INFECTION AND INFLAMMATION IN BOVINE *STAPHYLOCOCCUS AUREUS* MASTITIS

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Abstract

The aim of this project was to study the dynamics of intramammary infection and resulting inflammation in *Staphylococcus aureus* mastitis in cows. Four cows with naturally occurring subclinical mastitis in one or more quarters, caused by *S. aureus,* were used in this study.

The strains of *S. aureus* causing infection in the cows were determined by restriction enzyme fragmentation pattern (REFP) analysis. Two cows were infected with one strain of *S. aureus,* designated strain A, and two cows were infected with a different strain of *S. aureus*, designated strain B. The cows were subsequently challenged by the intramammary route in a novel cross-over design with large numbers (10^8-10^9) of either the indigenous strain or the non-indigenous strain of *S. aureus*. Quarters which were naturally infected with *S. aureus* and then challenged by the intramammary route with *S. aureus* remained persistently infected three weeks following challenge in three of the four cows, irrespective of the challenge strain. The fourth cow required treatment with antibiotics due to an increase in the severity of mastitis. The results of this study showed that in one cow, intramammary challenge of a subclinically infected quarter with the indigenous strain of *S. aureus* failed to induce an anamnestic immune response which might have instigated clearance of the original infection from the quarter; while in the other cow, the indigenous strain was replaced with three distinct isolates. It also showed that intramammary challenge of a subclinically infected quarter with the non-indigenous strain of *S. aureus*, in two cows, did not result in permanent replacement of the indigenous strain with the recently infused non-indigenous strain. In addition to the original strains A and B, eight distinct strains isolated from the four cows, were recognised by REFP analysis, indicating that a cow, or even a quarter, may be infected simultaneously by more than one strain of *S. aureus*. This study showed that the examination of multiple colonies per milk sample was required in order to identify the diversity of *S. aureus* strains present and to maximise the benefit of bacterial strain identification as an epidemiological tool in mastitis investigations. As REFP analysis is a time consuming method, a repetitive extragenic palindromic polymerase chain reaction (REP-PCR) was developed during this project to facilitate more rapid identification of the ten *S. aureus* strains originally identified by REFP.

To investigate the acute phase response as an indicator of inflammation in mastitis, mammary secretions from the four cows with naturally occurring subclinical mastitis caused by *S. aureus* were collected prior to, and following, intramammary challenge with *S. aureus*. Serum amyloid A (SAA) was isolated and purified from the serum of bovine clinical cases by ultracentrifugation, gel electrophoresis and electro-elution. Serum amyloid A was identified by enzyme linked immunosorbent assay and immunoblotting using a polyclonal rabbit anti-human SAA antibody. The purified SAA was used to immunise mice in an attempt to produce a monoclonal antibody specifically against bovine SAA but this proved to be unsuccessful even when the SAA was bound to a carrier protein. Increased levels of haptoglobin were identified in mammary secretions from a single quarter of one cow. This quarter had been originally uninfected and then challenged by the intramammary route with strain B. In the absence of a polyclonal or monoclonal antibody to bovine SAA, sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to identify proteins in mammary secretions with a similar molecular weight to SAA. A protein of similar molecular weight to SAA was identified in the same quarter in which the increased haptoglobin levels were measured.

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Chapter 1

Introduction

1.1 Mastitis

1.1.1 The Disease

Bovine mastitis is a serious welfare and economic problem in the United Kingdom. Mastitis is recognised as one of the major diseases adversely affecting dairy cow welfare and is the single most common cause of death in adult cows (Menzies *et al.*, 1995). Peracute and acute mastitis are recognised as significant causes of poor welfare in dairy cows (Logue and Hillerton, 1996), whereas the major impact of subclinical mastitis is in reduction of farm income (Blosser, 1979; Rebhun, 1995). Mastitis causes greater financial loss to the dairy industry than any other disease (De Graves and Fetrow, 1993), with 70% of this loss attributable to reduced milk production caused by cases of subclinical mastitis, many of which remain undetected for a considerable time (Sandholm and Mattila, 1986). Mastitis affects both the quality and quantity of the milk produced (De Graves and Fetrow, 1993; Blowey and Edmondson, 1995). The reduction in yield of milk is due to irreversibly damaged milk secretory tissue and the compositional changes in the milk which occur during a mastitic attack, which adversely affect the processing qualities of the milk and its products (Castle, 1985). Within the quarter, a low grade infection may steadily destroy the secretory cells, especially

the ducts, which are then replaced by scar tissue, thus reducing the capacity of the quarter to produce milk (Webster, 1993). Compositional changes in the milk vary depending on the severity of infection, the causative micro-organism involved, the stage of lactation of the cow and the susceptibility of the individual animal (Webster, 1993). Mastitic milk is unacceptable to the consumer and some mastitis pathogens constitute a public health risk, including some strains of *Staphylococcus aureus* which produce enterotoxins known to cause gastro-enteritis in humans (Batish *et al.*, 1988; Harvey and Gilmour, 1990; Orden *et al.*, 1992).

Mastitis, defined as inflammation of the mammary glands, produces clinically recognisable signs of heat, pain, swelling and inflammation of the affected quarters (Blowey, 1990). The majority of cases of bovine mastitis result from colonisation of the mammary gland by pathogenic bacteria (Sandholm and Mattila, 1986). Many different bacteria cause mastitis and the diversity of bacteria initiating infection is one of the major problems in rational treatment of the disease (Webster, 1993). Mastitis can be classified into peracute, acute, subacute, chronic and subclinical cases depending on the severity of the attack (Radostits *et al.*, 1994). In peracute mastitis, the udder becomes grossly inflamed and the resultant mammary secretions are considerably altered. The cow usually displays symptoms of severe systemic illness which can be fatal (Webster, 1993). Acute mastitis is less severe than peracute, however the udder is still swollen and inflamed and the animals temperature may be elevated, although the systemic symptoms are less marked than in the peracute form (Black, 1992). Subacute mastitis is characterised by mild inflammation, including abnormal milk secretion, whereas chronic mastitis is associated with recurrent attacks of inflammation (Radostits *et al.*, 1994). The cow with a chronically infected quarter is not only less productive, but acts as a continual source of infection to other quarters and cows (Webster, 1993). Subclinical mastitis is difficult to detect, with no visible abnormality of the milk or udder, and an elevated somatic cell count is taken as the usual indicator of infection. Somatic cells include epithelial cells, lymphocytes and monocytes but mainly consist of polymorphonuclear leucocytes (PMNL) (Lam, 1996). The main factor that determines the somatic cell count (SCC) is the

infection status (Shepers *et al.*, 1996) but other factors include the number of infected quarters, the causative organism, the age of the cow and the stage of lactation (Emanuelson and Funke, 1991).

1.1.2 Economics

Mastitis is one of the major causes of economic loss to the dairy industry. An average of 40 cases of mastitis per 100 cows occurs per year in UK herds and an annual incidence of mastitis of less than 30% in any herd is very rare (Esselmont and Spincer, 1992; Webster, 1993). Mastitis may present as either clinical or subclinical disease, or a combination of the two. It is, therefore, difficult to estimate the economic loss as there is an enormous variation within herds in levels of clinical and subclinical mastitis, so that a herd may have a high subclinical level but a low clinical level or *vice versa*. An estimate of the prevalence of subacute, acute and peracute mastitis cases based on figures suggested by Blowey (1986) is 70% for subacute cases, 29% for acute cases and 1% for peracute cases. The cost of an average case of mastitis in 1992 was £168 with a range of £60.46 to £2248, depending on the severity of the case (Esselmont and Spincer, 1992). An estimate of the number of dairy cows in the UK in 1991 was 2.6 million (June 1991, Agricultural and Horticultural Census), and calculations based on the prevalence values above, suggest that mastitis costs the UK dairy industry more than £100 million each year.

The cost of each episode of clinical mastitis results from: 1). discarded milk, 2). cost of intramammary antibiotics prescribed by a veterinarian; 3). the subsequent reduction in the milking potential of the affected quarters and poorer quality of the milk produced (Blowey and Edmondson, 1995). Antibiotic residues in milk and dairy products cannot be completely eliminated by pasteurisation or boiling (International Dairy Federation, 1979) and pharmacological legislation requires that milk be discarded during treatment and for a specified time period after intramammary infusion (Vautier and Postigo, 1986). Mastitis leads to a reduction in yield, in lactose and in butterfat content but milk protein levels increase slightly

with mastitis (Shuster *et al.*, 1991; Blowey *et al.*, 1992). For example, a herd with a somatic cell count of 750,000 cells/ml could be losing 750-900l of milk per cow per year, 50g/kg lactose per cow per year and 30g/kg of milk fat per cow per year (Blowey *et al.*, 1992). In severe cases of mastitis the costs may include the replacement cost of the cow due to premature culling or death during the mastitic episode (Esselmont and Spincer, 1992).

Farmers receive financial penalties or premiums based on the level of somatic cells in milk and the total bacterial count (TBC) of the milk. Somatic cell counts can be measured at the level of the bulk tank, the individual cow or the individual quarter.

Bulk milk somatic cell counts (BMSCC) have become universally adopted as a screening test for milk quality in herds. They are also useful in creating awareness of the existence of a mastitis problem in a herd, so that when a BMSCC exceeds permissible levels, further mastitis investigation using more specific techniques is often undertaken (Radostits *et al.*, 1994). The BMSCC is particularly useful as an indicator of subclinical infection within a herd (Booth, 1985; Blowey *et al.*, 1992). A BMSCC of 250,000 cells/ml is now considered to be a realistic upper limit for UK herds with good mastitis control (Blowey *et al.*, 1992). Field studies in Scotland show that the main cause of high BMSCC in herds in Scotland is subclinical mastitis, especially when caused by *Streptococcus agalactiae* and *S. aureus* (Logue *et al.*, 1995).

The individual cow SCC (ICSCC) is an indirect test based on the number of somatic cells in a composite sample including milk from all four quarters (Radostits *et al.*, 1994) and has been shown to be the most efficient way of identifying high SCC cows (Blowey and Edmondson, 1995). Individual cow SCC of less than 250,000 cells/ml are considered to be below the limit indicative of inflammation while counts of > 400,000/ml probably suggest the presence of infection.

Individual quarter SCC (IQSCC) is the collection and examination of milk from each individual quarter of the udder. Individual quarter SCC exceeding 240,000 cells/ml are considered to be excessive, although most healthy quarters show less than 100,000 cells/ml (Blowey and Edmondson, 1995).

Total bacterial counts in the milk are also used as indicators of intramammary infection. In the milk of acute clinical cases the TBC may be as high as 100 million bacteria/ml, subclinical quarters may contain between 1,000 and 10,000 bacteria/ml and normal quarters usually yield less than 1,000 bacteria/ml. The bulk tank TBC should be ideally < 10,000 bacteria/ml (Blowey and Edmondson, 1995).

The deregulation of the Milk Marketing Boards in 1994 was the biggest change within the UK dairy industry for over sixty years. The UK's five Milk Marketing Boards provided valuable information on national output in the last decade against quota; provided technical support, including mastitis control; carried out research and development on milk production and negotiated the best selling price for milk with the many milk buyers (Sumner, 1994). Under the Milk Marketing Scheme there was a barrier between producers and users, and industry management decisions were made by the milk boards who represented the producers, which resulted in a reduced producer awareness about the market place. On the 1st November 1994, the statutory requirement for the Milk Marketing Boards to buy all the milk produced was removed allowing direct purchasing from the farmers (Pearce, 1995). This allowed purchasers to influence the quality of the product and allowed full traceability of the supply to the producer.

The EC hygiene directive was implemented initially in the United Kingdom in May 1995 by the Dairy Products (Hygiene) Regulations 1995 (1). This replaced the existing milk hygiene legislation, The Milk and Dairies (General) Regulations 1959 (Madders, 1995). The directive sets maximum levels for SCC and TBC in milk. Initially this standard is being implemented at the level of bulk consignments to milk buyers however, from the 1st July 1997, the standard will apply at individual producer level. Supplies with a three month geometric mean SCC

higher than 500,000 cells/ml and a raw milk TBC of higher than 100,000 over a two month geometric mean will become unsaleable. From the 1st January 1998, the standard will be a maximum of 400,000 cells/ml with a raw milk TBC of less than 100,000 bacteria/ml (Baines, 1996). The implementation of EC 92/46 will produce some difficulties for UK milk producers, in particular for the minority of producers (approximately 10%), who have a BMSCC consistently over the accepted threshold of 400,000 (Logue *et al.*, 1995).

1.1.3 Pathogens

Organisms that cause mastitis can be divided into three main categories depending on their pattern of infection, i.e. contagious mastitis, environmental mastitis and summer mastitis pathogens (Blowey *et al.*, 1992).

Mastitis pathogens can also be subdivided into major and minor categories. The major pathogens are well-recognised as the cause of clinical or subclinical mastitis and include organisms such as the contagious pathogen *S. aureus* and the environmental pathogen *E. coli* (Shukken *et al.*, 1989). The role of minor pathogens is less clear with the possibility that they may either cause mastitis (Counter, 1981; Shukken *et al.*, 1989) or play a role in protecting the udder (Shukken *et al.*, 1989; Nickerson and Boddie, 1994; Pankey *et al.*, 1985; Lam *et al.*, 1996).

1.1.3.1 Contagious Pathogens

Primary udder pathogens, including *S. aureus* and *S. agalactiae*, are considered to be the major causes of contagious mastitis (Webster, 1993). Some authors consider *S. dysgalactiae* to be a contagious pathogen (Hillerton *et al.*, 1995), while others believe it should be classed as an environmental, and therefore, opportunistic pathogen (Smith *et al.*, 1985; Pankey *et al.*, 1987; Oliver *et al.*, 1993). Primary udder pathogens, which are usually transmitted between quarters

during the milking process, are natural inhabitants of the animals skin or udder, and are the predominant cause of subclinical mastitis (Fox and Gay, 1993).

Streptococcus agalactiae is found only within the udder (Fox and Gay, 1993). Infection with *S. agalactiae* causes acute inflammation of the affected quarter with clots forming in the foremilk, and often results in an elevation of the animal's temperature. *S. agalactiae* infection has remained relatively easy to control due to the micro-organism's continued susceptibility to antibiotic treatment (McKellar, 1996). Subsequent infections caused by *S. agalactiae* cause less acute inflammation and pain and thus infections may go unnoticed and continue to erode the milking capacity of the quarter.

Staphylococcus aureus is a natural inhabitant of the skin and accounts for 25-30% of bovine mastitis cases (Sutra and Poutrel, 1993). If the organism penetrates the teat sufficiently to cause infection, two main forms of staphylococcal mastitis occur (Webster, 1993). The peracute form occurs rarely, but usually occurs in early lactation when the defences of the mammary gland to infection are at their lowest. Peracute staphylococcal mastitis is extremely severe and can often result in the death of the animal within twenty four hours of the appearance of clinical signs. Chronic and subclinical infections with *S. aureus* are the most common result of infection (Sutra and Poutrel, 1993). No obvious clinical signs of infection or alteration in milk composition occur in subclinical cases. However, clots in the milk may be present during chronic infection (Radostits *et al.*, 1994). Staphylococci invade deep into the gland (Blowey, 1990) and can survive and multiply within phagocytes (Sandholm and Mattila, 1986), resulting in the host defence mechanisms and subsequent antibiotic treatment being incapable of completely eliminating the micro-organism from the udder. Treatment of staphylococcal mastitis is further complicated by the existence of many strains of *S. aureus* which can cause infection, each with variations in sensitivity to antibiotics (Webster, 1993; McKellar, 1996).

Streptococcus dysgalactiae is most frequently transmitted during the milking process and is found naturally in the udder and on skin teat lesions (Blowey,

1990), however, the method of transmission of *S. dysgalactaie* remains unclear (Lam, 1996).

