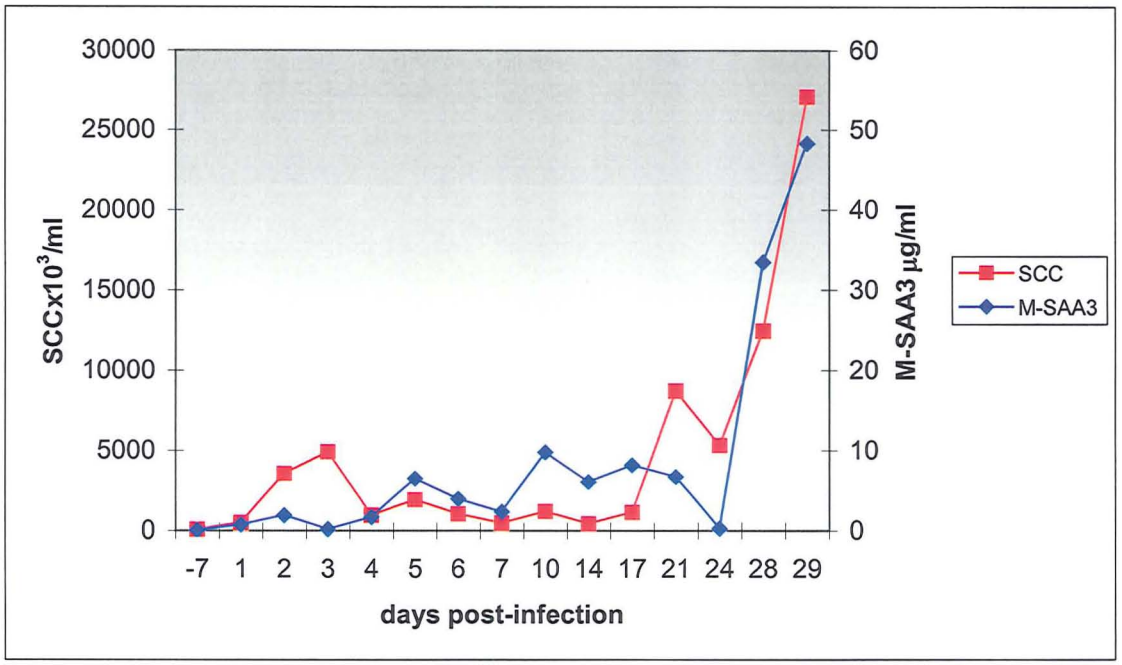


Figure 5-1: Profiles for somatic cell count (SCC) and mammary-associated serum amyloid A3 (M-SAA3) for cow 1, following inoculation on days 0 and 28 with *S. aureus*

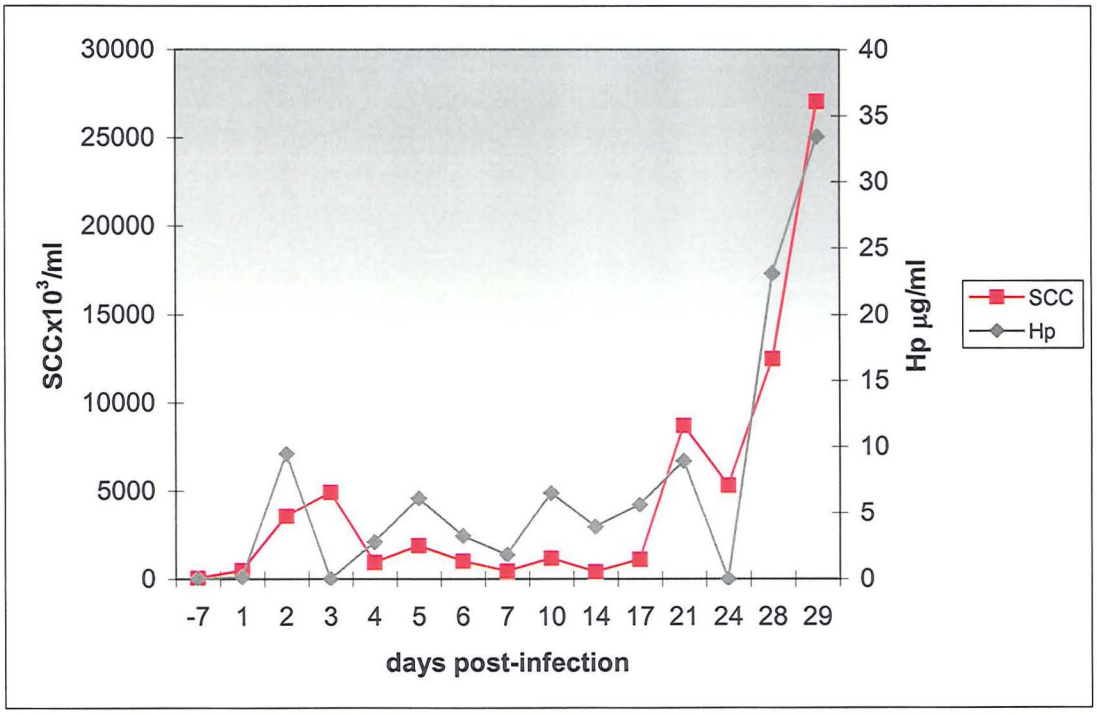


Figure 5-2: Profiles for somatic cell count (SCC) and mammary-associated haptoglobin (Hp) for cow 1, following inoculation on days 0 and 28 with *S. aureus*

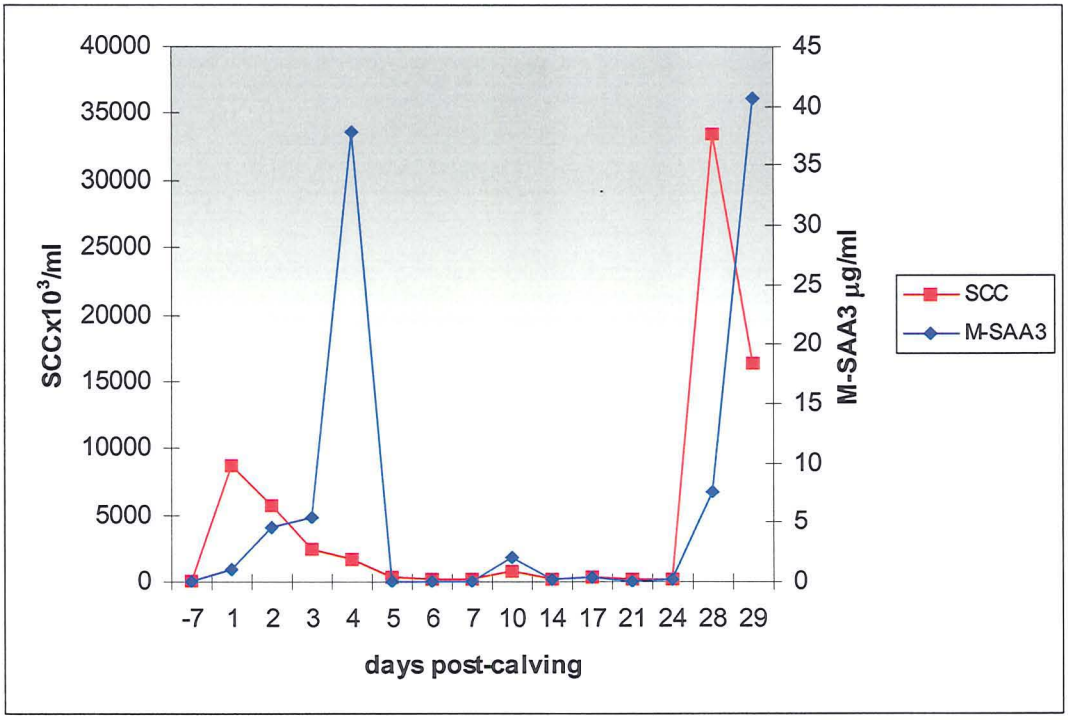


Figure 5-3: Profiles for somatic cell count (SCC) and mammary-associated serum amyloid A3 (M-SAA3) for cow 2, following inoculation on days 0 and 28 with *S. aureus*

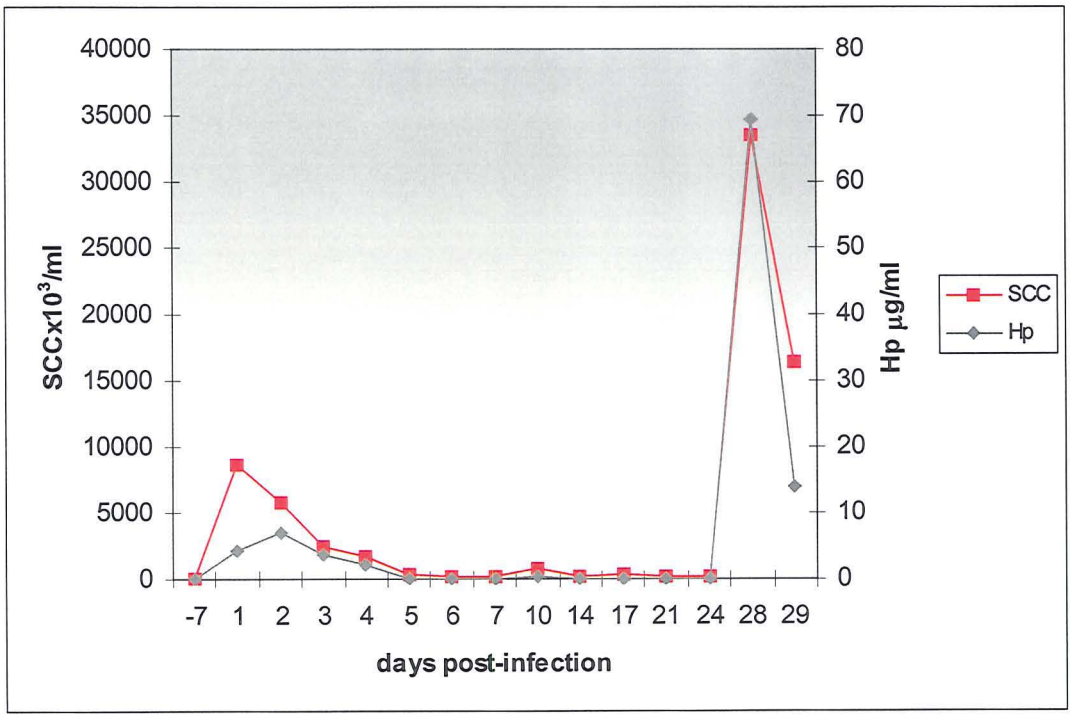


Figure 5-4: Profiles for somatic cell count (SCC) and mammary-associated haptoglobin (Hp) for cow 2 following inoculation on days 0 and 28 with *S. aureus*

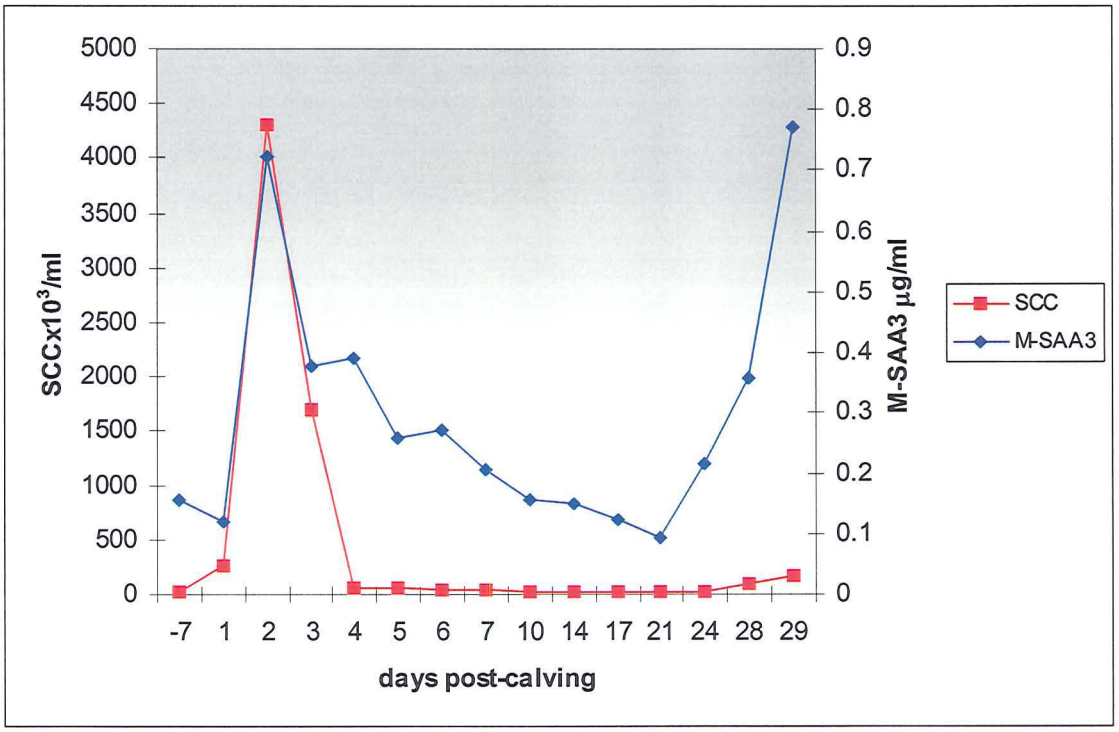


Figure 5-5: Profiles for somatic cell count (SCC) and mammary-associated serum amyloid A3 (M-SAA3) for cow 3, following inoculation on days 0 and 28 with *S. aureus*

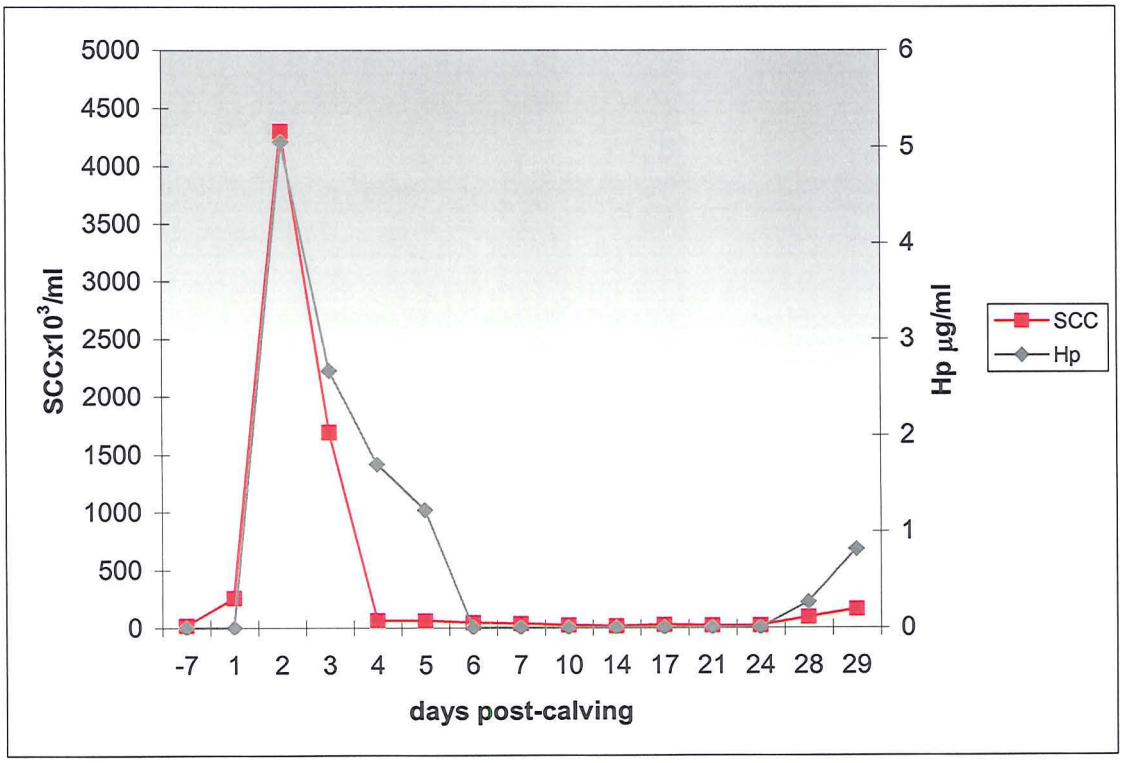


Figure 5-6: Profiles for somatic cell count (SCC) and mammary-associated haptoglobin (Hp) for cow 3 following inoculation on days 0 and 28 with *S. aureus*

5.3.3 Two-dimensional Electrophoresis

Milk whey proteins from 3 control cows and 3 cows with experimentally induced *S. aureus* mastitis were separated by 2-DE, at 7 time points (d –7, 1, 2, 3, 7, 28, 29). Gel images were first analysed manually by eye. The major milk proteins, casein, β-LG, α-LA and albumin, were present in all gels and were identified by comparison to the results in chapter 4. Examination of the gels from the 7 different time-points within the infected cows did show differences in the proteins expressed (Figures 5-7 to 5-9). However, there were no obvious changes to the major milk proteins. There was a much less dramatic reduction in β-LG and α-LA in the experimental samples compared to the clinically mastitic milk samples, used for optimisation of the method in chapter 4. Greater variation was observed in the less abundant proteins in milk but no consistent changes in the expression of a particular protein or subset of proteins were apparent through visual examination of the gels. Similarly, these differences were not consistent between animals, ruling out comparison between animal subsets.