1.1.3.2 Environmental Pathogens

Escherichia coli and *Streptococcus uberis* are the organisms most frequently associated with environmental mastitis (Smith *et al.*, 1985). Environmental pathogens are an extremely heterogeneous group of bacterial genera, species and strains which are naturally found in the faeces, bedding or on the skin of cows (Smith *et al.*, 1985).

The coliform, *E. coli*, is a natural inhabitant of the large intestine and is found surviving in faecal matter (Blowey, 1990). *Escherichia coli* mastitis can result in a wide range of clinical signs varying from mild to peracute cases of mastitis. The variation in clinical signs is dependent on the dose of organism and also the stage of lactation of the animal (Webster, 1993). In late lactation, or during the dry period, only mild infection usually occurs, whereas in early lactation, severe infection can result in peracute mastitis associated with a high mortality rate. The peracute mastitis caused by *E. coli* infection is difficult to distinguish from peracute contagious mastitis caused by *S. aureus* (Webster, 1993).

Streptococcus uberis is found naturally on mucous membranes, skin and in the faeces (Blowey, 1990) and generally causes less severe infection than *E. coli*. *Streptococcus uberis* can be isolated from many anatomical sites of the cow (Wilson, 1981). A survey of 400 herds in the UK showed that the prevalence of udder infection caused by *S. uberis* was approximately 15% (Wilson, 1981). *Streptococcus uberis* was a frequent cause of new intramammary infection during the non-lactating period and under natural conditions, existing infections in the dry period may act as the source of infection during lactation (Smith *et al.*, 1985). A large proportion of these infections was cleared by the animal shortly after parturition, with the organisms remaining sensitive to penicillin when treated (McKellar, 1996). *Streptococcus uberis* is now increasing in importance as a cause of mastitis as the levels of contagious mastitis decline (Pott, 1996).

Summer mastitis generally affects dry cows and heifers at pasture, especially in warm humid weather during the months of July to September (Rebhun, 1995). Several organisms can be isolated from infected quarters, although the major agent is *Actinomyces pyogenes* which can be isolated in 78% of summer mastitis cases (Blowey, 1990). Other organisms involved in summer mastitis include *S. dysgalactiae, S. indolicus,* and micrococci. Infection is transmitted to the damaged teat end by females of the head fly *Hydrotea irritans* (Webster, 1993). Most cases of summer mastitis are acute and severe with generalised toxaemia, signs of weakness and fever, and the udder is hard and swollen on palpation (Webster, 1993). Most cases of summer mastitis result in severe and permanent destruction of the affected quarter or occasionally death of the animal, while less severe cases can occur and cause scarring of the sinus and teat canal resulting in a blockage of secretion of milk when the cow calves (Radostits *et al.,* 1994).

Other environmental organisms which can cause mastitis include *Pseudomonas, Klebsiella, Bacillus, Pasteurella* species, *Enterococcus faecalis* and yeasts (Radostits *et al.*, 1994).

1.1.3.3 Minor pathogens

Micro-organisms classed as 'minor pathogens' are usually considered to be non-pathogenic and occur naturally in 'normal' udders (Bramley and Dodd, 1984). Minor pathogens include the coagulase negative *Micrococcaceae* and *Corynebacterium bovis* (Lam, 1996). Minor pathogens have been shown to have a potentially protective effect against infections with major pathogenic bacteria (Pankey *et al.*, 1985; Nickerson and Boddie, 1994; Lam *et al.*, 1996) and a higher prevalence of minor pathogens may be associated with a lower incidence of clinical mastitis (Schukken *et al.*, 1989). Minor pathogens have also been shown to be associated with cases of clinical mastitis (Counter, 1981; Schukken *et al.*, 1989) and induce a limited increase in the somatic cell count (Hogan *et al.*, 1987; Lam *et al.*, 1996 (b)). While some authors argue that the prevalence of minor pathogens, especially coagulase negative micrococcaceae should be reduced

(Myllys, 1995; Smith and Hogan, 1995), others believe that moderate levels of somatic cells induced by minor pathogens may play a role in protecting the gland against infection, particularly against coliform bacteria (Lam *et al.*, 1996).

1.1.4 Udder Defences

The bovine mammary gland has 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).

The teat duct provides the initial non-specific defence against bacterial penetration, acting as both a physical barrier and as a source of antimicrobial substances (Outteridge and Lee, 1988). The physical barrier is provided by the smooth muscle sphincter surrounding the teat canal which inhibits bacterial penetration by maintaining tight closure and limiting bacteria to the teat orifice (McDonald, 1975). The teat canal is partially occluded by keratin, a mesh-like substance derived from the teat canal lining which inhibits the progression of bacteria through the teat duct into the udder (McDonald, 1970). Keratin also provides the source of antimicrobial substances including lipids and proteins (McDonald, 1970). The importance of the teat duct keratin in protection against mastitis was demonstrated by Miller *et al.* (1991) when removal of 70% of teat duct keratin significantly reduced the ability of cows to resist mastitis for two days following its removal. Physical variations in teat canal and teat duct have also been shown to be important factors in mastitis susceptibility (Lacy-Hubbert and Hillerton, 1995).

1.1.4.1 Humoral Defences

This form of defence can be subdivided into a specific category due to the action of immunoglobulins and a non-specific category due to the action of cytokines and other proteins. Immunoglobulins form part of the specific humoral defences of the mammary gland. The immunoglobulin content of milk and colostrum also increases during inflammation (Nickerson, 1985). Immunoglobulins play a role in opsonisation and phagocytosis, toxin neutralisation, prevention of bacterial adhesion to tissues and direct bacteriolysis. IgG_1, IgG_2, IgM and IgA have all been clearly distinguished in bovine mucosal secretions (Duncan *et al.*, 1972). IgG_1 functions predominantly as an agglutinating, virus neutralising and antitoxic antibody (Colditz and Watson, 1985), but can also act as an opsonin (Nickerson, 1985) and is able to fix complement *in vitro* (McGuire *et al.*, 1979). IgG_2 is cytophilic for ruminant neutrophils (McGuire *et al.*, 1979) and has been shown to be particularly important in enhancing phagocytosis of *S. aureus* in cattle (Watson, 1976; Guidry *et al.*, 1993). The IgA class of immunoglobulins can inhibit binding of bacteria to epithelial tissue and may reduce absorption of antigen through the epithelium (Williams and Gibbons, 1972). IgM binds secretory component and may be secreted in a similar way to IgA (Butler, 1986). IgM has also been shown to play a role in opsonisation of bacteria by neutrophils (Williams and Hill, 1982). Immunoglobulin concentration in the mammary gland varies with the lactation cycle, the highest concentration occurring near parturition (Concha *et al.*, 1980) and at the end of lactation (Guidry *et al.*, 1980).

Non-specific defences include lactoferrin, lysozyme and lactoperoxidase and complement (Reiter, 1978). Cytokines may also be included as non-specific humoral factors as they can be produced by different types of stimuli, although in many cases their production may be induced by cells responding to specific immunological stimuli (Wood *et al.*, 1991).

1.1.4.2 Cellular Defences

Non-infected quarters usually have a SCC of 1-3 x 10^5 cells/ml milk and during infection this can rise to many millions per ml (Nickerson, 1985). The influx of bacteria and very high levels of SCC can be measured at 12-24 hours after infusion (Hill, 1981; Shuster *et al.*, 1996). A certain level of SCC in the gland may in fact be desirable as it is argued that the presence of cells in the secretion may in fact be protective against infection (Bramley, 1978). Non-specific leucocytosis, induced by the introduction of a polythene loop into the mammary gland, an intramammary device, has been shown to cause a mild chronic inflammation with maintenance of neutrophils within the gland resulting in a decreased likelihood of establishment of infection (Paape and Corlett, 1984). Quarters infected with minor pathogens, bacteria which are generally considered to be non-pathogenic, show increased resistance to superinfection (Bramley, 1978; Linde *et al.*, 1980) and attempts to intentionally infect quarters with minor pathogens to provide a stimulus to prevent infection against major pathogens have been made (Linde *et al.*, 1975; Brooks and Barnum, 1984).

Four cell types are present in normal bovine mammary secretions - neutrophils, macrophages, lymphocytes and epithelial cells and the proportion of these cell types varies between individuals and depends on the stage of lactation (Lee *et al.*, 1980). Phagocytosis of foreign material including bacteria is the primary function of neutrophils, although they are also involved in many processes including modulation of vascular permeability (Wedmore and Williams, 1981) and release of inflammatory mediators (Goetzl, 1980). In comparison to blood neutrophils, mammary neutrophils are less efficient at phagocytosis. This has been reported to be due to prior phagocytosis of casein micelles (Russel and Reiter, 1975) and fat globules (Paape and Guidry, 1977). Despite the factors limiting the phagocytic activity of mammary neutrophils, this activity is still thought to be the primary antibacterial defence mechanism of the mammary gland.

1.1.4.3 Lymphocytes

The percentage of lymphocytes in cells from mammary secretions are relatively constant throughout the lactational cycle (Lee *et al.*, 1980). The presence of both T and B cells in bovine milk was suggested by the work of Smith and Shultz (1977) as cells isolated from milk were shown to respond to T and B cell mitogens. Concha *et al.* (1978) isolated lymphocytes from milk and showed that the proportion of B and T cells was 20 and 45% respectively, similar proportions to those found in blood. Smith and Goldman (1968) demonstrated that human colostral lymphocytes were responsive to both phytohaemmagglutinin (PHA) and specific antigens. B lymphocytes and plasma cells are present in the mammary gland tissue and in the teat and play a role in synthesis of immunoglobulins (Nickerson and Heald, 1982; Collins *et al.*, 1986). T lymphocytes are involved in cell mediated immune response by producing lymphokines that act as chemoattractants for neutrophils and macrophages and for stimulating leucocyte microbial activity (Craven and Williams, 1985).

1.1.4.4 Macrophages

Cells of the monocyte-macrophage series are the major type in mammary secretions for most of the lactational cycle (Watson and Lee, 1978). They are also involved in the induction and development of the local immune responses (Concha and Holmberg, 1990). Mammary macrophages from involved ovine glands (Outteridge and Lee, 1981), lactating women (Mori and Hayward, 1982) and cows (Fitzpatrick *et al.*, 1995) have been shown to function as antigen- and mitogen-presenting cells in lymphocyte stimulation tests.

In spite of the presence of considerable numbers of immune cells in the local mammary gland environment, it has been suggested that the mammary gland is generally immunologically compromised when compared to the rest of the body (Hurley *et al.*, 1990). The activity of all types of white blood cells in milk, neutrophils, lymphocytes and macrophages, have each been shown to be reduced compared to cells isolated from blood (Paape *et al.*, 1975). The interaction

between cells in the local mammary gland environment is also important in induction of immunity and failure of cell co-operation is another possible mechanism whereby udder defences may be impaired.

1.1.5 Treatment and Control

All dairy farmers are encouraged to practice mastitis control, partly to reduce the wastage that the disease causes, but also to contribute to the maintenance of milk quality (Radostits *et al.*, 1994). Neave *et al.* (1969) introduced a standard mastitis prevention programme for contagious pathogens which has been continuously revised and developed, and has resulted in an approximately 50% reduction in udder disease over the past 25 years (Bramley and Dodd, 1984; Blowey and Edmondson, 1995). Great progress has been made in mastitis control in the UK during this period, mainly due to the uptake of the 'Five Point Plan' by dairy farmers (Blowey and Edmondson, 1995). The five point plan was outlined by the National Institute for Research in Dairying and the Central Veterinary Laboratory in 1969 and includes recommendations to: apply an approved teat disinfectant after every milking; treat clinical cases of mastitis promptly; infuse long-acting antibiotic into all quarters at drying off; test milking machines annually; and cull cows showing repeated cases of clinical mastitis (Blowey *et al.*, 1990).

The five point plan provides an economic advantage, is within the scope of the dairyman in terms of technical skill and understanding, can be easily introduced into the management system employed, and encourages farmers to persist with the plan by rapidly reducing the occurrence of clinical mastitis and the rejection of milk by milk processors on the grounds of quality (Blowey *et al.*, 1990).

The use of antibiotic therapy as a control measure for bovine mastitis is not ideal due to potential public health risks resulting from the use of intramammary antibiotics and the subsequent risk of antibiotic residues in meat and dairy products (Vautier and Postigo, 1986). The use of intramammary antibiotics can

adversely affect manufacturing processes for cheese and cultured milks (Sandholm and Mattila, 1986). To enable improved methods of treating and preventing mastitis to be developed, the basic defence mechanisms and immune responses within the bovine udder should be fully understood. A greater understanding of the individual micro-organisms which induce mastitis including strain diversity, toxin production, and their subsequent effects on the host should be determined to aid the development of future mastitis control programmes.

1.2 The Staphylococci

1.2.1 Genus

The staphylococci have been studied for over a century, during which time their pathogenic role in many diseases of man and animals has been established. The staphylococci are normally associated with the mucous membranes, skin and skin glands of warm blooded animals and are found frequently as aetiological agents causing mastitis, food poisoning and different forms of wound and systemic infections (Makaya, 1996). Currently the genus *Staphylococcus* is comprised of at least thirty one distinct species (Kloos and Bannerman, 1994). Some common staphylococcal species and their natural hosts are listed in table 1.1.

Members of the genus *Staphylococcus* are facultatively anaerobic, non-motile, Gram positive cocci which can occur singly, in pairs or irregular clusters (Kloos and Schleifer, 1986; Prescott *et al.*, 1990; Fox and Gay, 1993). The staphylococci, like the micrococci, are catalase positive. However, they differ from the micrococci in several ways: the staphylococci are oxidase negative, ferment glucose anaerobically, have a cell wall composition of peptidoglycan and techoic acids, and cell wall DNA with a lower guanidine and cytosine content (approximately 30-39% compared with at least 60% in the micrococci) (Prescott *et al.*, 1990).

Species	Coagulase	Common, Natural host
S. aureus	+	primates, humans, mammals, birds
S. intermedius	+	carnivores, mammals, birds
S. epidermidis	_	humans, domestic animals
S. xylosus	_	humans, birds, domestic animals
S. warneri	_	domestic animals, birds,
S. hyicus	+	swine, cattle, goats

Table 1.1: Common staphylococcal species and their natural hosts

The ability of the staphylococci to produce coagulase can be used to divide them into pathogenic and relatively non-pathogenic strains (Oeding, 1983). Coagulase positive strains often produce a yellow carotenoid pigment and can cause acute or chronic infections, whereas strains that do not synthesise coagulase are non-pigmented and generally less pathogenic (Prescott *et al.*, 1990). The most commonly known and well described coagulase positive staphylococci are *S. aureus* and *S. intermedius* (Sears *et al.*, 1993). *Staphylococcus aureus* is one of the most common staphylococci isolated from a wide range of animal species, either as normal flora or as an aetiological agent associated with purulent infections (Matsunaga *et al.*, 1993). The coagulase negative staphylococci make up the largest group within the genus and consist of more than thirty different species (Aarestrup, 1995). Non-haemolytic, coagulase negative staphylococci especially *S. epidermidis, S. warneri, S. hyicus* and *S. xylosus* are classified as minor mammary pathogens (Radostits *et al.*, 1994). Mastitis resulting from these organisms may be subclinical and result in decreased production of milk and an elevation in somatic cell count in infected glands (Rebhun, 1995).

1.2.2 Pathogenicity

1.2.2.1 The Staphylococci

A number of factors have been shown to be important in the pathogenicity of staphylococci, including the route of infection, enzyme or toxin production and components of the bacterial cell wall. The route of staphylococcal infection is important in determining the outcome of the invasion of the organism as generally only small numbers of staphylococci are required to produce infection of the skin or skin glands such as the sebaceous and mammary glands (Blowey *et al.*, 1992). For example, at least 10^9 colony forming units of some strains of *S. aureus* are required to kill mice by intraperitoneal or intravenous inoculation, whereas only nine colony forming units are required to kill mice by intramammary inoculation

(Anderson, 1986). The immediate availability of milk as a good growth medium as well as the absence of sufficient numbers of phagocytic cells, until they are recruited by chemotaxis, allows rapid multiplication of the staphylococci within the mammary gland (Anderson, 1986).