Differences in the gels from the control milk samples (Figures 5-10 to 5-12) were also observed, and again no consistent differences are obvious.

5.3.4 Image Analysis using Phoretix Software

Visual examination of the gels showed that there were pattern differences in the milk proteome in response to the infection, however no consistent changes were obvious. Much of the useful information in 2-DE is quantitative, such as changes in protein expression patterns and response. However, quantitative data can only be derived from gel analysis with computer assistance (Mahon and Dupree 2001). One infected cow, from this experiment was analysed by 'Phoretix 2D' image analysis software (Nonlinear Dynamics, Newcastle, UK), to try and quantify the changes taking place in the milk proteome throughout the course of infection, and to yield data on protein quantity. The pre-infection, (day -7) milk sample was used as the reference gel, and the milk samples from the other time-points (days, 1, 2, 3, 7, 28 and 29) were compared to the reference gel through creation of a difference map. Before the difference maps were created, all gels in the experiment first had to undergo spot detection, editing and filtering. The results for the computer analysis of the gels are detailed below.

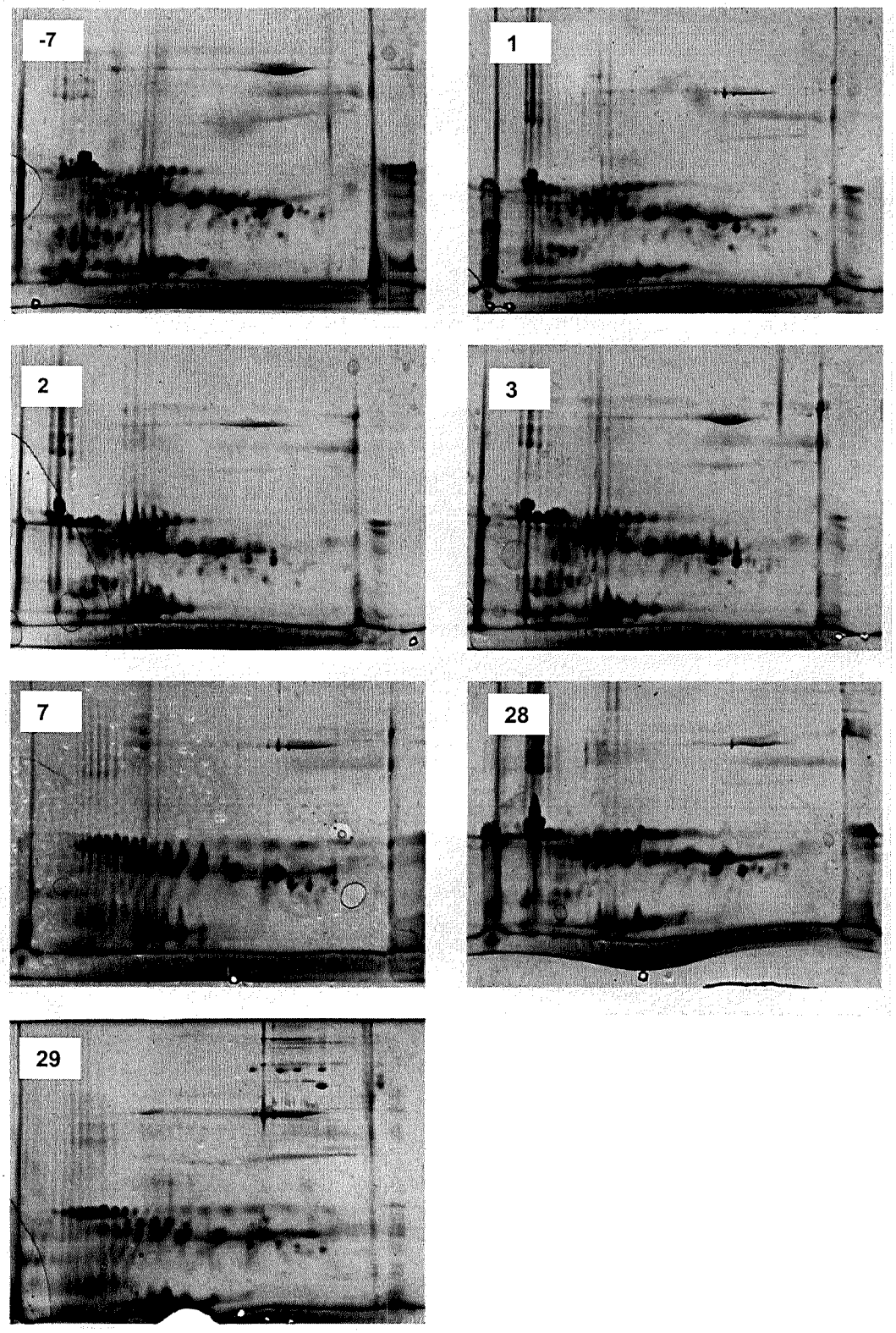


Figure 5-7: Two-dimensional gels for infected cow 1, for 7 selected time-points (days –7, 1, 2, 3, 7, 28 and 29) during the course of infection

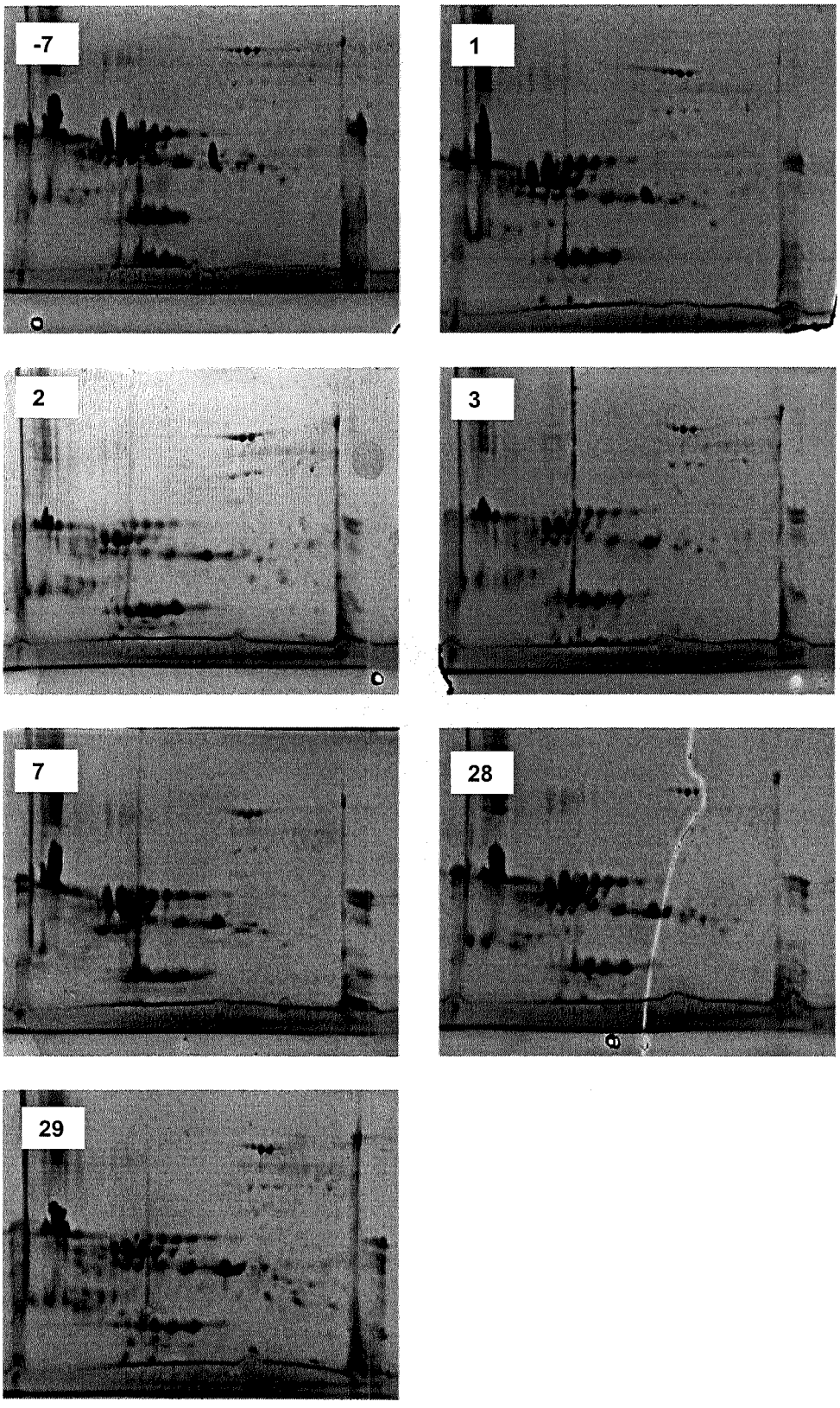


Figure 5-8: Two-dimensional gels for infected cow 2, for 7 selected time-points (days –7, 1, 2, 3, 7, 28 and 29) during the course of infection

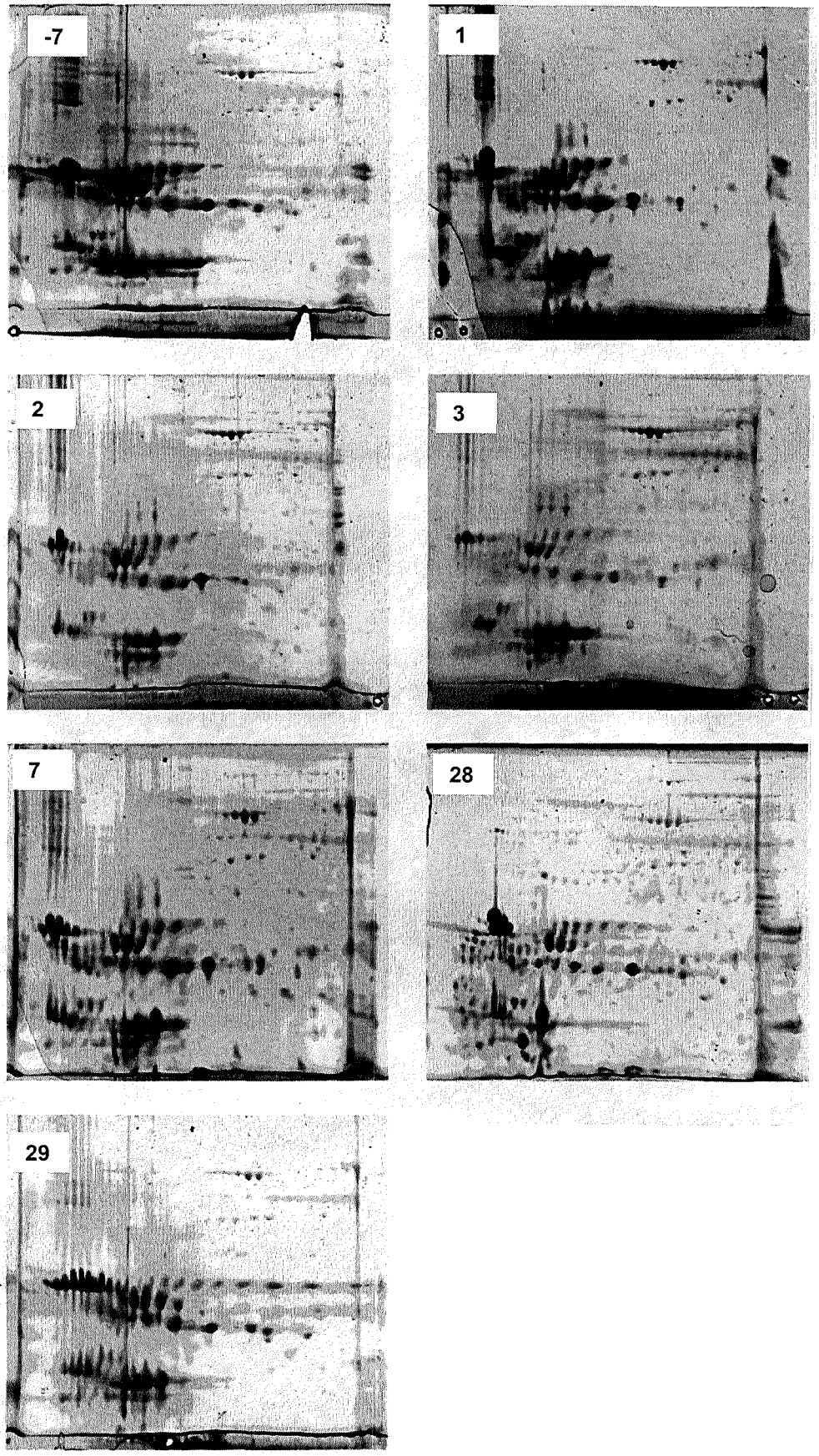


Figure 5-9: Two-dimensional gels for infected cow 3, for 7 selected time-points (days –7, 1, 2, 3, 7, 28 and 29) during the course of infection