Staphylococci induce infection through their ability to multiply and spread widely in tissues, which is aided by their production of many extracellular substances such as exotoxins or enzymes which are thought to be involved in invasiveness (Kotzin *et al.*, 1993; Lee, 1996). The pathogenicity of the staphylococci is related mainly to their synthesis of toxins such as haemolysin, leucocidin and enterotoxin, or of enzymes such as coagulase, hyaluronidase and penicillinase (Anderson, 1986). The function of these extracellular enzymes and toxins should be considered not only as destructive to the host, but as advantageous to the invading organism in providing low molecular weight nutrients for bacterial growth (Anderson, 1986; Makaya 1996). Coagulase, hyaluronidase, phosphatases, DNAase, proteinases, lipase, catalase, lactate dehydrogenase and penicillinase are the most frequent enzymes produced by the staphylococci, however, while not all enzymes are produced by every strain, every strain does produce catalase (Prescott *et al.*, 1996). Most of the enzymes are produced in the late logarithmic phase of growth with the exception of coagulase which is produced during early exponential growth (Arvidson, 1983). The main toxins produced are leucocidin, exfoliative toxin, toxic shock syndrome toxin (TSST-1), haemolysins and enterotoxins (Wood *et al.*, 1991; Kenny *et al.*, 1993). The enterotoxins are membrane-damaging exoproteins which can be divided into several different types, designated staphylococcal enterotoxins A-E (Wood *et al.*, 1991; Orden *et al.*, 1992). The function of alpha toxin, one of the enterotoxins, and leucocidin, is directed towards the destruction of cells and lysis of neutrophils (Anderson, 1986; Prescott *et al.*, 1990).

Several other substances which are involved in resistance to phagocytic ingestion are found within the cell wall structure of the staphylococci. Protein A binds immunoglobulin via the non-specific F_c receptor rather than the antigen specific F_{ab} terminal and thus activates complement which increases the resistance of the

staphylococci to phagocytic ingestion by masking the immunoglobulin F_c sites (Greenberg *et al.*, 1987; Roitt *et al.*, 1993; Lee, 1996). Peptidoglycan which is the major component of the staphylococcal cell wall can induce a number of endotoxin like effects, including pyrexia (Adlam and Easmon, 1983).

1.2.2.2 *Staphylococcus aureus*

Staphylococcus aureus is a particularly well adapted pathogen for the mammary gland (Pott, 1996). It frequently causes clinical mastitis, which is mainly a sporadic disease resulting from chronic subclinical infection.

The pathogenic capacity of a particular strain of *S. aureus* is due to the combined effect of extracellular factors and toxins, together with the invasive properties of the strain (Prescott *et al.*, 1990). *S. aureus* produces around thirty extracellular proteins, some of which have been shown to be virulence factors. Matsunaga *et al.* (1993) compared the distribution of virulence factors among *S. aureus* isolates from peracute, acute and chronic bovine mastitis cases and correlated the results with clinical presentation. These authors concluded that all *S. aureus* isolates from peracute cases produced TSST-1, staphylococcal enterotoxin C, alpha-haemolysin and beta-haemolysin, whereas none of the peracute isolates produced clumping factor or protein A, which were associated with chronic mastitis. However, there was no obvious variation in virulence factor production between isolates from acute cases or isolates from chronic cases which is in agreement with the findings of Jonsson and Holmberg (1981).

The initial line of defence against staphylococcal infection is the physiologically normal and intact skin. In terms of infection of the udder, the action of the teat duct sphincter (McDonald, 1975) and the antibacterial properties of the keratin lining the teat duct play an important role. Mechanisms such as flushing of the udder during milking also aid in preventing infection (Anderson, 1986). Attachment to ductal epithelial cells is an important step in colonisation of the mammary gland and surface epitopes are likely to be involved (Blowey *et al.*, 1992; Makaya, 1996). Antibody has been shown to play a role in preventing

adherance of bacteria to mammary gland epithelial cells (Kenny *et al.*, cited in Blowey *et al.*, 1992). Once the outer skin defences of the host are breached the staphylococci are killed almost exclusively by the phagocyte-opsonin system (Anderson, 1986). Many staphylococcal products are directly or indirectly chemotactic for neutrophils and macrophages (Kenny *et al.*, 1993). However, in small quantities, alpha toxin, leucocidin and peptidoglycan may be antichemotactic (Craven and Williams, 1985). Recruitment of neutrophils is generally not impaired, and ingestion by phagocytes is not a limiting factor as long as recruitment of neutrophils is sufficiently fast (Craven and Williams, 1985). The migration of neutrophils from the blood into the udder, involves the cells passing through the blood-milk barrier and is activated by chemotaxis (Kremer *et al.*, 1990). Once the neutrophils have passed into the milk in response to chemotaxis, they engulf the invading micro-organism. Inside the neutrophils, bacteria are destroyed by a system involving hydrogen peroxide (Blowey and Edmondson, 1995). Staphylococci are killed by neutrophils by discharge of the granular contents into phagosomes (Lee, 1996). *Staphylococcus aureus* avoids elimination from the udder by employing a number of features including the ability to survive inside white blood cells and to outlive these cells and thus survive within the udder. Host inactivation of strains of *S. aureus* is a delicate balance between staphylococcal growth and the phagocyte-opsonin system, and usually this balance reaches equilibrium at the level of intracellular survival of the staphylococci, which is a typical feature of chronic staphylococcal mastitis in cattle (Anderson, 1982). The inability of phagocytes to kill all intracellular staphylococci is not fully understood and may relate to the exhaustion of bactericidal substances, the metabolic dormancy of the organism or the low pH of the phagocytic vacuole (Rebhun, 1995). The development of methods to kill all intracellular staphylococci remains one of the most important and urgent problems in staphylococcal research (Anderson, 1986).

Abscess formation and fibrosis associated with staphylococcal infection reduces phagocyte infiltration and prevents antibiotic penetration, with multiple micro-abscesses forming throughout the mammary tissue in chronic mastitis (Sears and Heider, 1981; Makaya, 1996). When ingestion of invading organisms occurs,

many staphylococci are killed by the neutrophils, however sufficient levels of alpha toxin may have been produced to lyse the phagocytes and cause necrosis of the adjacent tissue (Anderson, 1986).

A major limitation in the effective control of staphylococcal mastitis remains its poor response to antibiotic therapy. There are a variety of reasons for this of which antibiotic resistance plays only a minor role (McDonald and Anderson, 1981; Newman *et al.*, 1985; Craven, 1987). The ability of bacteria to survive inside polymorphonuclear neutrophils, macrophages and epithelial cells, where they are protected from the action of certain antibiotics, may also contribute to their resistance to therapy (Sandholm and Mattila, 1986; Blowey *et al.*, 1992). At present, staphylococcal infections are treated most successfully with a bactericidal antibiotic such as the semisynthetic penicillins which have some ability to penetrate intracellular organisms surrounded by dense tissue (Anderson, 1986). However, Madgwick *et al.* (1989) concluded that penetration of certain antibiotics into bovine neutrophils can be altered by the presence of ingested staphylococci, and that high intracellular levels of antibiotics does not necessarily ensure effective intracellular activity against pathogenic micro-organisms.

Vaccination against staphylococcal bovine mastitis has been attempted but has met with limited success (Slanetz *et al.*, 1963). A *S. aureus* mastitis vaccine is commercially available in the United States of America which has been shown to cause decreased prevalence of *S. aureus* positive milk cultures and decreased persistence of intramammary infection following experimental infection (Williams *et al.*, 1966; Williams *et al.*, 1975). Anderson (1986) suggests that no single virulence determinant has been identified from which a vaccine against staphylococci can be developed. Many virulence factors play a role in the pathogenesis and, therefore, antibodies against them may have only a partial role in protection. Development of a multi-component vaccine comprising of capsular polysaccharide complexed with either a toxoided staphylococcal toxin or with a surface protein known to be important in colonisation of the mammary gland epithelial cells has been suggested by Foster (1991). The multiplicity of virulence factors produced by *S. aureus* in addition to this organisms ability to evade the

host immune system by a variety of mechanisms has resulted in *S. aureus* being a common mastitis pathogen which is particularly difficult to treat and control.

1.2.3 Identification

1.2.3.1 Methods

Species identification of a microorganism is important for diagnosis, treatment and prevention of disease. Identification and additional typing can be carried out on the basis of growth characteristics of an organism on selective media, the recognition of microbial antigens by monoclonal or polyclonal antibodies, antibiotic susceptibility, biochemical characteristics, susceptibility to infection by bacteriophages and general features such as odour and colony formation in addition to molecular typing techniques (van Belkum, 1994).

In bacterial typing, the reliability of a typing system depends on the bacterial characteristic on which the typing method is based. The choice of the characteristic depends on its stability within a strain and its diversity within a species (Eisenstein, 1990; Brown, 1991). Methods used for discrimination of strains within a species can generally be divided into phenotypic and genotypic procedures. The use of conventional phenotypic and molecular genotypic typing methods as epidemiological tools help in understanding infectious diseases (Kapur *et al.*, 1995). Control of communicable diseases, such as mastitis, can be improved by the use of typing methods which define sources of infection, mechanisms of transmission, and rates of spread within a population (Makaya, 1996).

Phenotypic procedures take advantage of biochemical, physiological and biological phenomena, whereas genetic procedures aim to detect polymorphisms at the level of nucleic acids or to detect allelic variation at the level of enzymes (Kapur *et al.*, 1995). Some of the commonly used conventional phenotypic

methods include antibiogram typing (Aarestrup *et al.*, 1995 (c)), biotyping (Devriese 1984), serotyping and phage typing (Guinee and Van Leeuwen 1978; Lipman *et* al., 1996; Makaya, 1996). These biological tests are laborious to perform and require sophisticated reagents which are often not commercially available. However, these methods can provide adequate epidemiological analyses of various organisms. Alternatively, protein analysis can be used to delineate the origin of a strain of microorganism and to establish relationships among isolates (Eisenstein, 1990). Techniques such as whole-cell protein profiling, outer membrane profiling, isozyme electrophoresis and various immunoblotting techniques are frequently employed (Eisenstein 1990). Phenotypic procedures are generally not used for discrimination between strains of different species, whereas genetic procedures are preferred when strain differentiation is required (van Belkum, 1994).

Genotypic methods are systems which have been developed more recently for use in bacterial typing. These methods are based on less environmentally influenced genetic bacterial elements, and as such, they have a higher typability, reproducibility and discriminating power compared to the phenotypic methods (Makaya, 1996). Commonly used genotypic methods are plasmid profiling (Aarestrup *et al.*, 1995 (c)), direct sequencing of DNA fragments, or variations of restriction fragment length polymorphism techniques such as ribotyping (Aarestrup *et al.*, 1995 (b)), pulsed field gel electrophoresis, polymerase chain reaction (PCR) -digest and random amplification of polymorphic DNA fragments (Wolcott, 1992; Olsen *et al.*, 1993; Matthews *et al.*, 1994; Aarestrup, 1995; Rantamaki and Muller, 1995; Lam *et al.*, 1996; Lipman *et al.*, 1996). However, genetic typing assays also have drawbacks. In general these procedures require relatively large amounts of high quality DNA or RNA and a high degree of technical skill. Therefore, simple procedures such as PCR, in which small amounts of relatively impure nucleic acid is required have been developed (van Belkum, 1994). PCR typing provides the potential to analyse most of the medically important organisms by a single technique. The bacterial genome harbours repetitive sequences that can be used for DNA typing (Versalovic *et al.*, 1993; Lupski and Weinstock, 1992). The characteristics of these sequences are

their restricted length and their widespread occurrence. Prokaryotic repetitive motifs occur throughout the entire genome but rarely within genes. Repeats were initially discovered in *E. coli* and *Salmonella typhimurium* (Higgins *et al.*, 1982; Gilson *et al.*, 1989), and were later identified in several other bacterial species. PCR assays aiming at prokaryotic consensus repeats, arbitrary sequences or sequences of the methicillin resistance gene complex detected nearly twice as much variation as phage typing, indicating that PCR fingerprinting enabled the detection of genetic variants in the homogeneous phage groups (van Belkum, 1994).

1.2.3.2 The Staphylococci

DNA restriction endonuclease fingerprinting (REF) measures restriction fragment length polymorphism and has been developed for many pathogens including the staphylococci (Burnie and Lee, 1988; Bialkowska-Hobrzanska *et al.*, 1990). Jayarao *et al.* (1991) reported that DNA REF was an effective method for differentiating closely related and unrelated strains of *S. uberis* isolated from bovine mammary secretions, which was in agreement with studies by Christ *et al.* (1988) and Hill and Leigh (1989). Another study by Matthews *et al.* (1992) examined 51 staphylococcal isolates by REFP and antibiogram typing and concluded that DNA REFP anlaysis successfully differentiated closely related strains of each species isolated. The ease by which REF analysis can be performed together with the reproducibility and clarity of REF patterns suggest that this technique is useful in the differentiation of closely related and unrelated strains of staphylococci isolated from bovine mammary secretions (Matthews *et al.*, 1992).

1.2.3.3 *Staphylococcus aureus*

Many phenotypic and genotypic methods have been used to study *S. aureus* strain diversity in cases of bovine mastitis (Fox *et al.*, 1990; Roberson *et al.*, 1994; Aarestrup *et al.*, 1995 (b); Lam *et al.*, 1996). A staphylococcal biotyping system was developed which differentiated *S. aureus* strains from man and animals into host-specific ecovars and biotypes which were not host-specific (Devriese, 1984). Problems with biotyping include the strict need for adherence to the described methods in order to obtain reproducible results. Multilocus enzyme electrophoresis (MLEE) has been used extensively to index allelic diversity among human and animal pathogens including the staphylococci. (Matthews *et al.*, 1994). Kapur *et al.* (1995) typed 90% of *S. aureus* isolates using MLEE, and clearly demonstrated the presence of genetic heterogeneity among strains isolated from a single herd. MLEE provides data from which statistical estimates of genetic diversity and overall chromosomal relationships can be obtained, however, levels of discrimination are often too low to be of epidemiological value when used alone.

Phage typing and genotyping by PCR using RAPD, ERIC1R and ERIC primers was used to differentiate 71 *S. aureus* strains isolated from bovine mastitis (Lipman *et al.*, 1996). The RAPD primers gave the most significant DNA polymorphism changes, with phage typing yielding no more additional information. ERIC primers were used to compare DNA polymorphism patterns of different isolates and identified 11 genotypes within 63 *S. aureus* isolates from five herds in a study carried out by Lam *et al.* (1996). Matthews *et al.* (1994) also used a PCR based DNA fingerprinting technique to type 75 *S. aureus* isolates into 19 distinct profiles and these authors concluded that this technique differentiated closely related strains within and between geographic locations.

PCR has been shown to be a reliable and practical method for identification of isolates associated with bovine mastitis although few publications describing the use of PCR in *S. aureus* mastitis are available. The genotype differentiation of *S.*

aureus strains is thought to be one of the most accurate methods to date (Wang *et al.*, 1993). Compared to existing typing methods, PCR based DNA fingerprinting is easy to perform and interpret (Matthews *et al.*, 1994), with only Saulnier *et al.* (1993) reporting that pulse-field gel electrophoresis was more discriminating. Most authors conclude that ribotyping is the most discriminatory typing method followed by biotyping and phage typing which can have a low level of discrimination due to the low typability of some bovine strains (Aarestrup *et al.*, 1995 (c, d); Makaya, 1996). Using more than one phenotypic or genotypic typing method for strain biotyping improves the discriminatory powers. Development of methods allowing maximum discrimination among *S. aureus* isolates should help in further understanding the ecology of these pathogens and the epidemiology of mastitis caused by them.

1.3 Inflammation

Mastitis is clinically recognisable by pain and inflammation of the udder (Webster, 1993). The heat and redness often associated with mastitis is caused by an increase in blood flow accompanied by leakage of cells and fluids into the tissues resulting in increased pain within the infected area (Janeway-Travers, 1994). Inflammation is a result of dilation of local blood vessels in conjunction with increased permeability to leucocytes and is a complex response to tissue injury. The response can be either local or systemic, or a combination of the two (Evans and Whicher, 1992). The main cell types involved in the inflammatory response are the polymorphonuclear neutrophilic leucocytes, macrophages, monocytes and a small proportion of lymphocytes (Gruys *et al.*, 1993; Travers, 1994).

Inflammation may be initiated in various ways through different pathways (Evans and Whicher, 1992). Simple trauma activates mast cells which release mediators to control vasodilation, vascular leakage and, in some circumstances, proteolytic

activation of biochemical mediator systems such as complement (Roitt *et al.*, 1993). The inflammatory response can be triggered directly by pathogens, especially in early infection where bacterial pathogens are the main initiating factors (Vander *et al.*, 1994). During the initial stages of infection, the inflammatory response is essential in attracting the non-specific inflammatory cells such as monocytes and neutrophils to the site of infection, where these cells release inflammatory mediators which activate other components of the immune response (Bornstein, 1982; Marinkovic *et* al., 1989; Travers, 1994). The cellular processes of inflammation fall into four major groups: 1). changes in blood flow caused by changes in smooth muscle cell function resulting in vasodilation; 2). alterations in vascular permeability caused by cytoskeletal contraction in endothelial cells; 3). migration of phagocytic leucocytes to the site of inflammation; 4). phagocytosis (Roitt *et al.*, 1993).

1.3.1 Acute Phase Response

The acute phase response (APR) in animals is a result of tissue damage and is particularly associated with inflammation (Eckersall and Conner, 1988). In addition to the local responses described above, there is also a systemic APR, which encompasses the animals initial reaction to infection, inflammation or trauma (Skinner *et al.*, 1991). The term 'acute phase' was introduced by Avery *et al.* as early as 1941. The APR is a non-specific response which commences within a few hours of infection, can last for several days and includes pyrexia, cachexia, and variations in serum hormone, trace element and protein concentrations (Eckersall, 1992 (b)).

The APR is controlled by a complex array of cytokines and hormones which produce multiple effects such as fever, lethargy, muscle proteolysis and leucocytosis (Evans and Whicher, 1992; Eckersall, 1992 (b)). The APR is initiated by cytokines such as interleukin-1 and interleukin-6 produced by white blood cells which alter the regulation of proteins secreted by the liver into the

bloodstream, with IL-6 acting specifically on hepatocytes (Janeway-Travers, 1994; Vander *et al.*, 1994). The APR is thought to be beneficial to the injured animal in acting to restore homeostasis and prevent microbial growth (Koj, 1974; Pepys and Baltz, 1983; Gruys *et al.*, 1994) and has been identified in cattle by measurement of acute phase proteins (APP) in a variety of clinical conditions such as salmonellosis, mastitis, acute severe metritis, pneumonia and traumatic pericarditis (Eckersall, 1992 (a)).

1.3.2 Acute Phase Proteins

During the APR a major alteration is found in the liver where the hepatocytes are stimulated to increase production and secretion of several plasma proteins (Vander *et al.*, 1994). These are termed positive acute phase proteins and increase in concentration in the serum during the APR. The hepatocytes also regulate the secretion of albumin, a negative APP, which decreases in concentration during the APR, along with several other negative APP such as transferrin (Eckersall and Conner, 1988; Gruys *et al.*, 1994). The APP, including haptoglobin, serum amyloid A (SAA), alpha-1-anti-trypsin, ceruloplasmin and fibrinogen are mainly glycoproteins secreted by the liver in response to IL-6, IL-1 and TNFα produced by leucocytes and macrophages during infection and inflammation (Bornstein, 1982; Laurell, 1985; Marinkovic, 1989). Regulation of synthesis of the APP appears to be a highly complex process which is modulated or controlled by several cytokines. The major cytokines IL-6, IL-1, TNF-α, interferon γ, leukaemia inhibitor factor and transforming growth factor-β, alone, or in combination, have been shown to influence the serum concentration of the acute phase proteins (Maurhaug and Dowton, 1984). Acute phase proteins have many roles within the body ranging from acting as scavengers, promoting immunoglobulin production or involvement in tissue repair (Whicher and Dieppe, 1985). As the circulating concentration of these proteins reflects the presence or extent of infectious or inflammatory lesions they can be used as markers of disease (Eckersall, 1992 (a)).

Acute phase proteins are classified according to the degree of increase in serum concentration as either major, moderate or minor APP: for example, in humans C-reactive protein (CRP) is classed as a major APP, as the serum concentration of CRP increases from a very low level of less than 1mg/l to a concentration of greater than 200mg/l within twenty four hours (Kushner *et al.*, 1978; Roitt *et al.*, 1993), whereas a moderate APP in humans, such as alpha-1-anti-trypsin, has only a 2-3 fold concentration increase within 3-5 days of infection (Eckersall, 1992 (a)). Major APP are useful markers of inflammation if they have negligible or low basal values with a narrow reference range which remains unchanged with age, sex, or genetic make-up of the animal. The concentration of a major APP should increase rapidly to very high levels (>100 times) in response to infection or inflammatory conditions, with the level of response ideally equivalent to the amount of tissue damage sustained (Kent, 1992). Non-inflammatory conditions, nutritional status, exercise, handling or other forms of minor stress should ideally not affect APP concentration, whereas a secondary infection or relapse in the inflammatory condition should result in an increase in plasma concentration of an APP being used to provide diagnostic information (Kent, 1992).

Major APP vary with species, in humans CRP and SAA are major APP (Pepys,1979), whereas CRP appears to be a normal constituent of bovine serum (Maudsley, 1985). In general, the APP of the dog and cow are similar to those in man, however significant differences do exist (Eckersall, 1988). As in man, canine CRP is a major APP with a normal serum concentration of less than 5mg/l increasing to over 200mg/l during the APR (Eckersall, 1988). Fibrinogen is probably the most universally measured APP in animal plasma (Schlam, 1970), however, it is not a major APP in many animal species, with the most accurate assay procedures being unsuitable for rapid analysis (Laurell, 1985). Haptoglobin is a major APP in cattle with a low basal level which increases dramatically during the APR, yet haptoglobin is a constitutive plasma protein in dogs and humans (Eckersall, 1988). Levels of haptoglobin have been shown to double in dogs and humans following surgery (Conner *et al.*, 1988). Haptoglobin and SAA are major bovine APP (Eckersall and Conner, 1988; Gruys *et al.*, 1994).

The time course of the APR also varies considerably with the APP studied. In humans, SAA and CRP increase in concentration very rapidly within hours of the initial infection, reaching maximum levels in 1-3 days and rapidly resuming basal levels after the APR has been controlled (Kushner, 1982; Emery and Luqmani, 1993). In contrast, fibrinogen may not reach maximum serum concentration until two weeks after the initial APR and requires a further seven days to return to normal levels (Blackburn, 1994). In cattle, SAA has been shown to become elevated in both spontaneous and induced APR within eight hours of infection (Alsemgeest *et al.*, 1993).

In addition to using APP levels as markers of disease in the live animal, measurement of APP may also be useful during meat inspection, for detecting infected carcasses (Saini and Webert, 1991). Many of the conditions which cause condemnation of meat are also conditions which would be likely to produce an APR and thus an elevation of the relevant APP (Saini and Webert, 1991). The assessment of APP of animals at slaughter could enable detection of the presence of inflammatory lesions in the carcasses and form an important element of future quality assurance schemes (Eckersall, 1992 (b)).

1.3.3 Major Acute Phase Proteins of Cattle

1.3.3.1 Haptoglobin

Bovine haptoglobin is a highly polymerised serum haemoglobin binding protein which has been identified as an acute phase reactant in cattle (Morimatsu *et al.*, 1992). Levels of haptoglobin increase in sera after inflammatory stimuli and has resulted in haptoglobin being used as an indicator of a variety of inflammatory conditions (Eckersall, 1988; Morimatsu *et al.*, 1992). The rapid increase in haptoglobin concentration provides the basis for the usefulness of haptoglobin in the early detection of the APR (Skinner *et al.*, 1991). Bovine haptoglobin differs considerably from human haptoglobin in terms of physiology and immuno-reactivity and is a major APP which is normally absent or present at low concentration in serum (Eckersall and Conner 1988).

Haptoglobin concentration in bovine serum was originally expressed as a percentage of haemoglobin binding capacity based on the ability of haptoglobin to bind haemoglobin and preserve peroxidase activity at low pH. However the purification of bovine haptoglobin by Eckersall and Conner (1988) has now allowed the quantification of serum haptoglobin in grams per litre. The use of the haptoglobin-haemoglobin assay in routine testing of bovine plasma is useful in monitoring the APR, however, as the function of haptoglobin is to bind and remove free circulating haemoglobin, the interpretation of results has to be cautious when a potential haptoglobin response is accompanied by haemolytic disease, when inaccurate results may be obtained (Eckersall, 1988; Skinner *et al.*, 1991). *In vitro,* the formation of the haptoglobin-haemoglobin complex is followed by the removal of this complex by the liver, resulting in reduced haptoglobin levels. One advantage of the haptoglobin assay over other APP assays is that it can be performed quickly and accurately and may, therefore, be preferable to an assay for other major acute phase proteins, such as SAA, which have lengthy and technically demanding assay procedures (Skinner *et al.*, 1991).

Several other assays for detecting elevation in haptoglobin values have been developed involving single radial immunodiffusion (Morimatsu *et al.*, 1992) and high performance liquid chromatography (Salonen *et al.*, 1996). These methods have also been shown to have improved sensitivity compared to the routine haptoglobin-haemoglobin assay, but are still affected by removal of haptoglobin in haemolytic conditions.

In serum of healthy cattle the normal level of haptoglobin is less than 0.10g/l, with the concentration increasing up to 100-fold within twenty four hours of infection (Conner *et al.*, 1988). The increase in bovine haptoglobin concentration from a basal level of less than 0.10g/l to greater than 4.00g/l within 48 hours of infection may indicate the severity of the physical stress or inflammation in diseases such as mastitis, pyometra and traumatic reticulitis, and as such, may be a valuable aid in diagnosis of clinical disease (Makimura and Suzuki, 1982; Skinner *et al.*, 1991).

1.3.3.2 Serum Amyloid A

Serum amyloid A was initially recognised in serum due to its cross-reactivity with antisera to the amyloid AA protein isolated from deposits of secondary amyloid (Levin *et al.*, 1973; Husby and Natvig, 1974). Serum amyloid A is a trace level constituent of normal serum found in the circulation bound to the high density lipoprotein apoSAA, and is a very sensitive APP which increases up to 1000 fold after tissue damage and infection in cattle, horses and humans (Kushner, 1982; Maurhaug, 1983; Alsemgeest *et al.*, 1993; Horodagoda, 1994).

Serum amyloid A has been identified as a very heterogeneous protein in all species studied to date and is regarded as representing a family of 12kDalton molecules with 104-112 amino acid residues, with a possible insertion of 8 amino acids at position 72 (Betts *et al.*, 1991). Bovine SAA has 112 amino acid residues and contains a 9 amino acid insertion between positions 69 and 70 (Rossevatn *et al.*, 1992). The majority of the SAA isotypes are involved in the APR while others are constitutive proteins (Whitehead *et al.*, 1992). All types of SAA are transported in serum as apoproteins in the high density lipoprotein (HDL) fraction

(Benditt and Eriksen, 1977). The association of SAA and HDL is likely to occur following the secretion of the SAA (Hoffman and Benditt, 1982), and in most species, two structurally similar SAA isotypes are expressed in the liver, while a structurally different form of SAA is independently regulated and expressed primarily in the extrahepatic tissues (Maurhaug and Dowton, 1984). The increase in serum concentration of SAA during inflammation is due to both the increase in synthesis and decrease in degradation of the SAA by the liver (Maurhaug and Dowton, 1984).

The function of SAA remains unclear, with original studies by Aldo-Benson and Benson (1982) indicating that SAA may be influencing lymphocyte responses to antigens. Other authors have suggested that the function of SAA is to redirect HDL to inflammatory cells such as macrophages, or to aid in cholesterol removal (Kisilesky, 1991). The former theory has been supported in studies which showed that when SAA is bound to the HDL portion, the affinity of HDL for macrophages increases 2-3 fold. Atherosclerotic lesions possess many features similar to those found in inflammatory reactions, however the role of SAA in pathogenesis remains unclear (Maurhaug and Dowton, 1984). Studies by Shainkin-Kestenbaum *et al.* (1991) have indicated that a possible feedback system may exist between SAA and the immunoregulatory cytokines, and as SAA increases in concentration after tissue destruction, a role in normal tissue repair may also occur.

Serum amyloid A is the most sensitive APP characterised to date, in both humans and many animal species, and has been used in the diagnosis and monitoring of inflammatory and infectious diseases. An increased concentration of SAA has also been associated with amyloidosis in the dog, mink, cat, rabbit, horse and cattle (Hol and Gruys, 1984). Serum amyloid A has been identified as the major APP in the horse and has been shown to be a reliable marker for assessment of tissue damage during training and racing (Pepys *et al.*, 1989). Recently SAA has been identified as an APP in cattle (Boosman *et al.*, 1990; Alsemgeest *et al.*, 1993) and monitoring bovine SAA concentration may serve as a specific and reliable marker for clinical inflammation and tissue injury (Boosman *et al.*, 1990;

Alsemgeest *et al.*, 1993). However, due to the unavailability of a rapid automated assay, the use of SAA as a routine indicator of inflammation has been limited (Maurhaug and Dowton, 1984). Measurement of SAA in cattle is hampered by the difficulty in isolation of the SAA when dissociated from the HDL fraction and also the poor immunogenicity of the protein for the production of antiserum (Eckersall, 1992 (a)).

Other problems in the development of a SAA assay have been lack of standards and monospecific antibodies for SAA (Maurhaug and Dowton, 1984). Improved methods for purification of SAA and the introduction of monoclonal or polyclonal antipeptide antibodies have aided in the development of current assays for human SAA (Casl and Grubb, 1993; Wilkins *et al.*, 1993).

The regulation of SAA and haptoglobin appear to be disease specific pathways. Serum amyloid A was found to be an indicator of acute inflammatory lesions, whereas haptoglobin indicated more chronic pathology (Alsemgeest *et* al., 1994). In human disease, SAA is a more sensitive indicator of inflammation than haptoglobin but studies in cattle have been limited due to the non-availability of a robust SAA assay. In man, the assay of APP has become an accepted and useful diagnostic aid for the detection and monitoring of conditions leading to an APR (Kushner and Mackiewicz, 1987). The development of APP detection assays in the assessment of the chronicity and severity of disease in cattle has considerable potential for diagnosis and prognosis. Limited studies have shown that the circulating concentration of bovine SAA correlates with the level of severity of disease status i.e. acute, subacute or chronic (Gruys *et al.*, 1994; Horadogoda *et al.*, 1994). In clinical diagnosis in cattle the levels of APP may aid in monitoring diseases such as hepatic lipidosis, inflammatory processes and periparturient diseases especially when both haptoglobin and SAA are measured (Gruys *et al.*, 1993).