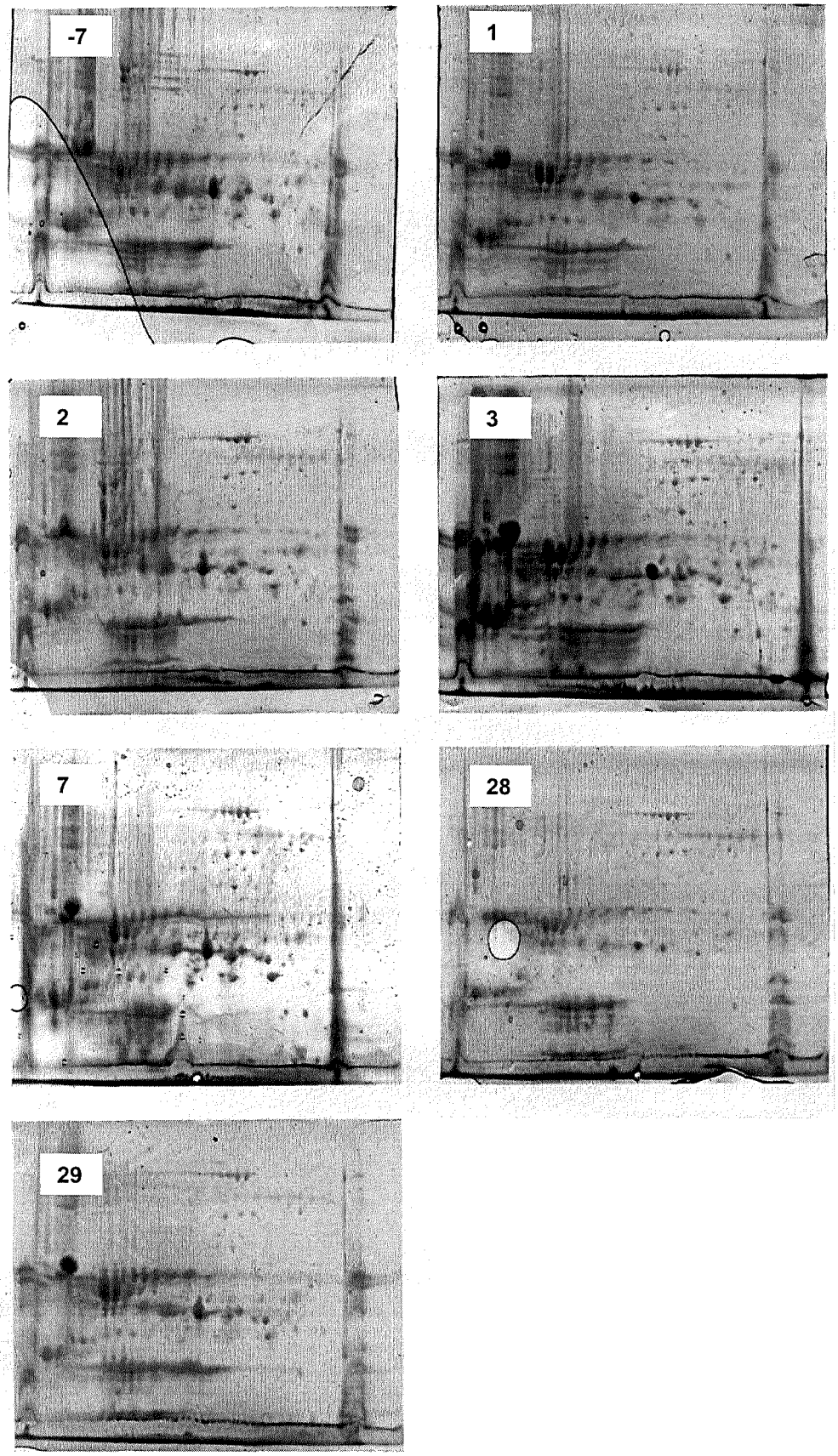


Figure 5-10: Two-dimensional gels for control cow 1, for 7 selected time-points (days –7, 1, 2, 3, 7, 28 and 29) during the course of the experiment

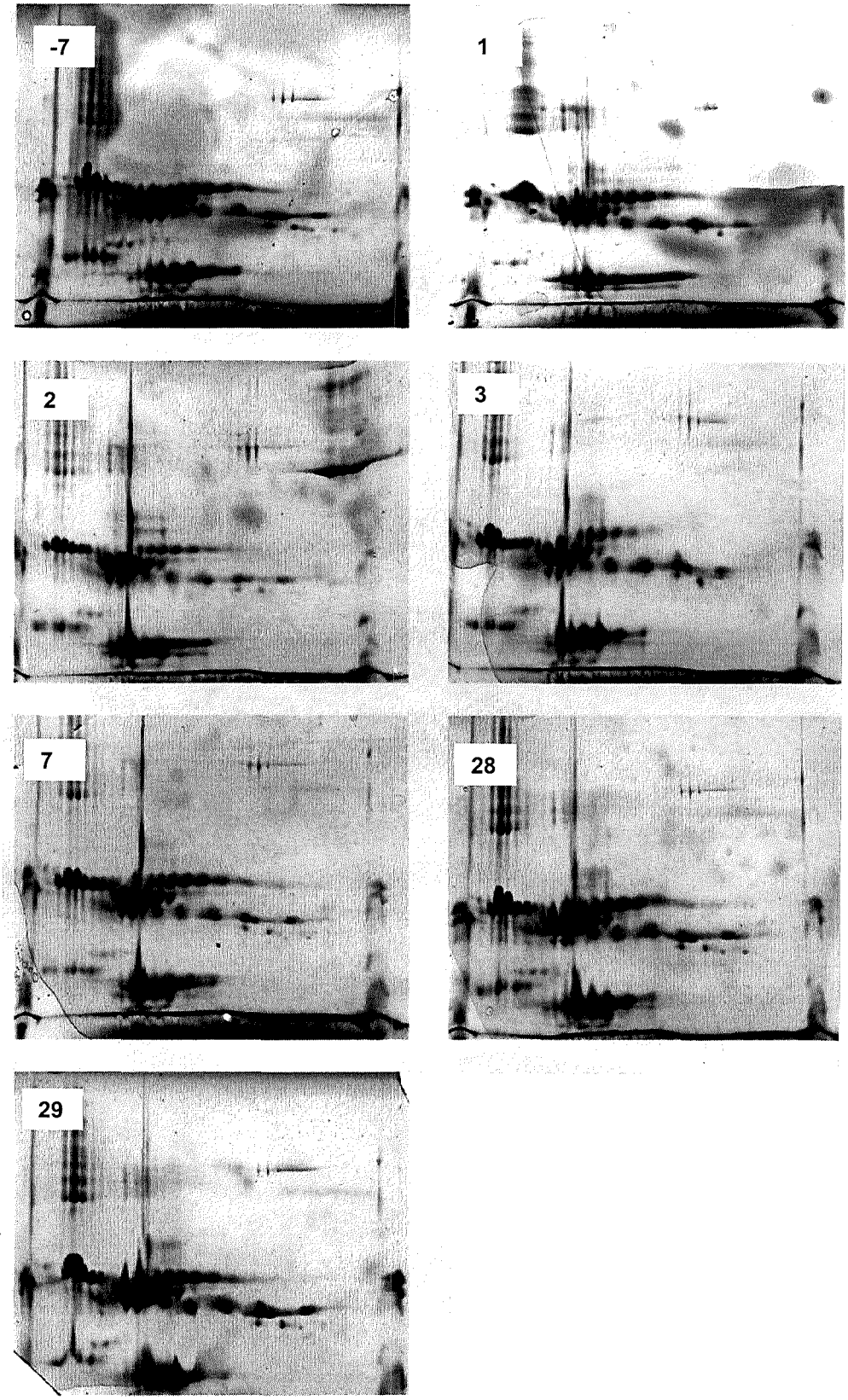


Figure 5-11: Two-dimensional gels for control cow 2, for 7 selected time-points (days –7, 1, 2, 3, 7, 28 and 29) during the course of the experiment

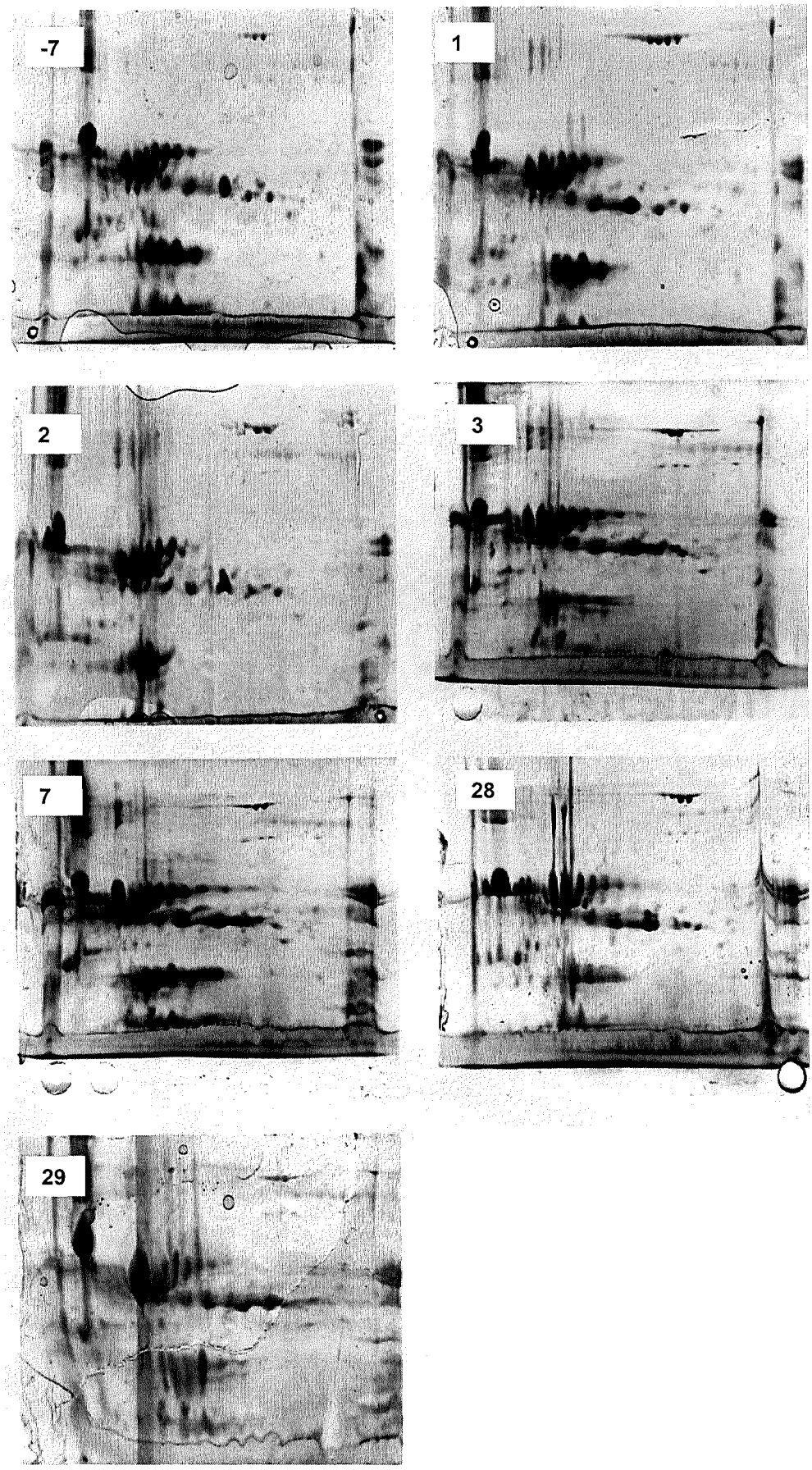


Figure 5-12: Two-dimensional gels for control cow 3, for 7 selected time-points (days –7, 1, 2, 3, 7, 28 and 29) during the course of the experiment

5.3.4.1 Spot detection

Spot detection was performed for all gels that were included in the analysis, (Figures 5-13 and 5-14). The automated spot detection wizard was used but all gel images were also scrutinised manually by eye for undetected or incorrectly detected spots. Some degree of manual intervention was required for accurate spot detection in most of the images.

5.3.4.2 Spot editing and filtering

Figure 5-15 shows an example of a gel image during spot filtering. All spots highlighted in blue will be removed from the selection, leaving only the spots highlighted in green, (Figure 5-16).

5.3.4.3 Reference gel and difference map

The milk sample taken on day -7 was used as the reference gel for the infected cow, selected for computer analysis. All other time-points included in the analysis were compared to the reference gel resulting in the creation of a difference map, which highlights all spots showing a differential increase in expression by two-fold or more, in green and those showing a differential decrease of two-fold or more, in yellow. Comparisons of spot intensity among the gels were made on the basis of spot volume, as described in section 5.2.5.5. Spot volumes reflect the relative abundance of a given polypeptide in milk. A small number of proteins spots, were highlighted using this tool

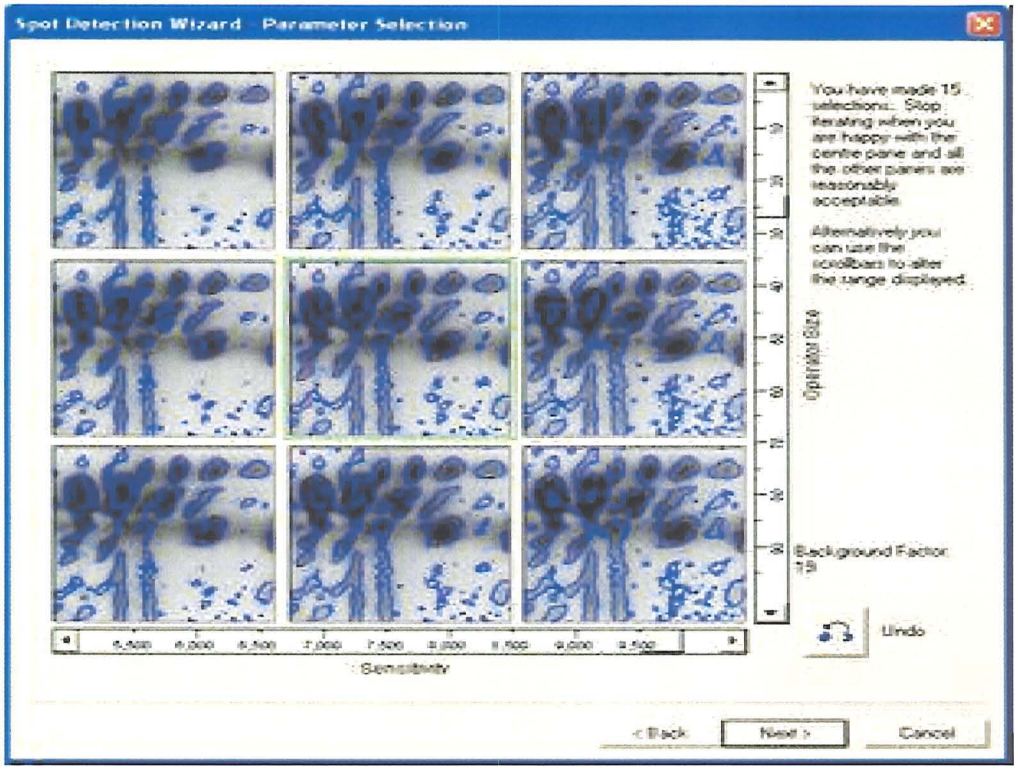


Figure 5-13: The spot detection wizard as used to determine the optimal sensitivity of spot detection for the entire gel. The aim was to achieve a similar appearance in all 9 panels.