1.3.4 Mammary secretions

1.3.4.1 Inflammation

Measurement of the SCC is an indirect test used routinely in the analysis of milk as an indicator of bovine mastitis (Sears and Heider, 1981). Indirect tests which determine alterations in milk composition, enzyme concentration and serum protein levels can also be used as indicators of inflammation (Huszenicza and Stollar, 1993). The NAGase test is based on the measurement of the cell associated enzyme N-acetyl-β-D-glucosaminidase and is reputed to be the most accurate of the indirect tests and as accurate as SCC in predicting the infection status of a quarter (Pyorala, 1988). Other indirect tests such as the estimation of serum albumin concentration, is indicative of damage to the mammary mucosa and increases according to the epithelial damage sustained (Radostits *et al.*, 1994). The antitrypsin test which measures the trypsin-inhibitor capacity of the milk has also been extensively used in diagnosis (Radostits *et al.*, 1994). The diagnosis of both clinical (Shuster *et al.*, 1991; Huszenicza and Stollar, 1993) and subclinical mastitis has been carried out using these methods of detection (Nizamilioglu *et al.*, 1989).

1.3.4.2 Acute Phase Response and Acute Phase Proteins

Limited work has been performed to date on the detection of the APR and APP in mammary secretions. Schroldt *et al.* (1995) described the first detection system for the APP CRP in milk as a novel indicator of bovine mastitis. Milk samples taken from healthy and mastitic cows gave CRP concentrations ranging from 65-103ng/ml and 93-417 ng/ml respectively. In some cases, as much as a tenfold increase in CRP levels was observed in milk following inflammation of the udder, suggesting the potential usefulness of CRP as an indicator of mastitis. CRP however is generally not considered as a major APP in bovine or equine serum (Kent, 1992). Levels of α_1 anti-trypsin, which is only a moderate serum APP in

the bovine, have also been shown to increase in serum and milk of cows with mastitis (Honkanen-Buzalski *et al.*, 1981; Sandholm *et al.*, 1984). The concentration of α_1 anti-trypsin was related to the presence of serum albumin and increased SCC in milk: as the severity of the mastitic attack increased, the levels of α_1 anti-trypsin in milk also increased, by 100 fold, while in some animals only a 4 fold increase was detected in serum (Honkanen-Buzalski *et al.*, 1981).

Acute phase protein assays may provide a valuable aid to prognosis and early diagnosis of conditions such as bovine mastitis (Conner and Eckersall, 1986), and detection of the major bovine APP, SAA and haptoglobin in milk may also form a part of future quality assurance schemes. Of these two APP, SAA would be the better analyte to quantify as it is not effected by haemolytic conditions and is more responsive to stimulation than is haptoglobin (Horadogoda *et al.*, 1994).

1.4 Aims of the Project

The overall aim of this project was to study infection and inflammation in experimentally-induced *S. aureus* mastitis in cows. A novel double-cross over design was employed for experimental intramammary challenge in this study. Udder quarters, with naturally-occurring subclinical mastitis caused by *S. aureus* were challenged by the intramammary route with very high numbers of either the indigenous or non-indigenous strain of *S. aureus*.

In studying infection due to *S. aureus*, the main aim of this part of the project was to study the dynamics of intramammary challenge in cows and to determine if infusion with large numbers of different *S. aureus* strains induced clearance, alteration or persistence of the original subclinical mastitis. Another aim was to determine whether variations in the strain of *S. aureus* were evident when more than one colony of *S. aureus* per milk sample was examined employing recent molecular techniques such as Restriction Enzyme Fragmentation Pattern (REFP) Analysis and Repetitive Extragenic Palindromic Polymerase Chain Reaction (REP-PCR).

In studying inflammation due to *S aureus,* the main aim of this part of the project was to investigate if SAA could be used as an indicator of inflammation in *S. aureus* mastitis and thus as a potential adjunct to future quality control programmes for dairy products. To facilitate this, the aim was to develop a monoclonal antibody raised specifically against bovine SAA by developing methods allowing improved purification of SAA.

Chapter 2

Infection in Bovine *Staphylococcus aureus* Mastitis

2.1 Introduction

One of the main aims of this part of the project was to investigate the effect of infusing *S. aureus* by the intramammary route into quarters already subclinically infected with *S. aureus*. It was considered necessary to infuse very large numbers of *S. aureus* bacteria into the quarters in order to provide sufficient challenge to the existing organisms colonising the tissue, and greater numbers of *S. aureus* were infused in this study than had been previously described in the literature.

To improve investigation of intramammary infection caused by *S. aureus*, it was decided to examine multiple colonies isolated from infected quarters and to use genetic fingerprinting to identify strain variation among cows and quarters.

Due to the time taken to perform the genetic fingerprinting using standard techniques, an attempt to develop a repetitive extragenic palindromic polymerase chain reaction (REP-PCR), which allowed more rapid identification of bovine *S. aureus* strains, was made.

2.2 Materials and Methods

2.2.1 Experimental Animals

To investigate the dynamics of intramammary infection with different strains of *S. aureus*, four adult Friesian cows with naturally occurring subclinical mastitis caused

by *S. aureus* were studied. The cows were initially selected as having high ICSCC (> 600,000/ml), and the infected quarters were subsequently identified by IQSCC (> 400,000/ml), and *S. aureus* was isolated on routine bacteriology from them. Two cows, both from the same farm, were shown to be infected with the same strain on the basis of genomic DNA fingerprints (described in section 2.2.5.1) and the strain was designated strain A. Two other cows, both from a different farm, were shown to be infected by the same method with a different strain, designated strain B.

2.2.2 Intramammary Infusion with *Staphylococcus aureus*

Each cow described above was challenged with either 10^8 *S. aureus* per quarter in the case of the non-infected quarter, or 10^9 *S. aureus* in the case of infected quarters in a double crossover design, such that one cow from each farm was challenged with the indigenous strain and one cow from each farm challenged with the non-indigenous strain (Table 2.1). Throughout the trial all four quarters were used and given the prefixes LF, RF, LH and RH referring to the left fore, right fore, left hind and right hind quarters respectively.

Staphylococcus aureus cultures containing 1.25×10^9 cells per ml in Brain Heart Infusion Broth (Oxoid, Basingstoke, Hampshire) were incubated overnight at 37^0C and then centrifuged at 5670g for 10 minutes. The supernatant was decanted, the pellet was resuspended in 15ml Ringers solution and centrifugation and resuspension of the pellet was repeated. One and a half millilitres of cell suspension at 1.25×10^{10} cells/ml was added to 20 ml Ringers solution and infused into infected quarters (i.e. a total of 10^9 bacteria), uninfected quarters had 20ml of a 1:10 dilution of the original cell suspension (i.e. a total of 10^8 bacteria) infused into them. Control quarters had only 20ml Ringers solution infused into them. The teats were prepared by washing and drying prior to thorough disinfection with surgical spirit. The first few draws of milk were discarded from each quarter immediately prior to intramammary infusion. The *S. aureus* cultures were infused using a 6FG dog urinary catheter inserted through the teat canal to which a 20ml syringe containing the bacterial culture was attached. Following infusion, the base of the quarter was massaged in an upward

Cow Number	Quarters infected prior to trial	Pre challenge strain isolated	Quarters challenged with 1×10^9 *S. aureus*	Quarters challenged with 1×10^8 *S. aureus*	Challenge strain
186	LF, RF, LH, RH	A	LF, RF, LH, RH		B
125	LF, RF, LH	A	LF, LH		A
520	RH	B	RH	RF	A
703	LF	B	LF	LH	B

Table 2.1: The double cross-over design for intramammary challenge with *Staphylococcus aureus*

motion while holding the teat duct closed, to ensure thorough dispersion of the infused material into the gland cistern. None of the infusion was lost via the teat canal and all teats were then dipped in an iodophor teat dip. The cows were examined clinically at regular intervals for 48 hours after the infusion procedure and were milked twice daily by machine.

2.2.3 Milk Sample Collection

Individual quarter milk samples were collected by hand stripping on day -4 (pre-challenge), and on days 3, 8 and 17 post-challenge.

2.2.4 Routine Bacterial Isolation from Milk Samples

Three millilitres of milk was mixed with 17ml of sterile distilled water and centrifuged at 4500g for 20 minutes. The cell pellet was resuspended in 100µl of lauryl broth, from which 50µl was spread onto columbia blood agar and mannitol salt agar plates which were incubated overnight at 37^0C. After incubation, 15-20 individual *S. aureus* colonies, identified by colony morphology, were subcultured individually onto columbia agar slopes and incubated overnight at 37^0C. Inoculates columbia agar slopes were then stored at 4^0C.

Further strain characterisation was employed and used restriction enzyme fragmentation pattern (REFP) analysis.

2.2.5 Identification of *Staphylococcus aureus* Strains

2.2.5.1 Restriction Enzyme Fragmentation Pattern Analysis

Subculture of *Staphylococcus aureus* Strains. Ten millilitres of BHI broth (Oxoid) was inoculated aseptically from columbia agar culture slopes and incubated overnight at 37^0C. Columbia blood agar streak plates were also prepared and incubated to confirm the purity of the initial culture.

Isolation of Genomic DNA

Purification of DNA. Purification of DNA was carried out over a period of two days. On day one of the purification procedure, overnight BHI broths were centrifuged at 4480g for 10 minutes and resuspended in 3ml Tris ethylene diamine tetra acetic acid sodium chloride buffer (TES) (50mM Tris-base, 50mM sodium chloride, 5mM disodium EDTA, (pH8.0)). The samples were then aliquoted into 3 eppendorf tubes and microcentrifuged at 13,800g for 30 seconds before being resuspended in 200µl TES which contained 50mM sucrose to prevent cell lysis. Twenty microlitres of the endopeptidase lysostaphin (Sigma, 1000units/ml) which is specific for the pentaglycine bridge involved uniquely in the cell wall structure of *S. aureus*, and 100µl lysozyme (Sigma, 40mg/ml) were added and mixed thoroughly before being incubated at 37^0C for 30 minutes. After incubation 20% SDS (15µl) was added and mixed by inversion, fifty microlitres of Proteinase K (10mg/ml) was also added before the sample was sheared through a 25 gauge needle to reduce the viscosity of the DNA and incubated for two hours at 37^0C. One hundred microlitres of TE_{10} (10mM Tris base, 10mM disodium EDTA, (pH7.8)) was added to reduce the DNA concentration before the initial purification stage using phenol chloroform (500µl). After mixing thoroughly, the samples were microcentrifuged at 13,800g for 10 minutes and the aqueous layer was transferred to another eppendorf and 500µl of isopropanol was added. The samples were left at room temperature for 60 minutes to precipitate the DNA, followed by centrifugation at 13,800g for 10 minutes. The pellet was resuspended in 100µl TE_{10} and triplicate tubes were pooled, 100µl of ammonium acetate (7.5M, pH8.0) and 600µl of 95% ethanol were added and samples were stored at -20^0C overnight, to precipitate the DNA.

On day two, further purification procedures were carried out. Samples were centrifuged at 13,800g for 10 minutes, resuspended in 300µl TE_{10} and 20µl RNAase (Sigma, 10mg/ml) was added. After incubation at 37^0C for 60 minutes, the phenol/chloroform, isopropanol and ethanol steps were repeated as above and the samples were again stored at -20 overnight.

Restriction Digestion of DNA. Restriction digestion was carried out on day three, the day immediately following the purification procedures. Samples were centrifuged at 13,800g for 10 minutes and resuspended in 60µl TE (10mM Tris base, 1mM disodium EDTA (pH8.0)). Restriction digestion of each sample using restriction enzyme *Hha* I (Gibco Life Technologies Limited, Paisley) was carried out, with bacteriophage λ DNA digested with *Hha* I, *Kpn* I and *Pst* I as controls. Restriction products mixed with loading buffer (25g sucrose, 60mg sodium acetate, 100mg SDS and 50mg bromophenol blue in 100ml) were electrophoresed in a 0.8% agarose gel (2:1 dilution with TBE:distilled water, (Tris base 89mM, boric acid 89mM disodium EDTA 1.25mM, pH8.0-8.4)) at 25mA overnight and the gel was stained using ethidium bromide in TBE (0.5-1.0ug/ml ethidium bromide). Following removal of excess background staining by rinsing the gel in distilled water for approximately 15 minutes, the gels were photographed on a transilluminator at 302nm using Polaroid film type 655.

Digitisation of Restriction Enzyme Fragmentation Pattern Results. Interpretation of electrophoretic gels was a skill-intensive task. Digitisation of gel photographs was an objective yet flexible technique that allowed the accurate assessment of electrophoretic data. The statistical analysis was used to assess the accuracy of calibration data, and by limiting the percentage fragment size variation, accurate between-gel comparisons were made. Data was stored in numerical form (molecular weight of restriction fragments). The graphical output was used to present the data in a familiar format which allowed visual comparisons with the original gel photograph. Digitisation of REFP isolates allowed gel to gel-comparisons of *S. aureus* strains. Strains were analysed using a commercially available programme (Platt and Sullivan, 1992) with restriction digested λ DNA using enzymes *Kpn* I and *Pst* I as calibration controls. Restriction fingerprints were compared using a coefficient of similarity calculated from the formula:

$$S_D(\%) = \frac{2m}{a+b} \quad X100$$

where 'm' was the number of fragments common to two fingerprints and 'a' and 'b' were the total number of fragments generated from each fingerprint respectively after digestion by the same restriction enzyme (Dice, 1945).

The molecular weight of the control fragments was fitted to a robust modified hyperbola, from which the size of restriction fragments in adjacent tracks was estimated. Numerical values were stored for subsequent graphical output which was on a logarithmic scale, the experimental variation in fragment size did not exceed 5% (Plikaytis *et al.*, 1986).

2.2.5.2 Repetitive Extragenic Palindromic Polymerase Chain Reaction

The repetitive extragenic palindromic polymerase chain reaction (REP-PCR) was used to obtain a rapid method for the identification of strain diversity in the bovine staphylococci. Genomic DNA was prepared by isolation of genomic DNA as described previously in section 2.2.5.1 following the purification stages carried out on day one and day two during REFP analysis. Fifty nanograms of genomic DNA was amplified in a total volume of 50µl containing 50pmols of primer REP 1R (5' 111 NCgNCgNCATCNggC3', R and D Systems Europe limited, Abingdon) and REP 2 Dt (5' NCgNCTTATCNggCCTAC3', R and D Systems), 250µM of dATP, dTTP, dCTP, and dGTP (Pharmacia, St. Albans, Herts.), 10mM Tris-HCL, 1.5mmol/l MgCl, 50mM/l KCL, 0.1mg/ml gelatin and 2.5 units of Dynazyme (Flowgen, Lichfield, Staffordshire). DNA samples were subjected to an initial denaturation of 94^0C for 7 minutes, followed by 32 cycles of 94^0C for 1 minute, 37^0C for 1 minute, 72^0C for 8 minutes followed by a final extension of 72^0C for 16 minutes using a Perkin Elmer 480 Thermocycler (Perkin Elmer, Warrington). Amplification efficiency was determined by agarose electrophoresis using a 1.2% agarose gel and viewed as described for REFP analysis. Initially, strain A, strain B and strain A* were used for the optimisation of the protocol, which involved reducing the primer annealing temperature by 3^0C to 37^0C, varying the target DNA concentration, primer and magnesium chloride concentrations.

Restriction Digestion of REP-PCR Products. Restriction digestion of the REP-PCR products was carried out to determine if any further strain pattern variation could be identified using this method. The digestion enzymes used were *Rsa* I, *Alu* I, *Stu* I, *Hind* III, *Pst* I, *Hae* III, *Eco* RI, *Bam* HI, *Hpa* I and *Kpn* I. Each digest contained 10µl of PCR product, 2µl of appropriate enzyme buffer, 3µl of enzyme and distilled water to give a total volume of 20µl. The samples were incubated overnight at 37^{0}C in a water bath. Following incubation the samples were electrophoresed on a 1.2% agarose gel, containing ethidium bromide.

2.3 Results

2.3.1 Infection in *Staphylococcus aureus* Mastitis

2.3.1.1 Isolation of *Staphylococcus aureus* from Milk Samples

Routine bacteriological analysis was carried out in the commercial laboratory at the Scottish Agricultural College, Auchincruive. Routine bacteriological analysis identified bacteria from each quarter of each cow on each sampling day. Quarters were recorded as either *S. aureus* positive or *S. aureus* negative and the presence of other bacterial species was also recorded. Initial identification of *S. aureus* was based on colony morphology and a positive coagulase test. The distribution of positive samples is shown in Table 2.2. Throughout the study, uninfected quarters were designated as control quarters and were analysed to confirm that cross-contamination had not occurred during the infusion procedure and subsequent sampling.

Cow Number	Sample Day	*S. aureus* Positive	*S. aureus* Negative
125	-1	RF, LF, LH	RH
125	3	RF, LF, LH	RH
125	8	RF, LF, LH	RH
125	17	LF, LH	RF, RH
186	-1	RF, LF, RH, LH	
186	3	RF, LF, RH, LH	
186	8	RF, LF, RH, LH	
186	17	RF, LF, RH, LH	
520	-1	RH	RF, LF, LH
520	3	RF, RH	LF, LH
520	8	RH	RF, LF, LH
520	17	RF, RH	LF, LH
703	0	LF	RF, RH, LH
703	3	LF, LH	RF, RH
703	8	LH	RF, RH, LH
703	17		RF, LF, RH, LH

Table 2.2: Routine bacteriological results from all quarters from cows 125, 186 and 520 on four sampling days i.e. day -1 (pre-challenge) and days 3, 8 and 17 post-challenge. Routine bacteriological screening from all quarters from cow 703 on four sampling days i.e. days 0 (pre-challenge), 3, 8, and 17 post-challenge.

2.3.1.2 Differentiation of *Staphylococcus aureus* Strains

Restriction Enzyme Fragmentation Pattern Analysis and Digitisation of Results. Bacterial isolates were fingerprinted and analysed by REFP from all four quarters of each cow on four occasions during the study i.e. day -4 (pre-challenge) and days 3, 8, and 17 post-challenge. A total of 400 cultures were studied by REFP analysis. The four cows studied were challenged by the intramammary route as shown in table 2.1.

Following digestion of bacterial isolates with restriction digest *Hha* I, a strain with 25 fragments ranging from 2.92 kilobases to 9.59 kilobases in size was identified and was designated strain A (figure 2.1, Lanes 6-14). Digitisation of the REFP results is shown in figure 2.2, Lanes 6-14. A molecular variant of strain A (A*) was also recognised; 25 fragments were evident following digestion with restriction digest *Hha* I. Twenty-four of the 25 fragments matched those of strain A (figure 2.1, Lanes 1-5). Digitisation of the REFP results is shown in figure 2.2, Lanes 1-5. The only difference between strain A and A* was in the largest fragment, with strain A fragment size being 9.59 kb and strain A* fragment size being 9.32 kb.

Following digestion of bacterial isolates with restriction digest *Hha* I, a strain with 30 fragments ranging in size from 2.92-9.59 kb was identified and was designated strain B (figure 2.3, Lanes 3, 4, 6, 8-15). Digitisation of the REFP results is shown in figure 2.4, Lanes 3, 4, 6, 8-15.

Seven genetically unrelated strains were recognised by REFP analysis as strains other than strain A, strain A* or strain B and were designated strains R_1 to R_7 (figure 2.7, Lanes 5-11). As an example of an unrelated strain, R_1, is shown in lane 8 in figure 2.5 and following digitisation in figure 2.6. The REFP results from additional strains were digitised (figure 2.7, lanes 5-11) and the total number of fragments identified and the size of each determined. These additional strains ranged in fragment number from 20-36 fragments with sizes of 2.86-15.88 kb and were isolated both pre and post-challenge (Table 2.3).

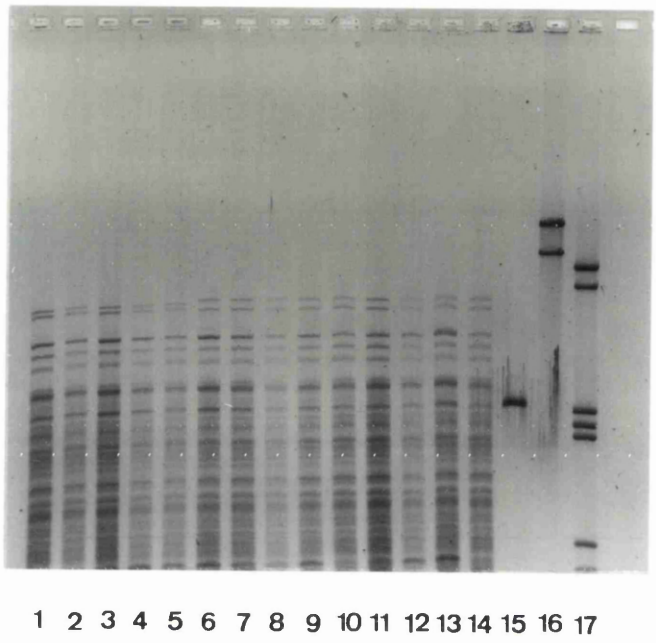

Figure 2.1: REFP of bacterial isolates from cow 186 on day -4 (pre-challenge) from RH and LH quarters; ethidium bromide (0.5-1.0 μg/ml) stained 0.8% agarose gel. DNA was digested using restriction endonuclease *Hha* I. Lanes 1-5 show isolates from RH identified as A*. Lanes 6-14 show isolates from LH identified as strain A. Bacteriophage λ DNA controls were digested with *Hha* I, *Kpn* I and *Pst* I and are shown in Lanes 15, 16 and 17 respectively.

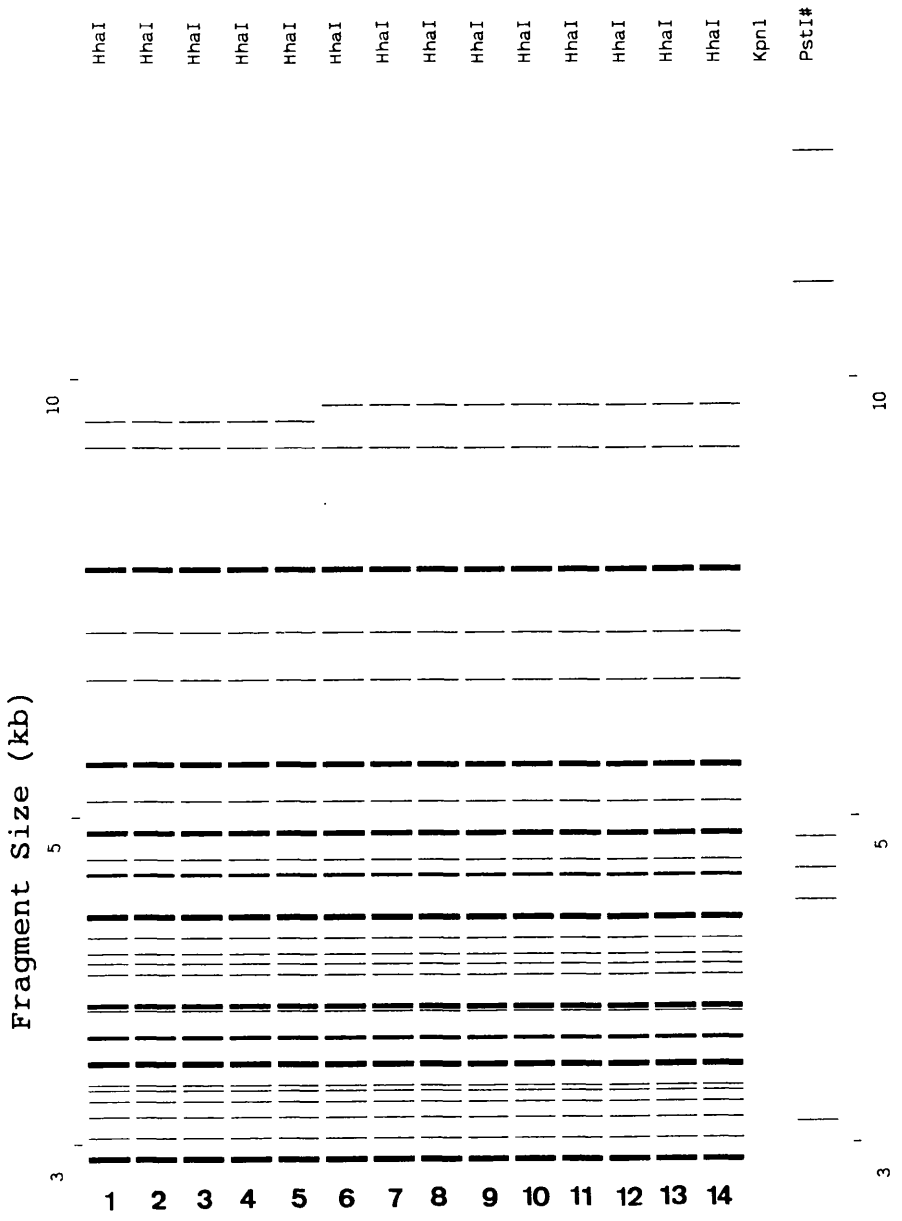

Figure 2.2: Graphical output of digitised image from figure 2.2. Lanes 1-5 show isolates from RH identified as A*. Lanes 6-14 show isolates from LH identified as strain A. Bacteriophage λ DNA controls were digested with *Hha* I, *Kpn* I and *Pst* I. Lanes 15 and 16 show *Kpn* I and *Pst* I respectively.

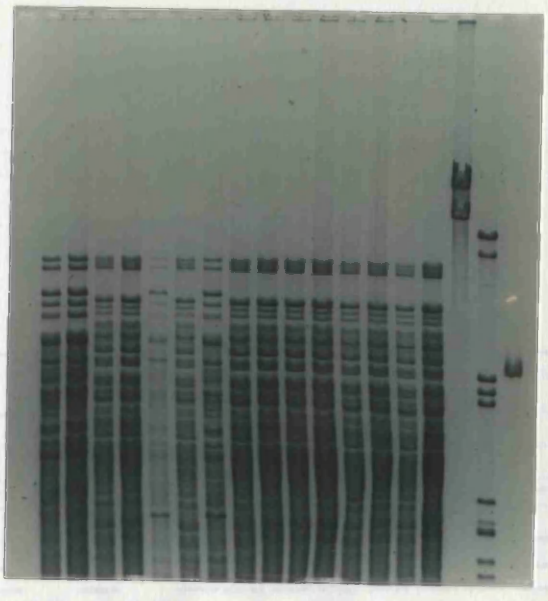

Figure 2.3: REFP of bacterial isolates from cow 520 on day 17 post-challenge from RF and RH quarters after digestion with *Hha* I; ethidium bromide (0.5-1.0μg/ml) stained 0.8% agarose gel electrophoresis. Lanes 1-7 show isolates from the LH quarter. Lanes 1, 2, 5 and 7 show strain A whereas lanes 3, 4 and 6 show strain B. Lanes 8-15 show isolates from the RH quarter and were strain B. Bacteriophage λ DNA controls were digested with *Kpn* I, *Pst* I and *Hha* I and are shown in lanes 16-18 respectively.

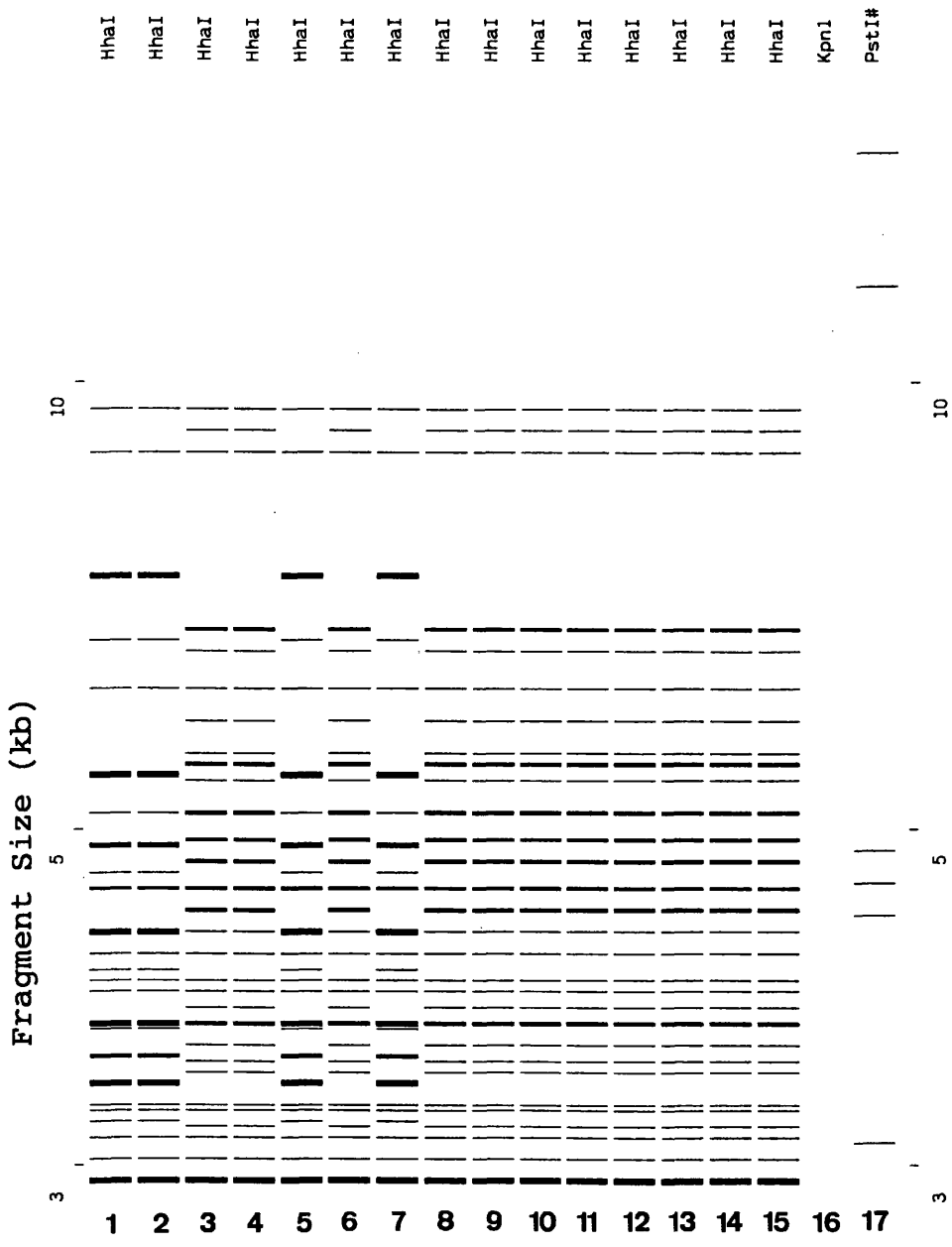

Figure 2.4: Graphical output of digitised image from figure 2.3. Lanes 1-7 show isolates from the LH quarter. Lanes 1, 2, 5 and 7 show strain A whereas lanes 3, 4 and 6 show strain B. Bacteriophage λ DNA controls were digested with *Kpn* I, *Pst* I and *Hha* I. Lanes 15 and 16 show *Kpn* I and *Pst* I respectively.