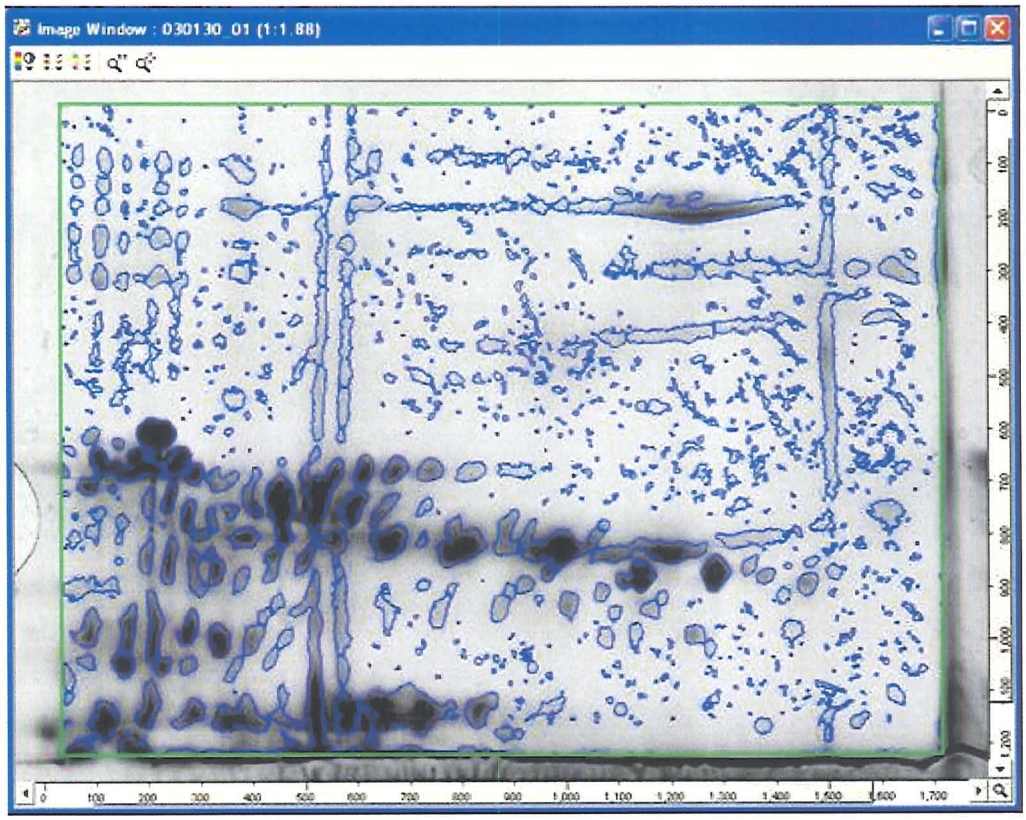


Figure 5-14: Spot detection for the entire gel is achieved after optimisation using the spot detection wizard

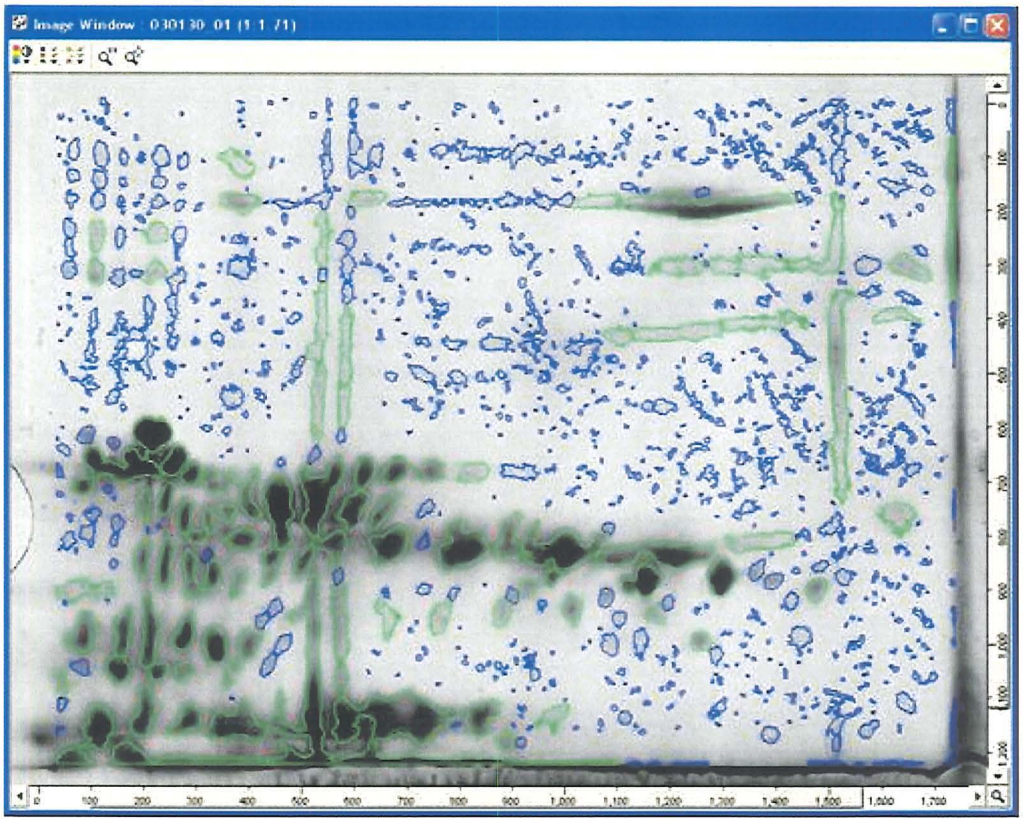


Figure 5-15: Spot filtering was used to remove all spots below the threshold volume (shown in blue)

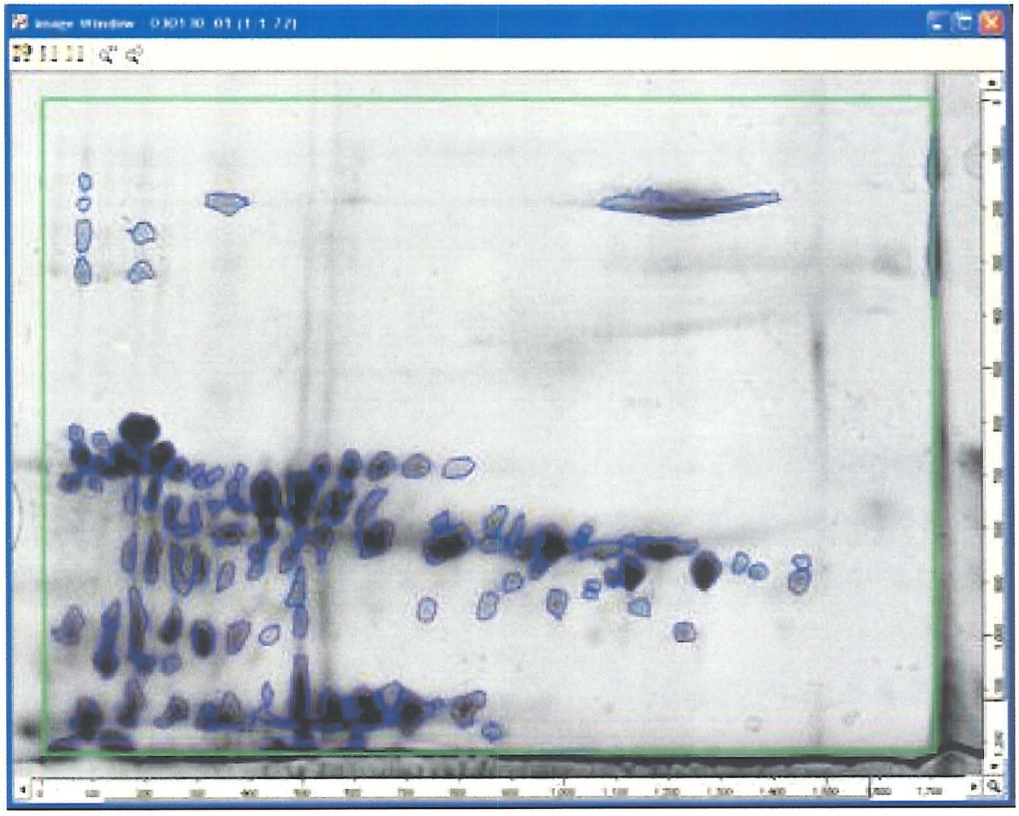


Figure 5-16: Final spot selection after editing and filtering has been performed

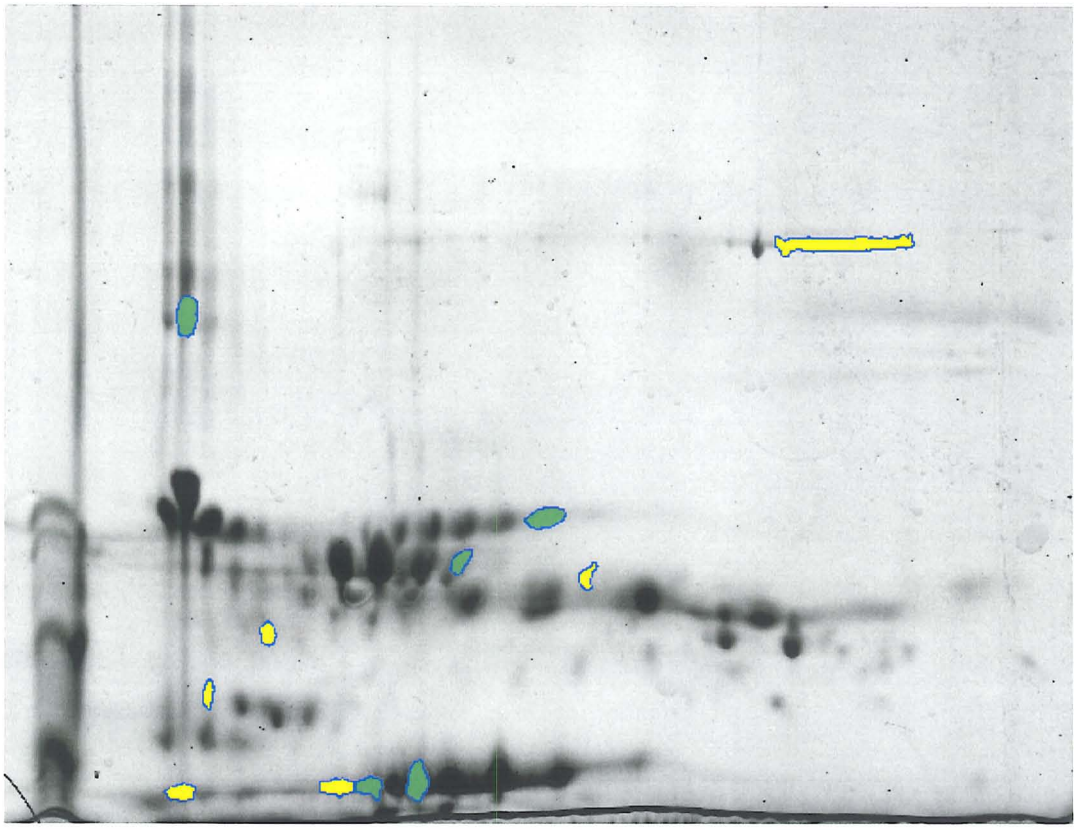


Figure 5-17: A difference map was created to highlight the spots that show more than a two-fold increase (green)or decrease (yellow) in volume at day 2 post-infection to the equivalent spot in the reference gel.

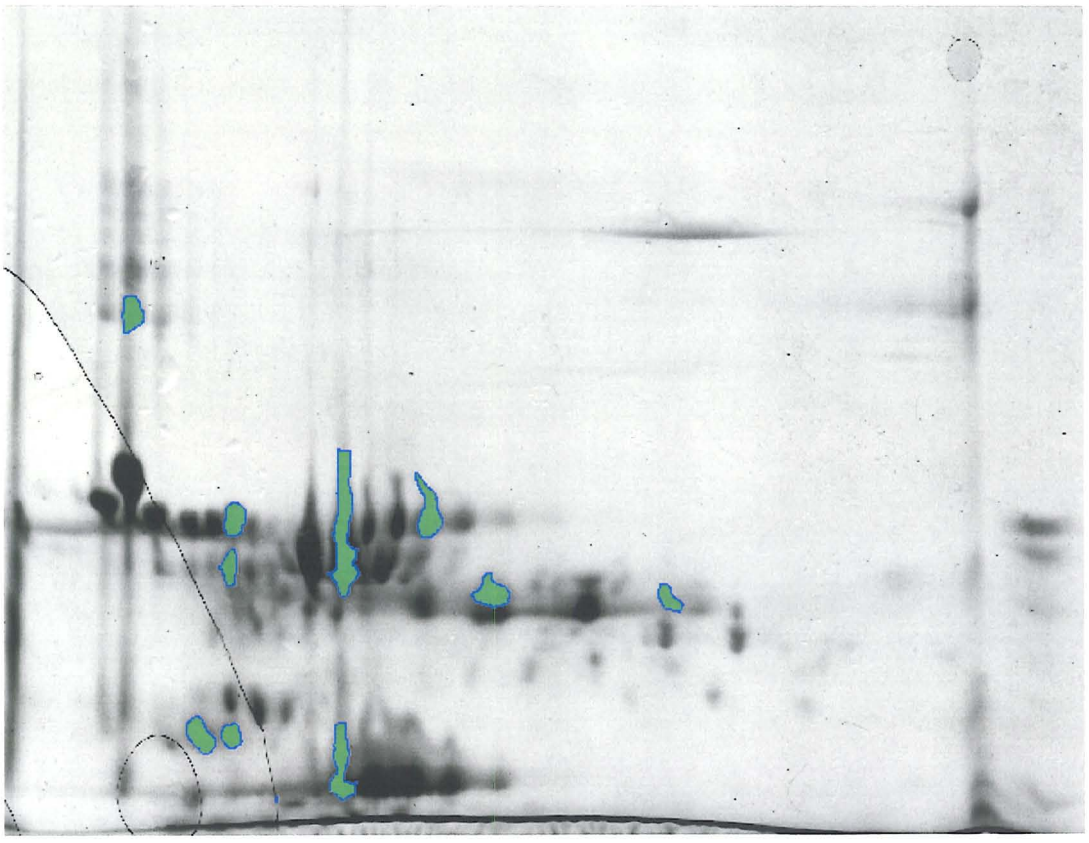


Figure 5-18: A difference map was created to highlight the spots that show more than a two-fold increase (green) in volume at day 3 post-infection to the equivalent spot in the reference gel.

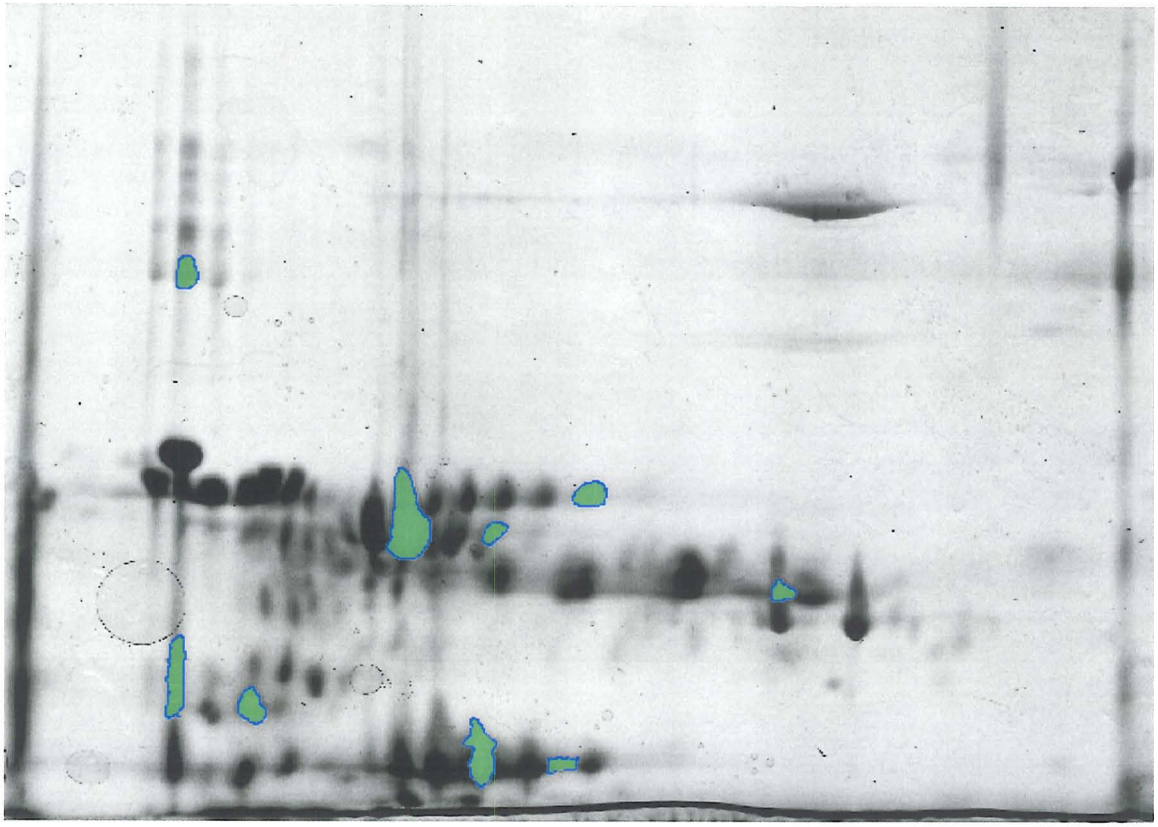


Figure 5-19: A difference gel was created to highlight the spots that show more than a two-fold increase (green) in volume at day 7 post-infection to the equivalent spot in the reference gel.

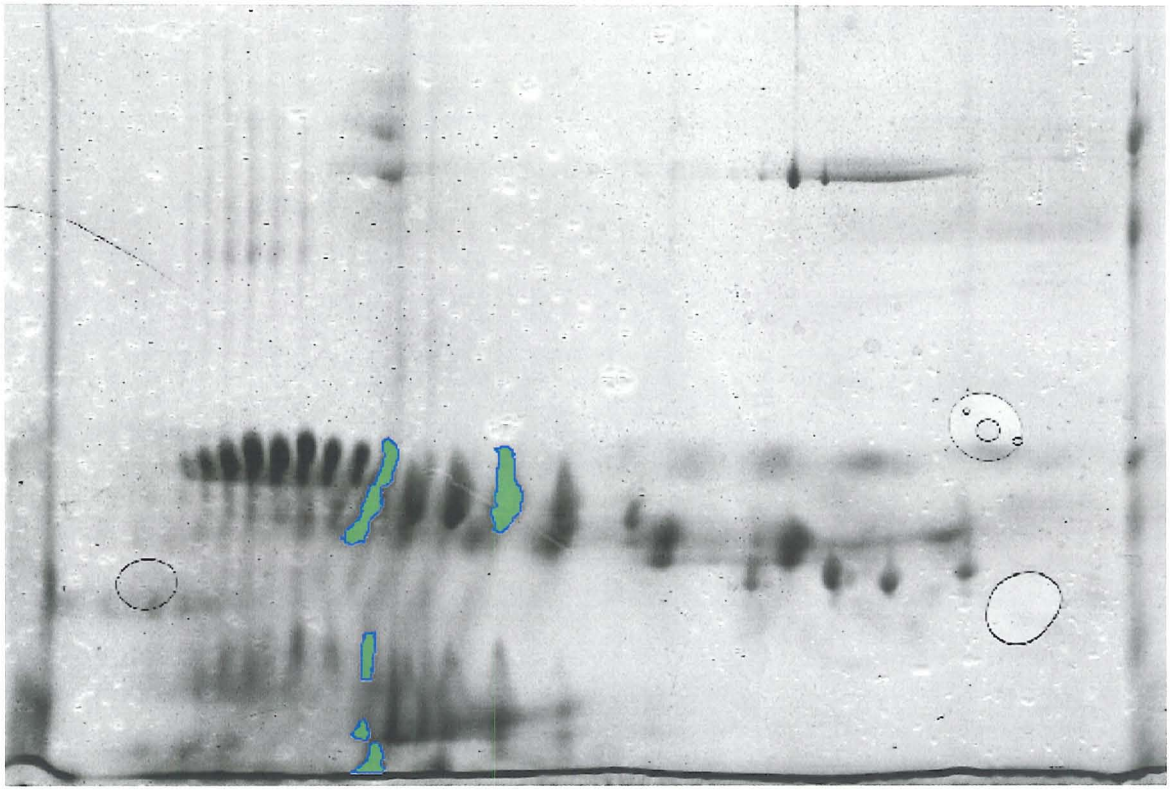


Figure 5-20: A difference gel was created to highlight the spots that show more than a two-fold increase (green) in volume at day 29 post-infection to the equivalent spot in the reference gel.

5.4 Discussion

Previous work (chapter 4) showed that there were obvious differences in the milk proteins of healthy cows and those suffering severe clinical mastitis. Major whey proteins such as α-LA and β-LG were diminished, while other proteins, notably serum protein such as albumin and serotransferrin were increased.

The goal of this study was to extend this work to the examination of an experimental model of *S. aureus* mastitis, designed to induce a mild form of infection that did not cause visible signs of the disease. Changes to the milk proteome during the course of experimental infection in quarters of dairy cows with a defined strain of *S. aureus* were investigated by comparing the whey proteome prior to and during intramammary infection, to identify protein spots in the 2-D gels that change in concentration in a way that correlates with disease status or progression.

The changes obtained in this experimental infection model equated to a mild sub-clinical disease as confirmed by the results for milk APP and SCC. As a result the changes in the milk proteome of healthy and infected milk were much less obvious than those previously seen during severe clinical mastitis.

Detailed image analysis of the 2-DE gels was carried out with Phoretix 2-D advanced software. Two-dimensional electrophoresis induces unavoidable technical differences from gel run to run, arising from sample preparation, gel preparation and staining. For this reason gel analysis could not be performed between animal subsets. Computer analysis was therefore, only performed as a time-course experiment within the one animal. After spot detection, and background subtraction, 2-D gels were edited, filtered and the quantitative determination of the spot volumes was performed. To compare spot volumes across different gels, the protein spots detected in each experimental gel were matched to the corresponding protein spots within the reference protein gel. Background intensity was subtracted from the spot volumes. Computer analysis of the gels confirmed that there was little variation in concentration of the major milk proteins between the different time-points of infection. Some differences were observed, but these did not show correlation to the infection status of the gland.

The proteomic method used in this study was developed and optimised for the study of severely mastitic milk. It is likely that the method requires further optimisation to increase the sensitivity and enable the analysis of smaller changes occurring as seen with subclinical infection. A number of approaches could be considered in future studies that should lead to improvement of the current method.

Many of the emerging technologies for the global evaluation of protein expression are being applied in the field of biomarker discovery for disease progression. However, these analyses can be made difficult by variation between samples that arises from both technical and non-disease related physiological causes (Steel et al 2003). Indeed, the main obstacle in this study was that there was an inherent variability in the resolution of proteins from the milk samples across cows.

This issue of inter-animal variation could be addressed through adopting a different experimental approach in future studies. A possible approach could be to use composite gels, generated from pooling samples from animals in the infected and control groups, in which robust biomarkers should still stand out. Protein spots showing significant variation when comparing the composite gels would then be further investigated.

Second, consistency in the method could be improved through the use of plastic-backed pre-cast gels for the second dimension. Variations in casting of SDS-PAGE gels can contribute to difficulties in gel comparison studies (Wang et al 2003). Additionally automated staining would eliminate any differences in exposure times to the stain, and hence standardise the background levels of the gels (Wang et al 2003).

The very wide range of protein abundance found in milk samples makes separation by 2-DE of unfractionated samples complicated particularly on mini gels. Although the caseins were partially removed, it is evident that this approach must be extended for future studies.

Although they were not consistent, pattern changes were observed to a greater degree in the low molecular weight (<15,000 KDa) and more basic pI (pH 7 to 10) region of the gels. However examination of such proteins and thereby the changes within is made difficult due to the obscuring effect by the major milk proteins.

This study has further emphasised that pre-fractionation of milk is a prerequisite prior to examination of its minor proteins and further preliminary investigations have demonstrated approaches as to how this can be achieved. This could be achieved through use of a pre-

fractionation apparatus, based on pI, that has recently become available. Pre-treatment of milk samples by isoelectric separation in solution using an IEF, zoom fractionator (Invitrogen) can subdivide whole milk samples into 5 fractions and as the major milk proteins have similar pIs in the region of pH 4.5 to 5.5, these can be isolated in one or at most two fractions. By effectively concentrating the major milk proteins into fractions containing proteins of low pI (pH 4.7 to 6.1), disease related alteration in low abundance proteins with pI's outside this range could be more easily identified.

Matching and comparing hundreds of protein spots across gel images taken from a large number of different cows is extraordinarily time-consuming, even with specialized software designed to accommodate some variation.

The ease of use of the software is an important criterion in any proteomics experiment, since gel analysis can be a very slow and laborious procedure. The user must make many choices in operation of 2-D gel analysis software. Editing is one of the most labour-intensive aspects of gel analysis. There is always variability in values of spot volumes determined from samples analyzed in duplicate gels. Some variation will result from gel preparation, and sample handling; some will arise from the analysis procedure. Quantitation and observation errors are the 2 most common problems in 2-D gel image analysis. First, there are spots that are not detected by the software. Second there are spots that are reproducibly observed and yet their spot volumes have some intrinsic variation between the two gels. Background noise from scanning of the images can disrupt spot detection and thus smaller spots are more likely to be undetected (Mahon and Dupree 2001).

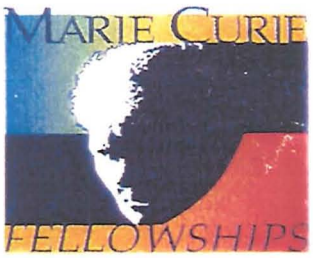
It is important to note that there is a large variability in the relationship between the spot volume derived from Phoretix 2-D and the protein quantity in the original sample, because of variable recovery in the gel and variable staining, in addition to software derived differences in spot measurement.

The original goal of this study was to identify protein spots in the 2-D gels of milk that change in abundance in a way that correlates with disease status or progression, during experimentally induced *S. aureus* mastitis.

Significant biological sample variation from cow to cow, lengthy steps in performing 2-DE and intricate gel analysis, all contributed to the difficulties encountered in trying to differentiate authentic differences caused by disease, with those artefacts caused by sample

variations, running conditions of 2-DE and staining. In conclusion, the variation in sample from cow to cow was too wide to permit conclusive associations between time-points of infection and disease progression.

Marie Curie Fellowship



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Chapter VI

Expression of Mammary-associated Serum Amyloid A3 in Bovine Mammary Tissue

6.1 Introduction

The plasma concentration of the APPs SAA and Hp have been shown to be elevated during mastitis (Conner et al 1986; Hirvonen et al 1996; Hirvonen et al 1999; Eckersall et al 2001). Eckersall and others (2001) were first to demonstrate that the concentration of SAA and Hp increased not only in the plasma during mastitis but also in the milk. There has since been a number of studies investigating milk APPs, all of which discuss the likelihood of SAA being produced locally (Pedersen et al 2003; Gronlund et al 2003a; Nielsen et al 2004). Serum amyloid A present in mastitic milk may be a result of locally produced M-SAA3 pooled with hepatically derived SAA present in the milk through leakage across the blood-mammary barrier.

Extra-hepatic expression of SAA has been documented in a variety of tissues and these are detailed in section 1.3.12. The SAA genes are highly conserved through evolution and have been identified in a wide range of vertebrate species including higher mammals, marsupials, birds and fish (Uhlar et al 1994; Thorn and Whitehead 2002).

Serum amyloid A1, SAA2 and SAA3 isotypes all show profound increases in expression during inflammation (Marhaug et al 1997). All 3 isotypes are expressed in the liver, but, SAA3 appears to be the predominant isoform expressed extrahepatically (Marhaug et al 1997). Serum amyloid A 3 synthesis has been reported at extra-hepatic sites including the ovary, spleen, kidney and lung (Meek et al 1994).

Recent studies have reported the presence of genes expressing SAA in the epithelium of involuting mammary tissue (Kho et al 2000; McDonald et al 2001), and during infection (Molenaar et al 2002). The milk isoform has a unique 4 residue aa motif TFLK within the first 8 residues and is termed mammary-associated serum amyloid A 3 (M-SAA3). This motif has not been reported in hepatically produced SAA. A number of roles have been suggested for SAA. The constitutive expression of SAA in non-hepatic cell lines may

suggest a housekeeping role for the protein. Additionally it could have a role as an immunological defence molecule, produced at local sites to protect against injury caused by inflammation. Rabbit SAA produced by synovial cells has been implicated in tissue repair. Hence it is very interesting and timely to study the expression of M-SAA3 in the bovine mammary gland during the inflammatory condition of mastitis.

The study presented herein investigates the presence of M-SAA3 in bovine mammary tissue during infection, and exploits the small difference in amino acid sequence of this isoform to design primers that have the ability to distinguish between M-SAA3 and hepatically derived SAA.

6.1.1 Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) represents a powerful tool for analysing ribonucleic acid (RNA) (Erlich et al 1991; Freeman et al 1999). The polymerase chain reaction (PCR) is a technique that allows the amplification of a specific sequence of DNA and is effective even when the target DNA is present in very low concentration. The technique was developed by Mullis *et al* (1986), and allows large amounts of a single copy gene to be generated from genomic or viral DNA (Mullis et al 1986; Mullis and Faloona 1987). The initial procedure used the Klenow fragment of DNA polymerase I. This enzyme needed to be replaced at each cycle as it was denatured by the high temperature reaction conditions. Efficiency was greatly improved by introduction of a thermostable *Taq* polymerase isolated from *Thermus aquaticus*, as this allowed automation of the procedure (Saiki et al 1988).

PCR allows the amplification of any unknown DNA sequence by the simultaneous extension of primer pairs flanking the unknown sequence, each complementary to opposite strands of the DNA. An overview of PCR and its applications is available (Innis and Gelfand 1990).

6.1.1.1 The coding region of the gene

In eukaryotes most of the gene protein coding region is not a continuous DNA sequence reading out co-linearly into messenger RNA (mRNA) but is composed of an alternating array of coding sequence, called exons separated by intervening pieces of non-protein coding DNA referred to as introns. The removal of these intronic sequences from the initial RNA transcript and the joining of all exons in a head to tail arrangement occurs, through a

precise mechanism mediated by the splicing machinery in the nucleus (Weiner 1993), to form a mRNA molecule. Genomic DNA containing introns can be used to express the gene of interest but these sequences can span up to hundreds of kilobases and are therefore impossible to clone into the currently available mammalian expression vectors. Alternatively cDNA, which is a direct copy of the mRNA transcript, can be made *in vitro*, and used to directly transcribe and translate genes to a functional protein. Thus, the cDNAs encoding the bovine genes SAA and M-SAA3 have been used in this chapter.