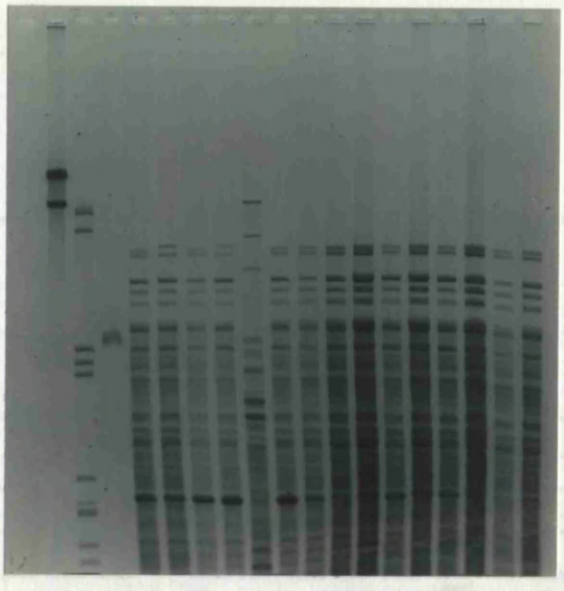

Figure 2.5: REFP of bacterial isolates from cow 186 on days 3 and 17 post-challenge from RH quarter; ethidium bromide (0.5-1.0μg/ml) stained 0.8% agarose gel electrophoresis. Samples were digested using restriction endonuclease *Hha* I. Bacteriophage λ DNA controls were digested with *Kpn* I, *Pst* I and *Hha* I and are shown in lanes 1, 2 and 3 respectively. Lanes 4-10 show isolates from RH quarter on day 17 post-challenge. Lanes 4, 6, 9 and 10 show strain A*, whereas lanes 5 and 7 show strain A. Lane 8 shows strain R_1. Lanes 11-18 show isolates from RH day 3 post-challenge and were identified as strain A*.

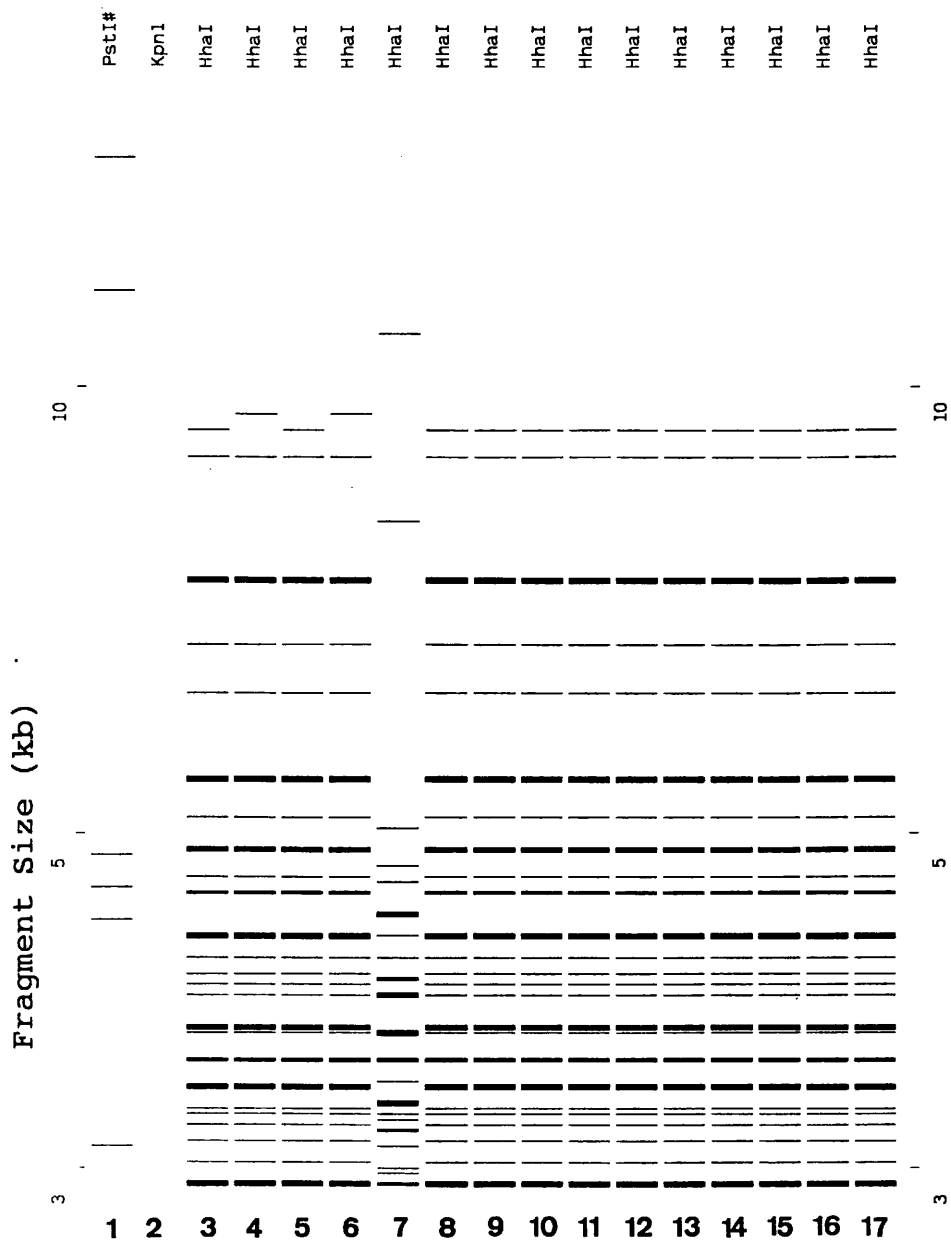

Figure 2.6: Graphical output of digitised image from figure 2.5. Bacteriophage λ DNA controls were digested with *Kpn* I, *Pst* I and *Hha* I. Lanes 1 and 2 show *Pst* I and *Kpn* I respectively. Lanes 3-9 show isolates from RH quarter on day 17 post-challenge. Lanes 3, 5, 8 and 9 show strain A*, whereas lanes 4 and 6 show strain A. Lane 7 shows strain R_1. Lanes 10-17 show isolates from RH day 3 post-challenge and were identified as strain A*.

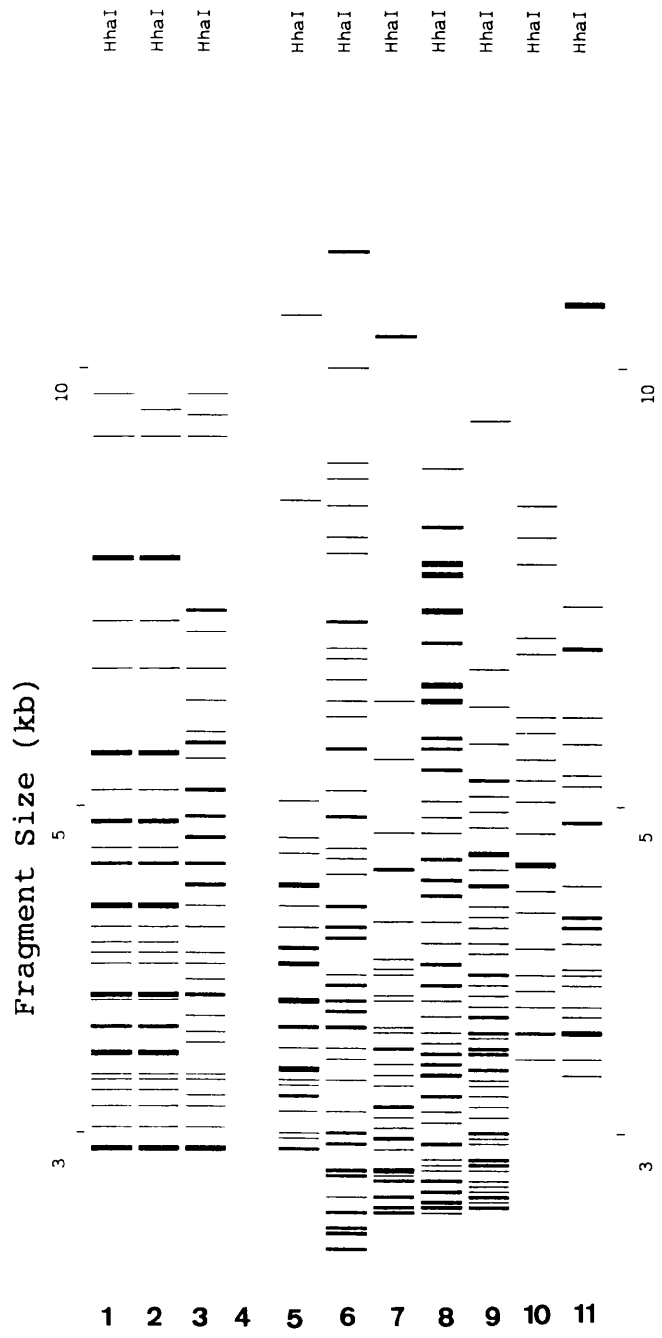

Figure 2.7: Graphical output of digitised image of REFPs after *Hha* I digestion of genomic DNA. Strain A (Lane 1), strain A* (Lane 2) and Strain B (Lane 3). Additional distinct isolates R_1-R_7 are shown in lanes 5-11 respectively.

Isolate	Cow No.	Sample Day	Quarter	Total Number of Fragments	Range of Fragment Sizes (kilobases)
R_1	186	17	RH	22	2.93-15.88
R_2	125	17	LF	33	2.95-12.01
R_3	703	8	LF	22	2.92-10.49
R_4	703	8	LF	33	2.89-8.52
R_5	125	-4	LH	36	2.86-9.19
R_6	703	8	LF	20	3.38-8.06
R_7	703	8	LF	20	3.30-10.99

Table 2.3: Strains, unrelated to strain A, strain A*, or strain B, recognised by REFP analysis from cows 125, 186 and 703 and designated R_1 to R_7.

Coefficient of Similarity between Strains. The similarity coefficient between strain A and strain A* was 96% (24 of the 25 fragments matching). A similarity coefficient of 83.6% was shown between strain A and strain B with 23 of the 30 fragments matching. Strain A* and strain B also had a percentage similarity coefficient of 83.6% with 23 of the 30 fragments matching. Strain A and strain A* showed between 48.9-73.7% similarity to strains R_1 to R_7, whereas strain B had between 52.0-83.9% similarity to strains R_1 to R_7 (Tables 2.4 and 2.5).

2.3.1.3 Discrimination Between Strains of Bovine *Staphylococcus aureus* by Polymerase Chain Reaction

REP-PCR amplification using REP-1 and REP-2 primers on strain A genomic DNA generated two products of approximately 1,300 base pairs and 2,000 base pairs. The same method using strain B genomic DNA as the template, generated products of 1,100 base pairs and 2,000 base pairs (figure 2.8). The two strains were, therefore, distinguished by this method. However, using this method, no difference in the size amplification of products was observed between strain A and strain A* and identical banding patterns were seen (figure 2.8).

Under the same REP-PCR conditions and cycling parameters, genomic DNA from strains R_1 to R_7 gave entirely different banding patterns to strains A, B or strain A* (Figure 2.9).

Restriction Digestion of REP-PCR amplification products. Restriction endonuclease digestion was carried out to identify any variation between REFP fingerprinted genomic DNA from strain A and strain A*. The digestion enzymes *Rsa* I, *Alu* I, *Hin*d III, *Pst* I, *Hae* III, *Eco* RI, *Bam* HI and *Kpn* I all gave no variation in amplification product between strain A and strain A*. Only restriction endonuclease *Stu* I gave a slight variation in amplification products between strain A and strain A* (Figure 2.10, Lanes 6 and 7). The variation was the presence of an additional band in strain A in lane 6. Digestion enzymes *Rsa* I, *Alu* I, *Hin*d III, *Pst* I and *Hae* III are shown as examples of the absence of variation in restriction digestion of REP-PCR amplification products in figure 2.10 (lanes 2-5, 8-13).

Isolate	Cow No.	Sample Day	Quarter	% Similarity with Strain A	% Similarity with Strain B	% Similarity with Strain A*
R_1	186	17	RH	63.8	61.5	63.8
R_2	125	17	LF	72.4	66.7	72.4
R_3	703	8	LF	68.1	69.2	68.1
R_4	703	8	LF	73.7	83.9	73.7
R_5	125	-4	LH	71.2	75.0	74.6
R_6	703	8	LF	53.3	52.0	53.3
R_7	703	8	LF	48.9	64.0	48.9

Table 2.4:The percentage similarity between strains R_1-R_7, identified by REFP analysis and analysed following digitisation and strain A, or strain B, or strain A*. Isolates were from cows 125, 186 and 703.

Isolate	Cow No.	Sample Day	Quarter	No. of matching fragments with Strain A	No. of matching fragments with Strain B	No. of matching fragments with Strain A*
R_1	186	17	RH	15 out of 25	16 out of 30	15 out of 25
R_2	125	17	LF	21 out of 25	21 out of 30	21 out of 25
R_3	703	8	LF	16 out of 25	18 out of 30	16 out of 25
R_4	703	8	LF	21 out of 25	26 out of 30	21 out of 25
R_5	125	-4	LH	21 out of 25	24 out of 30	22 out of 25
R_6	703	8	LF	12 out of 25	13 out of 30	12 out of 25
R_7	703	8	LF	11 out of 25	16 out of 30	11 out of 25

Table 2.5: The number of matching fragments between strains R_1-R_7 identified by REFP analysis and analysed following digitisation between strain A, or strain B or strain A*. Isolates from cows 125, 186 and 703.

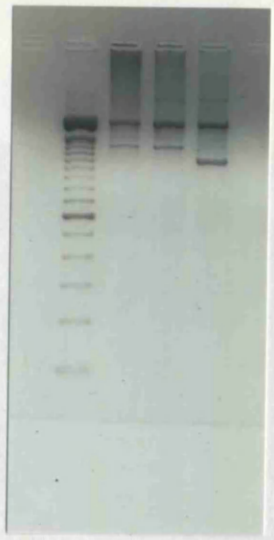

Figure 2.8: PCR amplification products obtained using REP-1 and REP-2 primers after agarose electrophoresis using a 1.2% agarose gel. Lane 1 shows 100 base pair ladder. Lane 2 shows strain A PCR amplification products of 1,300 and 2,000 base pairs. Lane 3 shows strain A* amplification products of 1,300 and 2,000 base pairs. Lane 4 shows strain B amplification products of 1,100 and 2,000 base pairs.

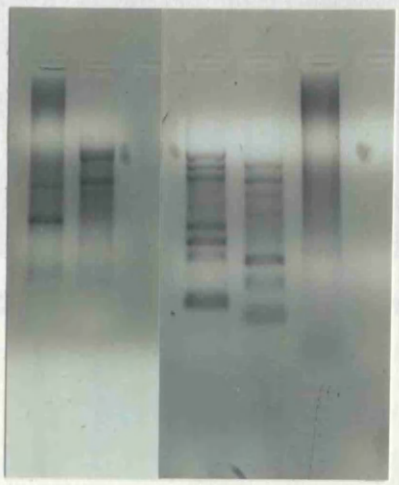

Figure 2.9: PCR amplification products obtained using REP-1 and REP-2 primers after agarose electrophoresis using a 1.2% agarose gel. Lanes 1-5 show amplification products obtained from additional isolates R_1 to R_7, previously identified by REFP analysis.

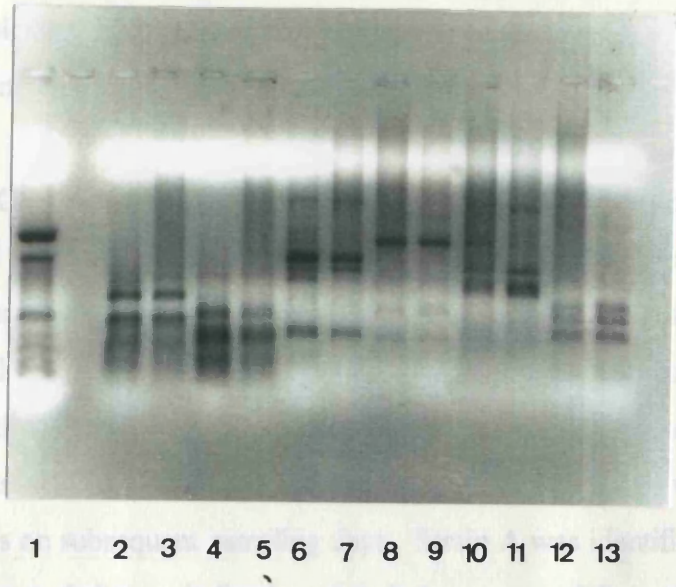

Figure 2.10:Restriction digestion of REP-PCR amplification products after agarose electrophoresis (1.2% agarose). Lane 1 shows 100 base pair ladder. Lane 2 shows strain A digested with *Rsa* I. Lane 3 shows strain A* digested with *Rsa* I. Lane 4 shows strain A digested with *Alu* I. Lane 5 shows strain A* digested with *Alu* I. Lane 6 shows strain A digested with *Stu* I. Lane 7 shows strain A* digested with *Stu* I. Lane 8 shows strain A digested with *Hind* III. Lane 9 shows strain A* digested with *Hind* III. Lane 10 shows strain A digested with *Pst* I. Lane 11 shows strain A* digested with *Pst* I. Lane 12 shows strain A digested with *Hae* III. Lane 13 shows strain A* digested with *Hae* III.