6.1.1.2 The immediate 5′ UTR

The 5′ untranslated region (UTR) of the gene is important for the initiation of gene transcription. The process of transcription involves the binding of RNA polymerase to the initiation site, under the influence of the 5′ regulatory region, followed by the addition of nucleotides in a 5′ to 3′ fashion to form the primary RNA transcript. The most 5′ nucleotide of the transcript is usually modified post-transcriptionally by the addition of a methylated G (guanine) residue linked by a 5′-to 5′- triphosphate bridge. This feature, referred to as a 'Cap' structure, is essential for efficient initiation of protein synthesis.

The 5′ UTR is also important in the translation process of most mammalian mRNAs to protein. Translation is initiated by the binding of the numerous protein factors including the 40S ribosomal subunit to the region 5′ to the AUG. The ribosomal component moves to the first AUG where the 60S ribosomal subunit joins to produce the first peptide bond (Kozak, 1991). Translation initiation from the mRNA template is the rate limiting step in protein synthesis and is controlled by the context and position of the AUG initiation codon with its surrounding secondary structure and length of 5′ untranslated region (UTR). The optimal context for initiation is 5′ GCC(A/G)CC**AUG**G 3′ where the **A** of the **AUG** codon is referred to as the +1 nucleotide. The most critical residues controlling the efficiency of initiation are purines at positions –3 and +4, usually an 'A' and 'G' respectively. The presence of multiple upstream initiation sites serves to suppress the initiation efficiency from the correct site. Position of the AUG codon is also extremely important since approximately 90% of vertebrate mRNAs are initiated from the first AUG codon without giving preference to an optimal context.

6.1.1.3 The immediate 3′ UTR

The 3′ UTR, defined as the mRNA sequence following the termination codon (TAA, TAG) is thought to play a role in mRNA transcript stability, providing a determinant of mRNA levels and also plays a role in protein synthesis through the control of translation (Jackson and Standart 1990). The half-life of a mRNA molecule determines the length of time that the template is available for protein synthesis (Peltz et al 1991), which can vary from several minutes for some cytokines to 100 hours for stable mRNAs. However, the half-life may not always be constant and will change with the conditions of the cell. The rapid turnover of mRNA has been linked to cis-acting instability elements present within the RNA sequence. One of these instability elements is in the class of AU-rich elements (ARE) and contains the pentameric motif AUUUA in a U-rich context sufficient for rapid mRNA degradation (Vakalopoulou et al 1991). This rapid degradation process is important for transient induction of molecules and rapid cellular changes that occur in response to the environment providing the fine-tuning of protein levels within the cell.

The minimal, mammalian poly(A) signal, located in the 3′ UTR, is composed of an AAUAAA motif 20-30 nucleotides and serves to control the termination and processing of the primary RNA transcript with the formation of the poly(A) tail on the mRNA transcript (Proudfoot and O'Sullivan 2002).

6.2 Materials and Methods

6.2.1 Sample material

The tissue samples used in this experiment were obtained from an experiment using a *S. aureus* experimental infection at Glasgow Veterinary School, taken 24h after the second inoculation. The experimental infection is described in detail in chapter 5.

6.2.1.1 Mammary tissue

Udder tissue samples were collected aseptically from slaughtered cows within 5 to 10 minutes after killing. The udder skin was washed with hibiscrub and incised with a disinfected knife (70% alcohol). A small tissue section (1 by 1 by 1cm) was removed from the epithelial lining of the gland and preserved in 'RNA later' (Qiagen, Hilden, Germany)

for RNA analysis. 100 mg of tissue (wet weight) was used as the starting material for RNA isolation.

6.2.1.2 Isolation of total RNA

The total RNA was isolated from mammary tissue using TRIzol (Gibco Brl., Life Technologies Inc., NY, USA). RNA concentrations were determined by UV spectrophotometry. Two μg of isolated RNA was separated on a 1% agarose gel to assess the integrity of the 28S and 18S ribosomal RNA as an indication of RNA quality.

6.2.1.3 Dnase treatment

Prior to first strand cDNA synthesis the total RNA was treated with Dnase I (DNA-free kit, Invitrogen, Breda, The Netherlands) to prevent amplification of genomic DNA. In a simple two-step reaction 1 μg of total RNA was incubated for 15mins at room temperature with the DNAse I and the enzyme – specific reaction buffer. The reaction was terminated by adding 1 μl of 25mM EDTA and heating for 10min at 65°C.

6.2.1.4 First strand cDNA synthesis

Analysis of gene expression requires accurate determination of mRNA levels, but as PCR is based on amplification of DNA, the process of amplifying RNA sequence requires an initial step of conversion of RNA to cDNA by reverse transcription. The Molony-Murine Leukaemia Virus (M-MLV) reverse transcriptase enzyme (Invitrogen, Breda, The Netherlands) was used to carry out this conversion (Brooks et al 1995). Oligo-dT primers (Amersham Biosciences, NJ, USA) pair with the poly A sequence found at the 3' end of most eukaryotic mRNA molecules and initiate the synthesis of cDNA strands in the presence of dNTPs.

The specific conditions used involved a 3-step reaction as follows:

1) 1 μg of total RNA, 1 μl of oligo $(dT)_{12-18}$ primers, 1 μl of 10mM dNTP were added to a nuclease-free microcentrifuge tube, made up to a total reaction volume of 12 μl with ddH_2O and heated for 5 min at 65°C.
2) 2) 4 μl 5X first-strand buffer, 2 μl of 0.1M DTT and 1 μl of RNaseOUT recombinant ribonuclease inhibitor (40 units/ μl) were added to the tube and incubated for 2 min at 37°C.

3) 3) The contents of the tube were mixed and 1 μl of M-MLV RT was added and the mixture was heated for 50 min at 37°C. Finally the reaction was terminated by heating for 15 min at 70°C.

6.2.2 PCR amplification of M-SAA3 cDNA in bovine mammary tissue

RT-PCR analysis on RNA extracted from mammary gland epithelium of 4 control cows and 4 with experimentally induced *S. aureus* mastitis was carried out to determine a) if the SAA or M-SAA3 genes are expressed in the mammary tissue and b) if they are expressed, is their expression up or down-regulated during mastitis.

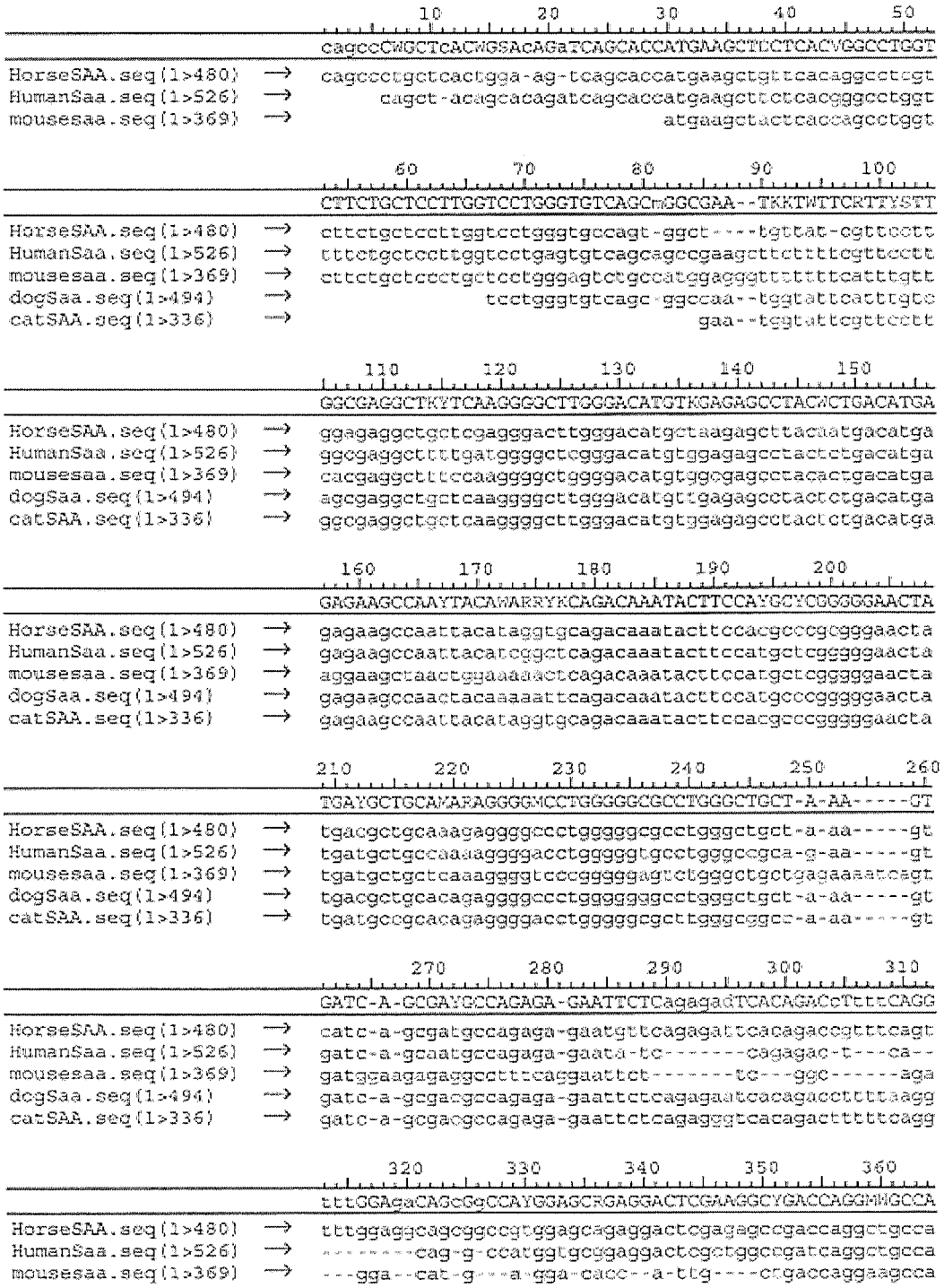
6.2.2.1 Primer design

Primers were designed following the guidance of a standard text (Innis and Gelfand 1990). The goal of primer design is to produce an oligonucleotide that will hybridise efficiently with the DNA sequence of interest, and not hybridise with any other sequence. The amount of sequence permissible between primers is flexible, and up to 10kb is possible, however beyond 3kb efficiency is decreased (Jeffreys et al 1988). The annealing portion of primers should generally be between 18 and 30 nucleotides in length; any increase in size beyond this is unlikely to improve primer specificity significantly and this size of primer is sufficient for sequence as complex as the human genome. Primer GC content should match that of the template as closely as possible. For any given pair of primers, the annealing temperatures ™ and GC content were balanced.

It was essential that primers were designed that could distinguish between hepatically derived SAA and the mammary isoform M-SAA3. Mammary-associated serum amyloid A3 contains a unique four amino acid motif TFLK within the first 8 residues (McDonald et al 2001). The M-SAA3 primer was designed by back-translating this amino acid sequence to include the nucleotides encoding for this motif in the M-SAA3 forward primer (bovM-SAA3) (Table 6.1). Serum amyloid A has a high degree of conservation across species (Uhlar and Whitehead 1999), so SAA primers was designed according to regions of sequence homology between different species. A sequence alignment was performed on the cDNA sequences of horse, human, mouse, dog and cat using the Seqman computer programme (DNASTAR Inc.,WI, USA) (Figure 6-1). A degenerate primer was designed for the SAA forward primer (bov SAAF) (Table 6.1) as they allow a degree of flexibility when the exact sequence of the target gene being amplified is not known

Primer name	Primer sequence (5' to 3')	TM (°C)
bovMAAF	GATGGGGGACATTCCTCA	65.5 °C
bov SAAF	CGAATGGTWTTCRTTYTTTGGC	65.1 °C
bovSAAconR	ATCACTTTAGCAGCCCAGGC	65.3 °C

Table 6-1: Sequence and melting points for the primers used for RT-PCR


10 20 30 40 50
cagccCWGCTcACWGSAcAGaTCAGCACCATGAAGCTBCTCACVGGCCTGGT
HorseSAA.seq(1>480) → cagccctgctcactgga-ag-tcagcaccatgaagctgttcacaggcctcgt
HumanSaa.seq(1>526) → cagct-acagcacagatcagcaccatgaagcttctcacgggcctggt
mousesaa.seq(1>369) → atgaagctactcaccagcctggt
60 70 80 90 100
CTTCTGCTCCTTGGTCCTGGGTGTCAGCmGGCGAA--TKKTWTTCRTTYSTT
HorseSAA.seq(1>480) → ctttctgctccttggtcctgggtgccagt-ggct----tgttat-cgttcctt
HumanSaa.seq(1>526) → tttctgctccttggtcctgagtgtcagcagccgaagcttcttttcgttcctt
mousesaa.seq(1>369) → ctttctgctcctgctcctgggagtctgccatggagggtttttttcatttgtt
dogSaa.seq(1>494) → tcctgggtgtcagc-ggccaa--tggtattcacttgtc
catSAA.seq(1>336) → gaa--tggtattcgttcctt
110 120 130 140 150
GGCGAGGCTKYTCAAGGGGCTTGGGACATGTKGAGAGCCTACWCTGACATGA
HorseSAA.seq(1>480) → ggagaggctgctcgagggacttgggacatgctaagagcttacaatgacatga
HumanSaa.seq(1>526) → ggcgaggcttttgatggggctcgggacatgtggagagcctactctgacatga
mousesaa.seq(1>369) → cacgaggctttccaaggggctggggacatgtggcgagcctacactgacatga
dogSaa.seq(1>494) → agcgaggctgctcaaggggcttgggacatgttgagagcctactctgacatga
catSAA.seq(1>336) → ggcgaggctgctcaaggggcttgggacatgtggagagcctactctgacatga
160 170 180 190 200
GAGAAGCCAAYTACAMARRYKCAGACAAATACTTCCAYGCYCGGGGGAACTA
HorseSAA.seq(1>480) → gagaagccaattacataggtgcagacaaatacttccacgcccgcgggaacta
HumanSaa.seq(1>526) → gagaagccaattacatcggctcagacaaatacttccatgctcgggggaacta
mousesaa.seq(1>369) → aggaagctaactggaaaaactcagacaaatacttccatgctcgggggaacta
dogSaa.seq(1>494) → gagaagccaactacaaaaattcagacaaatacttccatgcccgggggaacta
catSAA.seq(1>336) → gagaagccaattacataggtgcagacaaatacttccacgcccgggggaacta
210 220 230 240 250 260
TGAYGCTGCAMARAGGGGMCCTGGGGGCGCCTGGGCTGCT-A-AA-----CT
HorseSAA.seq(1>480) → tgacgctgcaaagaggggccctgggggcgcctgggctgct-a-aa-----gt
HumanSaa.seq(1>526) → tgatgctgccaaaaggggacctgggggtgcctgggccgca-g-aa-----gt
mousesaa.seq(1>369) → tgatgctgctcaaaggggtcccgggggagtctgggctgctgagaaaatcagt
dogSaa.seq(1>494) → tgacgctgcacagaggggccctgggggggcctgggctgct-a-aa-----gt
catSAA.seq(1>336) → tgatgccgcacagaggggacctgggggcgcttgggcggcc-a-aa-----gt
270 280 290 300 310
GATC-A-GCGAYGCCAGAGA-GAATTCTCagagagTCACAGACCcTttCAGG
HorseSAA.seq(1>480) → catc-a-gcgatgccagaga-gaatgttcagagattcacagaccgtttcagt
HumanSaa.seq(1>526) → gatc-a-gcaatgccagaga-gaata-tc-------cagagac-t---ca--
mousesaa.seq(1>369) → gatggaagagaggcctttcaggaattct-------tc---ggc------aga
dogSaa.seq(1>494) → gatc-a-gcgacgccagaga-gaattctcagagaatcacagacctttaagg
catSAA.seq(1>336) → gatc-a-gcgacgccagaga-gaattctcagagggtcacagacttttcagg
320 330 340 350 360
tttGGAgaCAGcGgCCAYGGAGCRGAGGACTCGAAGGCYGACCAGGMWGCCA
HorseSAA.seq(1>480) → tttggaggcagcggccgtggagcagaggactcgagagccgaccaggctgcca
HumanSaa.seq(1>526) → --------cag-g-ccatggtgcggaggactcgctggccgatcaggctgcca
mousesaa.seq(1>369) → ---gga--cat-g---a-gga-cacc--a-ttg----ctgaccaggaagcca

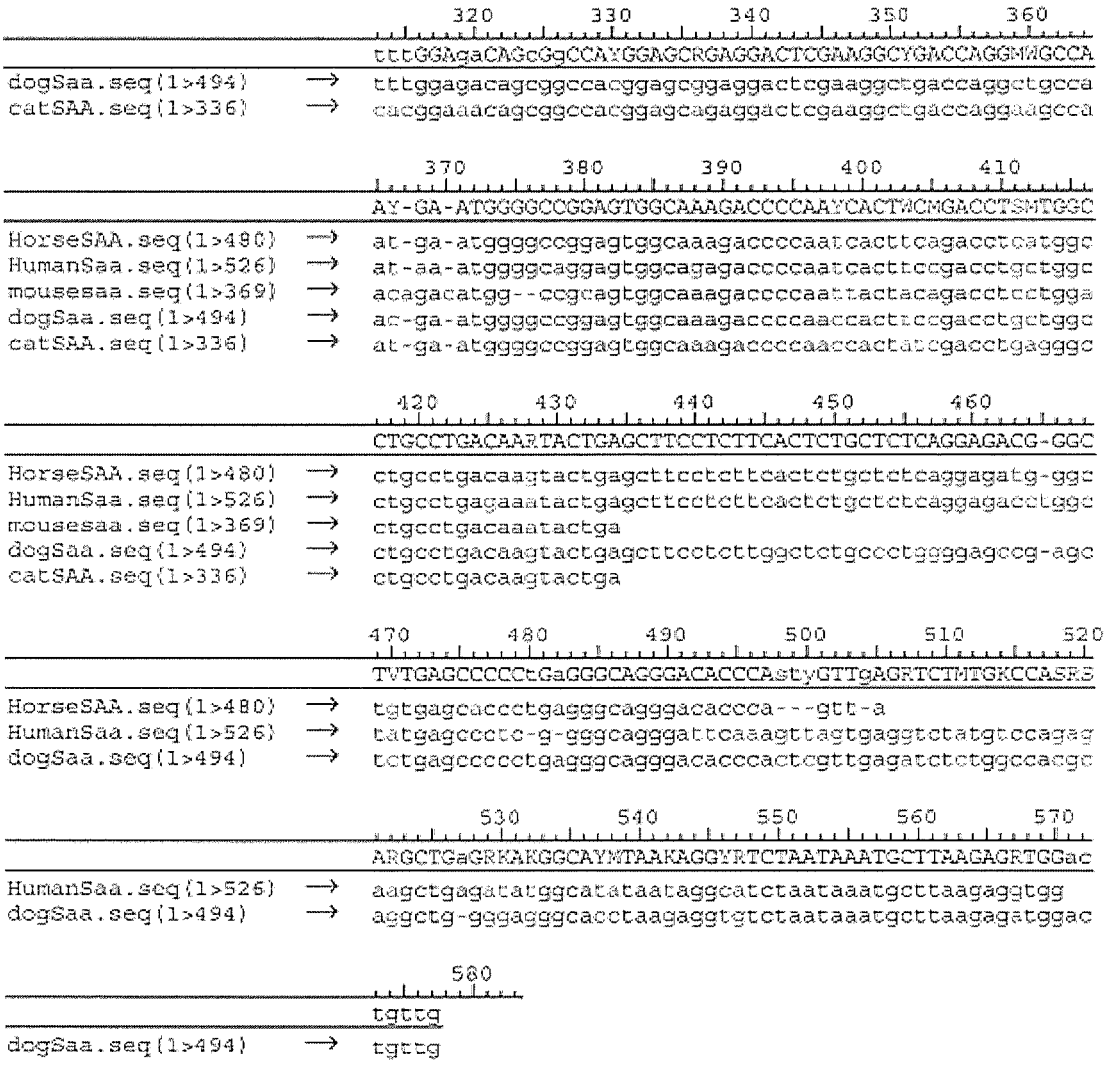


Figure 6-1: An alignment with the nucleotide sequences of serum amyloid A (SAA) for horse, human, mouse, dog and cat.

(Kwok et al 1994; Linhart and Shamir 2002). The difference in the sequence of M-SAA3 occurs at the 5' end of the sequence and as a result the same reverse primer (bovSAAconR) (Table 6.1) designed from the consensus sequence can be used in both reactions. A final alignment of the bovM-SAA3 primer sequence against the consensus sequence was carried out to check that the M-SAA3 primer did not match. The primer sequences are detailed in table 6.1. With these primers, a M-SAA3 specific cDNA product of 169bp was expected.

6.2.2.2 RT-PCR reaction

The sensitive nature of PCR means that very stringent steps must be taken if contamination is to be avoided. First, physical separation of the PCR area from bench space used for other work is recommended. In the case of PCRs carried out in this project, a dedicated PCR suite isolated from the main laboratory was used. In addition, a set of instruments used to aliquot reagents (micropipettes and their tips) were kept within the PCR suite and restricted to PCR use. Filter tip pipette tips were used to reduce the chance of carryover from one step to the next, and master mixes of reagents were used whenever possible to reduce the number of pipetting steps required per reaction. Reaction components, including primers, were aliquoted prior to use and stored at -20°C. PCR reactions were performed using 1µl of bovine cDNA sample in a total volume of 50µl containing 1µl (10pmol) of both sense primer (bovMAAF or bov SAAF) and anti-sense primer (bovSAAconR), 1µl of dNTPs, 0.2µl of *Taq* DNA polymerase (Promega, WI, USA) and 5µl of $MgCl_2$ containing reaction buffer (Promega, WI, USA) . The mixture was placed in a Perkin-Elmer 480 thermal cycler. Samples were subjected to an initial denaturation at 94°C for 2min followed by 40 cycles of amplification, each cycle consisting of a denaturation step of 94°C for 2min and annealing temperature of 60°C for 30s, followed by an elongation step of 72°C for 30s. A final extension step at 72°C for 7mins completed the reaction. Samples were kept at 4°C after the reaction finished. A negative control containing all PCR components (primers, dNTPs, PCR buffer and Taq polymerase) but using ddH_2O instead of template was included to ensure that there was no contamination of the PCR reactions.

As an internal endogenous control for mRNA/cDNA integrity, an RT-PCR of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed.

6.2.2.3 Assessment of PCR products

An aliquot of the PCR product was assessed by 1% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. The molecular weight of the fragments was deduced through comparison to a ladder of molecular weight markers (SmartLadder, Eurogentec, The Netherlands).

6.2.2.4 Extraction and sequence analysis

The resulting PCR products were extracted from the gel using the QIAEX II Gel Extraction Kit (Qiagen, CA, USA), and ligated in a PGEM-T Easy vector (Promega, WI, USA) at 16°C overnight following manufacturer's instructions. *E. Coli* bacteria were transformed by electroporation of the ligated vector. After gel purification the gel products were directly sequenced with an ABI 310 Prism Genetic Analyzer (Perkin Elmer Applied Biosystems, CA, USA), using the Big Dye Terminator Cycle Sequencing Ready Kit (Perkin Elmer Applied Biosystems, CA, USA).

The 'BLAST' search engine within the NCBI database (www.ncbi.nih.gov/BLAST/) was used to analyse sequence data.

6.3 Results

6.3.1 Analysis of RNA quantity and quality

Full-length, total RNA was isolated from bovine mammary tissue from 4 control cows and 4 cows inoculated with *S. aureus*. Good quality RNA samples were obtained as determined by ultraviolet spectrophotomtery (A260/A280 value >1.6) and agarose gel electrophoresis assessment (Figure 6.2); the RNA migrated through the 1% agarose gel revealing intact 18s and 28s ribosomal RNA components.

6.3.2 PCR amplification of bovine M-SAA3 cDNA

RNA samples (1µg) were subjected to first strand synthesis using M-MLV reverse transcriptase and random primers to produce bovine cDNA. The cDNA samples were used as the template for the amplification of M-SAA3 using sense (bovMAAF) and antisense (bovSAAconR) oligonucleotide primers and *Taq* DNA polymerase. The PCR products obtained migrated on a 1% agarose gel to show a DNA fragment of approximately 160 bp

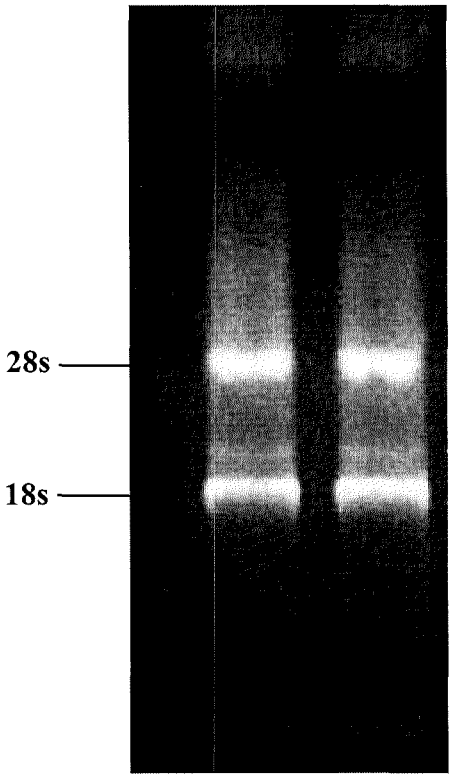


Figure 6-2: Ethidium bromide stained agarose gel showing the integrity of the 18s and 28s ribosomal RNA components.

(Figure 6-3). The expected product visualised in lanes 3 and 4 corresponds to the portion of M-SAA3 amplified by RT-PCR using primers specific for M-SAA3. Lanes 1 and 2 show that there was no amplification of hepatically derived SAA or M-SAA3 when the bov SAAF primer was used in the reaction instead of the bovMAAF. Lane 4 shows that the PCR product is upregulated in mastitic udder tissue as compared to the control tissue (lane 3). There were no bands present in the negative control reaction (not shown).

Variation in the level of RNA between samples was assessed by using an endogenous RNA internal control GAPDH was used to assess the quality of the cDNA, but no significant changes in GAPDH levels were observed (not shown). Bovine mammary cDNA was amplified by RT-PCR to produce a 169bp product.

6.3.2.1 Sequencing of PCR product

The nucleotide sequences determined from the genetic analyzer were assessed using the 'BLAST' search engine within the NCBI database. The nucleotide sequence was compared to every gene sequence within the data bank to identify those genes which shared the greatest homology with the 169 bp fragment obtained in this experiment. The results revealed that the nucleotide sequence showed 100% homology to bovine SAA3 (Figure 6-4), and therefore confirmed the local production of M-SAA3 in the bovine mammary gland and its up-regulation during bovine mastitis.

6.4 Discussion

RT-PCR analysis was performed on RNA extracted from the mammary gland epithelium of 4 control cows and 4 cows with experimentally induced *S. aureus* mastitis. The aim of the study was to determine if the M-SAA3 gene was expressed in bovine mammary tissue and if so, is its expression up or down-regulated during mastitis.

Primers were carefully designed, to include the nucleotide bases encoding for the unique 4 amino acid motif, TFLK present within the first 8 residues of the M-SAA3 sequence (McDonald et al 2001). No product was visible when the primers derived from the consensus sequence of SAA were used in the RT-PCR reaction, indicating that only the unique M-SAA3 isoform is produced locally in the bovine mammary gland. Mammary-associated serum amyloid A3 mRNA, was detected in epithelial tissue from control cows, but was up-regulated during mastitis.

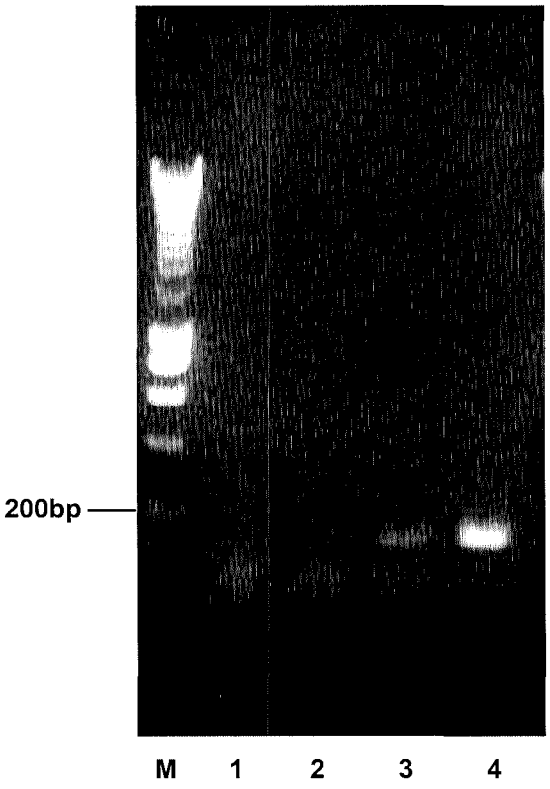


Figure 6-3: Ethidium bromide stained agarose gel electrophoresis showing the RT-PCR products of total RNA from control (lanes 1 and 3) and mastitic mammary tissue (lanes 2 and 4) amplified using primers that can distinguish between SAA and M-SAA3. The SAA primers were used in the reaction shown in lanes 1 and 2 and the M-SAA3 primers were used in the reaction shown in lanes 3 and 4. Markers of a DNA ladder are shown in lane m.

```
Query: 1   gatgggggacattcctcaaggaagctggtcaaggggctaaagacatgtggagagcttacc 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 59  gatgggggacattcctcaaggaagctggtcaaggggctaaagacatgtggagagcttacc 118

Query: 61  aagacatgaaagaagccaactacaggggtgcagacaaatacttccacgcccgtggaaact 120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 119 aagacatgaaagaagccaactacaggggtgcagacaaatacttccacgcccgtggaaact 178

Query: 121 atgacgctgcccgaaggggacctgggggtgcctgggctgctaaagtgat 169
           |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 179 atgacgctgcccgaaggggacctgggggtgcctgggctgctaaagtgat 227
```

Figure 6-4: The nucleotide sequence of the 169-bp fragment amplified by RT-PCR showed 100% homology to bovine M-SAA3 Genbank accession number: AF335552. Underlined in red are the forward and reverse primers used for the RT-PCR

Previous studies examining the extrahepatic expression of SAA in mice, reported increased expression of SAA mRNA in extra-hepatic sites following LPS stimulation, but SAA mRNA was either not detectable or present in low levels in all saline infused control tissue sites (Meek and Benditt 1986; Kalmovarin et al 1991). The results in this study were in agreement with these previous findings, with M-SAA3 mRNA expression being detected at low levels in mammary tissue from control cows as compared to tissue from *S. aureus* infused cows (McDonald et al 2001; Molenaar et al 2002).

The high nucleotide sequence conservation between different species directly reflects the amino acid sequence and the latter is closely related to biological function. Therefore it is predicted that M-SAA3 will share the same basic biological functions as the SAA3 isoform in other species. M-SAA3 has considerable amino acid homology to rabbit SAA3 produced extrahepatically by synovial fibroblasts (Brinckerhoff et al 1989), which has been shown to induce the production of collagenase and stromelysin. This assigned function to rabbit SAA3 may be important in localized tissue remodelling during tissue regeneration and repair that occurs in wound healing. McDonald et al (2001), speculate that M-SAA3 may play a role in mammary gland tissue remodelling during lactation-associated changes. During mastitis the integrity of the blood-mammary barrier can be severely disrupted and it is possible that M-SAA3 may play a role in modulation of connective tissue repair.

The principal function of SAA remains elusive, and little is known concerning the precise localization of SAA bio-synthesis outside the liver in mammals (Marhaug et al 1997). However SAA expression in the mucosa of the intestine (Meek et al 1989), as well as in the endometrium may reflect a role in surface epithelial protection for this protein. Additionally, evidence that SAA is expressed in endothelial cells (Meek et al 1994) lends support to the theory that SAA may be a factor in barrier functions of epithelial and endothelial cells.

The APP, SAA has been considered to be expressed primarily in the liver until recent years (Urieli-Shoval et al 1998). Why these extrahepatic tissues express mRNA encoding the SAA genes is unclear, but the induced expression probably results from the reaction of certain cytokines or hormones with their cellular receptors in response to inflammatory signals. Although the extrahepatic expression of the SAA3 isoform is usually in association with inflammation, its high abundance in colostrum of healthy cows suggests that M-SAA3 may play an important functional role associated with the well-being of the newborn and possibly mammary tissue remodelling (McDonald et al 2001).

Additional studies will be necessary to clarify further the role of M-SAA3 in the bovine mammary gland, but based on the sequence homology of M-SAA3 with SAA3 it is possible that it may have a role in the modulation of numerous immunological responses that are essential to protect the host and restore homeostasis. Expression of M-SAA3 on surface epithelia indicates a defensive role for the protein, situated in a position to communicate with the external environment and protect local tissues from potential threats such as microorganisms.

This important APP, M-SAA3 has been shown to be produced locally in the bovine mammary gland and there is evidence that it is up-regulated during mastitis. Additional work is needed to further investigate this finding. *In-situ* hybridisation should be performed to give a more detailed analysis of the tissue localization of M-SAA3 in the bovine udder. Quantitative real-time PCR could be used to measure the degree of up-regulation of M-SAA3 in the bovine mammary gland during mastitis.

Chapter VII

General Discussion

The aims of this thesis were outlined in chapter 1 and the relevant conclusions have been detailed in the appropriate chapters. This chapter serves to summarise the most significant findings and highlight the areas which have been recognised as worthy of future research.

Acute phase proteins are produced in response to a variety of disease conditions stimulated by the pro-inflammatory cytokines and in response to infection, trauma or inflammation. The measurement of APPs in serum of animals has been widely used over the past two decades, for disease diagnosis in the field of veterinary medicine (Gruys et al 1994; Eckersall 1995b; Horadagoda et al 1999). Eckersall *et al* (2001) were first to demonstrate significant increases in the concentration of APPs in the milk of cows with clinically mastitis. This initial finding spurred the research described in this thesis, during the period of which there have been further studies carried out involving investigations of milk APP (Pedersen et al 2003; Gronlund et al 2003a; Gronlund et al 2003b; Nielsen et al 2004).

Many gaps exist in our knowledge and understanding of mastitis, as a result it remains a major source of economic lose to the dairy industry worldwide. Increasing the insight into the pathogenesis of mastitis will help to develop new strategies for control and prevention of bovine mastitis. Early diagnosis of mastitis is paramount if economic loses arising from this disease are to be reduced. There are a number of strategies currently employed for the diagnosis of mastitis, and these were reviewed comprehensively in chapter 3. It was recognised that there are many limitations surrounding the use of standard parameters such as SCC and bacteriology for mastitis diagnosis. Therefore the potential of measuring APP in milk as a diagnostic tool for bovine mastitis was investigated in this study.

In validation of any new diagnostic test, it is essential to compare the new method with the existing method. It was therefore fundamental in this thesis to establish the association of APP in milk with SCC, the conventional markers of mastitis and a large proportion of the work undertaken focussed on this. The analysis of APP in milk demonstrated that they are comparable to and complementary to SCC. Many farmers now measure composite-quarter milk SCCs, however this means that a moderate SCC increase in one quarter may go unnoticed if the other three have normal SCCs. Analysis of quarter milk SCC, is a

possibility to monitor udder health closely and to investigate subclinical mastitis, but would be very expensive.

The methods used to analyse APPs have developed considerably over the past few years and commercial tests are now available for all the major APPs (Eckersall 2004). Mammary associated SAA3 was measured in milk by modification of the commercially available ELISA for SAA. However, an ELISA based method had to be developed to measure milk Hp as the existing method for measuring serum Hp was not suitable and the SRID method used previously for the measurement of milk Hp lacked sensitivity. The work carried out in chapter 2 of this thesis has led to the development of the first ELISA to measure milk Hp. The method described is sensitive even at low levels and is more expeditious than existing methods.

This newly developed milk Hp assay was employed to measure the concentration of Hp in milk from an experimental model of *S. aureus* mastitis. In the experimental model both M-SAA3 and Hp were sensitive markers of acute clinical mastitis, but it was concluded that only M-SAA3 could be used to detect chronic subclinical mastitis. Haptoglobin reached higher values in the milk during the acute phase of infection than M-SAA3.

Chapter 3 investigated further the pathophysiology of the milk APR using a field study of naturally occurring mastitis designed to establish the earliest time-point of appearance of APP in the milk. The results showed that both M-SAA3 and Hp showed similar kinetics to SCC and can be concluded as sensitive and reliable markers of clinical mastitis. Furthermore, this study showed that the APP responses differ significantly between individuals. In some cows the concentration of M-SAA3 reaches higher levels than that of Hp, whereas in others the situation is reversed and Hp reaches higher concentrations than M-SAA3, the latter being in agreement with previous studies (Eckersall et al 2001; Pedersen et al 2003; Gronlund et al 2003a; Nielsen et al 2004).

Of greater relevance to clinical practice is the possibility that once the presence of inflammation has been identified, assays for the APPs can indicate the nature of the condition, including pathogen type, severity and whether it is a clinical or subclincal infection that is present. Acute phase protein measurement in serum has the ability to differentiate between chronic and acute inflammation (Horadagoda et al 1999).

The implication from the results of the work in chapter 3, that the levels of APP in milk may be indicative of the pathogen responsible for mastitis is very encouraging. Much interest in deciphering pathogen type has been generated in mastitis research by awareness that different pathogens require different treatment. Additionally knowledge of pathogen type is useful as it may help predict and influence the probability of infection or cure rate. Of further interest was the kinetics of APP observed across a time-course and the pathogen-specific effects. In *E. coli* mastitis both Hp and M-SAA3 increased sharply prior to the onset of clinical signs and decreased rapidly post-infection. Acute phase proteins in milk display similar kinetics to SCC and it has previously been shown that different mastitis causing pathogens are associated with different baseline levels for SCC (de Haas et al 2002). The kinetics of SCC in *E. coli* infections have been investigated and a similar pattern of response to that seen for APPs was reported (Peeler et al 2003).

Although for veterinarians and skilled stockmen, clinical mastitis is not difficult to diagnose, a more refined approach is needed to detect chronic subclinical mastitis. At present, increased SCC is often the only characteristic of chronic subclinical mastitis. Acute phase proteins, M-SAA3 and Hp were also detected in subclincal mastitis but at much lower concentrations to those found in clinical mastitis. Further study is required to confirm the ability of milk APPs in identification of subclinical mastitis.

The increased use of automatic milking systems has created a need for new monitoring systems for early and accurate detection of mastitis. Electrical conductivity of milk has been used for the automatic detection of mastitis, but is not considered sufficiently sensitive or reliable for use routinely in robotic milking systems for the detection of both clinical and subclinical mastitis in dairy cows (Nielen et al 1995; Mottram 1997). Further field studies are needed to see whether measurement of APP in milk might meet the requirement.

Prevention of mastitis is preferable over cure especially in developed countries where consumer concern over animal welfare and food safety is an issue. Reducing the use of antibiotics would be beneficial for the health and welfare of humans and cows alike, and early diagnosis could contribute to this being achieved. Dairy farming is an economic activity, so a diagnostic test is only successful when it is cost-effective.

The ready availability of milk offers excellent opportunity for its analysis in disease diagnosis. The mammary glands can be accessed with ease keeping stress levels to a minimum which is a very important consideration when lactating dairy cows are involved.

Acute phase protein research in milk is at an early stage but there is considerable scope for further development. Extending the investigation of the pathophysiology of the APP response in milk should be undertaken to determine for example the relation between severity of infection and type of pathogen, and also to assess the effects of concurrent inflammatory diseases common in dairy cows e.g. lameness and metritis. It would be of value to establish if the response in individual cows is variable between individuals and consistent within an individual, and if this has a significant genetic component. Further fundamental questions should be addressed relating to function of the APPs in milk during mastitis, for instance do they have an anti-bacterial role and do they affect mammary cell function. If the outcomes of such studies are positive then the next logical and feasible step would be the development of new, robust, rapid and inexpensive methods of analysis, suitable for use in on-line milking systems.

Changes taking place in milk during mastitis have been well documented (Kitchen 1981; Watanabe et al 2000). In an effort to identify milk polypeptides whose presence or absence correlates with the presence of mastitis, a proteomic strategy was developed. The entire protein complement of bovine milk during severe clinical mastitis was compared to that of milk from healthy cows using 2-DE and MALDI-MS. The advantage of this approach was that all the proteins in milk could be analysed at once. Obvious differences were apparent in the milk proteins of healthy cows and those suffering from mastitic infection. During mastitis the major milk proteins were diminished while other proteins, notably serum protein such as albumin and serotransferrin were increased. Although this research produced no new findings as such, the investigations herein are among the first few attempts reported to analyse bovine milk using proteomic methods and in particular to use proteomics to investigate the differential changes induced in bovine milk during mastitis.

Moreover the optimisation of a method for proteomic investigation of milk during mastitis was a valuable outcome of the project. This study has successfully established the methodology for studying the milk proteome in mastitis and may be used for further studies in different disease states. Such studies would also be of value in relation to the assessment of milk quality and in cheese production.

The extension of the proteomic method developed in chapter 4 was applied to analyse the changes taking place during a mild form of experimentally induced *S. aureus* mastitis. It was anticipated from the previous work that differentially expressed proteins would be observed in response to infection. However the mild subclinical nature of the infection model meant that alteration to the major protein fractions in milk were only minimal and

consequently changes in the minor fractions were difficult to identify. Whilst the results of this study (Chapter 5) were disappointing in that infection did not have any discernable and consistent effect on the major milk proteins, this was in effect a pilot study and lays the groundwork for possible future investigations. The next logical step would be to analyse the changes in milk proteins during a moderate experimental model of mastitis.

Investigations into the bovine milk proteome are in their initial stages and common to other systems the over abundance of a few major proteins cause a problem in detecting and monitoring changes in less abundant proteins in milk such as APP. Consequently the proteomic analysis performed in this study was biased towards highly abundant and soluble proteins. The limitation of spatial resolution which causes spots to overlap in 2-D gels was first reported in 1982 for albumin (Bjellqvista et al 1982). The use of narrow pH range IPG strips has been shown to increase the separation and resolution of protein spots. However even the use of narrow range pH strips has been reported to still only allow detection of abundant proteins (Gorg et al 2000). Proteomic study has further emphasised that pre-fractionation of milk is a pre-requisite prior to examination of its minor proteins and there are possible approaches as to how this might be achieved.

To overcome the problem of the overabundance of the major milk proteins during investigation of mastitis, pre-treatment of the milk samples with a recently available apparatus would be desirable. The IEF Zoom Fractionator (Invitrogen, Paisley, UK) can subdivide the whole milk samples into 5 fractions and as the major milk proteins have similar pIs in the region of pH 4.5 to 5.5; these can be isolated in one or at most 2 fractions. Subsequently the fractions would then be examined by 2-DE using narrow range IPG strips over the pH range appropriate to each fraction. The benefit of this method is that spatial resolution should be greatly enhanced and with the confinement of the highly abundant milk proteins to, at most 2 fractions, identification of the more minor proteins in milk should become feasible.

Small peptides are notoriously difficult to analyse in conventional 2-DE based proteomic approaches (Jenkins and Pennington 2001). Surface enhanced laser desorption/ionisation time-of flight mass spectrometry (SELDI-TOF-MS) provides a methodology to extend examination of the milk proteome and to include these smaller peptides which are likely to be the product of bacterial proteases but could still have value as biomarkers of disease. Unlike MALDI-MS the surface in SELDI-MS plays an active role in the extraction, presentation and enrichment of the sample eliminating the need for meticulous sample

preparation including desalting, that is necessary prior to MALDI-MS (Jenkins and Pennington 2001).

The complete proteome analysis suffers from a number of technical limitations. Briefly, the process is time-consuming, labour intensive and requires significant technical expertise if reproducible gels and quantitative data are to be generated. It is probable that a number of differentially expressed proteins between the infected and control samples were not identified. Fluorescent two-dimensional difference gel electrophoresis (DIGE) allows samples labelled with different dyes to be co-separated simultaneously on the same gel, thereby eliminating the gel to gel variation. Images of the 2D gels are acquired using as many as three different filters and the ratio of the differently coloured fluorescent signals is used to identify protein differences between the samples (Patton 2002). This technique would be very useful in quantitative and comparative proteomic studies and should be employed in future studies to enhance the analysis of differentially expressed proteins in bovine milk during mastitis.

Unfortunately, the proteomic methods developed lacked the sensitivity to detect APPs in the milk. It is likely that Hp and M-SAA3 were not identified on the 2-DE gels because they are present at concentrations below the limit of sensitivity of the colloidal Coomassie stain employed in this investigation. As antibodies are available to these proteins it is possible that immunoblotting of the 2-D gels could be used in future studies to locate these proteins.

The extrahepatic expression of APP, now documented in a wide variety of tissue opens a further field of investigation into both the local control mechanisms and the functions of the locally produced APPs. The recent recognition of a milk isoform of SAA, namely M-SAA3 (McDonald et al 2001) required further investigation to elucidate the implications this protein may have on the pathogenesis of bovine mastitis. Our studies, confirm the local production of M-SAA3 in the bovine mammary gland and its up-regulation during bovine mastitis, providing further insight into a possible role for this protein. Future work in this area should employ *in situ* hybridisation to define the precise location of this protein in the bovine mammary gland.

There is a very real need for the discovery of markers that will detect disease at an earlier stage. The discovery of biomarkers in milk would be a very desirable achievement due to the ease with which this fluid can be collected. All of the work detailed within this thesis strives towards this similar goal. The emergence of new methodologies for the large-scale

analysis of differentially expressed proteins in body fluids is presenting opportunities in the search for novel diagnostic markers of disease. The development of proteomic technologies with improved sensitivity could be applied to identify APPs as biomarkers of mastitis in milk, hence linking the major findings in this thesis and demonstrating the close connection between this laboratory research and the potential for commercial application.

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