2.3.2 Dynamics of Intramammary Infection Following Experimental Challenge with *Staphylococcus aureus*

2.3.2.1 Cows Challenged with the Indigenous Strain of *Staphylococcus aureus*

Multiple colonies from each *S. aureus* positive quarter were analysed by REFP on each sampling day (tables 2.6 and 2.7)

Cow 125. Cow 125 was identified originally as naturally infected with strain A in the LF, LH and RF quarters and was challenged with the indigenous strain A into the LF and LH quarters. In the LH on day -4 (pre-infusion) isolates R_5 and R_2 were identified although the LH quarter had been identified as having been naturally infected with strain A on initial screening prior to this sample date. The challenge strain A was infused into the LF and LH quarters and strain A was isolated in these two quarters on subsequent sampling days. Strain A was identified in the LH quarter on day 3 post-challenge indicating that it has successfully colonised this quarter, remaining until day 17 post-challenge. It is possible that infusion of large numbers of strain A into the LH quarter may have replaced the R_5 and R_2 strains as the persisting organisms. Alternatively, failure to isolate strains R_5 and R_2 may have been simply due to a dilution effect where the presence of large numbers of strain A made random sampling of other strains less likely. The RF quarter which was naturally infected with strain A and did not receive any bacterial infusion was still infected with strain A at the end of the experiment. In the LF and RF quarters on day 17 post-challenge, while strain A predominated, unrelated strains R_2 (LF) and R_5 (RF) were also isolated as single colonies.

Cow 703. The LF quarter of cow 703 was naturally infected with strain B and she was subsequently challenged with strain B in the LF and LH quarters. Strain B was isolated on day 3 post-challenge in the LF quarter. On day 8, strains other than strain A or B were isolated in the LF quarter and were designated strains R_3, R_4, R_6 and R_7. Strain B was isolated as a homologous population on day 3 and day 8 from the LH quarter (Table 2.7). On day nine post-challenge cow 703 suddenly developed signs of

Staphylococcus aureus strains isolated from each quarter of cow 125				
SAMPLE DAY	**LF** Infected A Challenged A	**RF** Infected A	**LH** Infected A Challenged A	**RH** Control
Day -4 (pre-challenge)	AAAAAAAAA (9)	AAAAAAAAA A (10)	R_5 R_2 (2)	NT
Day 3 (post challenge)	NT	NT	AAAAAAAAA A (10)	NT
Day 8 (post challenge)	NT	NT	NT	NT
Day 17 (post challenge)	AAAAAAA R_2 (8)	AAAAAAAAA R_5 (10)	AAAAAAAAA (9)	NT

Table 2.6: *Staphylococcus aureus* strains identified by REFP analysis, from four quarters on four sampling days i.e. day -4 (pre-challenge) and days 3, 8, and 17 post-challenge. Total numbers of colonies subcultured are shown in brackets. A = Strain A, B = Strain B, A* = Variant Strain A. R_2 and R_5 = additional isolate. NT = not analysed. LF, LH, RF, and RH denotes the left fore, left hind, right fore and right hind quarters respectively.

Staphylococcus aureus strains isolated from each quarter of cow 703				
SAMPLE DAY	**LF** Infected B Challenged B	**RF** Control	**LH** Challenged B	**RH** Control
Day -4 (pre-challenge)	BBBBBBBBB (9)	NT	NT	NT
Day 3 (post challenge)	BBB (3)	NT	BBBBBBBBB (9)	NT
Day 8 (post challenge)	R_3R_3 R_4 R_6 $R_7R_7R_7R_7R_7$ (8)	NT	BBBBBBBBBB (10)	NT
Day 17 (post challenge	NT	NT	NT	NT

Table 2.7: *Staphylococcus aureus* strains identified by REFP analysis, from four quarters on four sampling days i.e. day -4 (pre-challenge) and days 3, 8, and 12 post-challenge. Total number of colonies sub-cultured are shown in brackets. A = strain A, B = strain B. R = additional strain. NT= not analysed. LF, RF, LH and RH denotes the left fore, right fore, left hind and right hind quarters respectively.

severe mastitis including reddening and swelling of the quarters. The cow was pyrexic and anorexic. Under Home Office Regulations the cow was treated with flunixin meglumine (Finadyne, Schering-Plough Limited), a non-steroidal anti-inflammatory and an antibiotic amoxycillin/clavulanic acid (Synulox, Pfizer Limited). No samples were therefore obtained from day 17 post-challenge for cow 703 as alteration in milk composition prevented the isolation of bacteria for subculture and REFP analysis.

2.3.2.2 Cows Challenged with the Non-Indigenous Strain of *Staphylococcus aureus*

Multiple colonies from each *S. aureus* positive quarter were analysed by REFP on each sampling day (table 2.8 and 2.9)

Cow 186. All four quarters of this cow were thought to be naturally infected with strain A on initial screening, however, while the other three quarters were shown to be infected with strain A, the RH quarter was shown to be naturally infected with strain A*. Following challenge of all four quarters with the non-indigenous strain B, REFP analysis showed that strain B was not isolated from any quarter post-challenge. Strain A was isolated from the LF, RF and LH in all the sampling days post-challenge (days 3, 8 and 17). Strain A* was isolated from the RH quarter on day -4 (pre-challenge), and on day 3 (post-challenge) as a homogeneous population. However, on day 17 post-challenge the RH quarter contained a mixed population of strain A, strain A* and one other isolate which was not strain A, strain A*, R_2 or R_5 (identified in cow 125 previously) and was designated strain R_1 (Table 2.8).

Cow 520. Cow 520 was naturally infected with strain B in a single quarter, the RH, and was challenged with strain A in both the RH and RF quarters. Strain B was confirmed as the only isolate from the RH quarter pre-challenge (day -4). Strain A was identified as a homogenous population on all isolates tested on day 3 post-challenge from the RH quarter, but by day 8 post-challenge, strain B was again identified in all isolates tested from the RH quarter and again on day 17 post-

Staphylococcus aureus strains isolated from each quarter of cow 186				
SAMPLE DAY	**LF** Infected A Challenged B	**RF** Infected A Challenged B	**LH** Infected A Challenged B	**RH** Infected A Challenged B
Day -4 (pre-challenge)	AAAAAAAA (8)	AAAAAAAAA AAA (12)	AAAAAAAAA (9)	A*A*A*A*A* A*A*A*A*A* (10)
Day 3 (post challenge)	AAAAAAAAA AAAAAA (14)	AAAAAAAAA AAAAAAAA (17)	AAAAAAAAA (9)	A*A*A*A*A* A*A*A*A*A* A*A*A*A*A* A*A* (17)
Day 8 (post challenge)	AAAAAAAAA AAAAAAAA (17)	NT	NT	NT
Day 17 (post challenge)	AAAAAAAAA AAAAAAAA (17)	AAAAAAA (7)	NT	AA A*A*A*A* R_1 (7)

Table 2.8: *Staphylococcus aureus* strains identified by REFP analysis, from four quarters on four sampling days i.e. day -4 (pre-challenge and days 3, 8, and 17 post-challenge. Total numbers of colonies sub-cultured are shown in brackets. A = strain A, B = strain B and A* = variant strain A. R = additional isolates. NT = not analysed. LF, RF, LH and RH denotes the left fore, right fore, left hind and right hind quarters respectively.

Staphylococcus aureus strains isolated from each quarter of cow 520				
SAMPLE DAY	**LF** Control	**RF** Challenged A	**LH** Control	**RH** Infected B Challenged A
Day -4 (pre-challenge)	NT	NT	NT	BBBBBBBBBB BBBBBBBBBB (20)
Day 3 (post challenge)	NT	AAA (3)	NT	AAAAAAAAA AAAAA (14)
Day 8 (post challenge)	NT	AAAAAAAAA (9)	NT	BBB (3)
Day 17 (post challenge)	NT	AAAA BBB (7)	NT	BBBBBBBBBB B (11)

Table 2.9: *Staphylococcus aureus* strains identified after REFP analysis, from four quarters on four sampling days i.e. day -4 (pre-challenge) and days 3, 8, and 17 post-challenge. Total numbers of colonies sub-cultured are shown in brackets. A = strain A, B = strain B. NT = not analysed. LF, RF, LH and RH denotes the left fore, right fore, left hind and right hind quarters respectively.

challenge. In the RF quarter, strain A was isolated on days 3 and 8 post-challenge. This result indicates that challenge with strain A resulted in persistence of this strain in this quarter for the duration of the study. However by day 17 post-challenge, both strain A and strain B were isolated from the RF quarter (table 2.9).

2.4 Discussion

The accurate determination of intramammary infection status is essential in conducting mastitis research and herd control (Sears *et al.*, 1990). There are a variety of diagnostic tests available for identifying mastitis and detecting infection levels in dairy herds. Methods for diagnosis have classically been divided into two major categories 1.) the detection of alteration in milk as a consequence of pathophysiologic changes due to an inflammatory response, 2.) the direct detection of microorganisms in milk by culture (Sears and Heider, 1981). However, the continued high incidence of chronic mastitis indicates that current control measures are sub-optimal and that the development of effective methods will require a more precise understanding of both the epidemiology of chronic mastitis within and between herds, and also a clearer view of the host-bacteria relationship within an immunological framework.

In recent years, new bacteriological and epidemiological techniques have become available to study patterns of infection in bovine mastitis (Lam, 1996). With modern techniques such as DNA fingerprinting and amplification of DNA by PCR, information for both diagnosis and epidemiology of intramammary infections may be provided quickly and reliably to allow detailed studies of natural bovine mastitis infections (Matthews *et al.*, 1994).

The present study followed the development of a strategic approach to REFP analysis based on restriction enzymes that recognise a 4-base rather than 6-base cleavage site in DNA. This technique provides a large amount of information, amenable to computer analysis and adaptable to a wide range of micro-organisms (Platt *et al.*,

1994). Firstly, from case control studies of human *S. aureus* infection, variation in 1-3 restriction fragments constituted clonal variation and that unrelated strains differed in ≥ 7 fragments. Secondly, in studies of bovine *S. aureus* in Scottish herds there was evidence of clonal variation within individual herds. This observation was based on the analysis of a single colony from each sample tested as in the standard diagnostic procedure. This raised the important question of whether strain variation reflected the choice of colony for study, genotypic diversification within the herd, or whether individual cows harboured one or more variants. Furthermore, the chronicity of bovine *S. aureus* mastitis raises the possibility that a cow may acquire different strains of *S. aureus* at different times that remain co-resident within the udder and would have consequences for the host-parasite relationship immunologically. Thus one of the initial aims of this study was to determine whether strain variation was evident within an individual quarter or cow and to what extent when multiple colonies were analysed from the same milk sample. Another aim of this project was to study the dynamics of intramammary challenge with *S. aureus* in four cows with subclinical *S. aureus* mastitis in one or more quarters. A double cross-over design was employed such that one cow from each source was challenged with the indigenous strain and the other cow was challenged with the non-indigenous strain. This approach allowed us to determine if the indigenous strain was displaced by the non-indigenous strain, or whether superinfection with the indigenous strain altered or had no effect on the original infection status of that quarter. This study also used a novel approach for intramammary infusion with *S. aureus*. Two concentrations of *S. aureus* were used for intramammary challenge depending on the original infection status of the quarter: $1.0x10^8$ c.f.u/ml. were infused into non-infected quarters, whereas $1.0x10^9$ c.f.u/ml. were infused into infected quarters. The high concentration of bacteria infused into the mammary gland in this study was in contrast to a study by Sears *et al.* (1990), which observed the shedding pattern of *S. aureus* from bovine intramammary infections. In that study a challenge dose which ranged from 20-2,000 c.f.u/ml was infused by the intramammary route (Sears *et al.*, 1990). However in the cited study the strain used was a particularly virulent *S. aureus* and of 21 previously uninfected glands challenged with *S. aureus*, 19 became infected. In the present study, the strains selected were isolated from cows with subclinical mastitis which are presumably less virulent than strains involved in clinical mastitis. It was considered

that it was necessary to infuse large numbers of bacteria into an infected gland in this study in order to provide a significant challenge to the existing organisms.

Although bacteriological culture of milk is commonly utilised for diagnosis of intramammary infection (Sears *et al.*, 1991), the milk collection technique, transportation and culture methodology should be taken into account when interpreting the microbial growth from a milk sample. Routine bacteriological screening generally involves single colony analysis. Many authors including Aarestrup *et al.* (1995(a)), Lam *et al.* (1996) and Birgersson *et al.* (1992) based their findings on the single colony analysis of an 'apparently pure culture'. These apparently pure cultures were identified by basic methods such as colony morphology, Gram-stain, coagulase, catalase test, lack of oxidase activity, antibiotic sensitivity and antibiogram typing. These methods of identification may however fail to identify all strains of *S. aureus* especially if they occur at low frequency.

In the present study, routine bacteriological investigation in the commercial laboratory resulted in *S. aureus* being isolated from the challenged quarters in three of the four cows (125, 186 and 520) whether the quarters were previously infected or uninfected on the majority of occasions. However on three separate occasions (cow 125, day 17, RF; cow 520, day 8, RF; cow 703, day 8, LF) bacteriological screening identified these individual quarters as *S. aureus* negative. These quarters were subsequently identified as *S. aureus* positive following REFP analysis and strain identification in the research laboratory, which may reflect differences in primary isolation techniques between the two laboratories. The failure to identify several infected quarters as *S. aureus* positive following routine bacteriological screening confirms that routine bacteriology is not 100% effective in the identification of intramammary infection. The study by Sears *et al.* (1990) concluded that routine bacteriological screening from a single quarter milk sample was only 75% sensitive in the diagnosis of intramammary

infection caused by *S. aureus*, thus 25% of infected cows may have a 'false' negative single culture result. Routine bacteriological investigation did not result in *S. aureus* isolation from both the challenged quarters (LF, LH) of the fourth cow (703) on all

the sampling dates. Cow 703 was naturally infected in the LF quarter with strain B and subsequently challenged in this quarter with strain B. The LH quarter which was originally uninfected was also challenged with strain B. *S. aureus* was not isolated in any quarters from day 10 post-challenge although it was detected on days 3 (LF and LH quarters) and 8 post-challenge (LH only). Failure to isolate *S. aureus* from this cow from day 10 post-challenge onwards was probably the result of systemic antibiotic therapy with amoxycillin/clavulanic acid (Synulox) which was administered on day 9 post-challenge. Failure to isolate the bacteria following therapy probably indicates that the antibiotic was either effective in killing the *S. aureus* in the mammary gland or reduced the shedding of the organism into the milk for the period of the study.

Restriction enzyme fragmentation pattern analysis was used to determine strain variation of *S. aureus* genotypically. REFP was shown to discriminate between both similar and unrelated strains of *S. aureus* in this study. REFP allowed discrimination of two similar strains of *S. aureus*, strain A and strain A* in the cows. Strain A and strain A* had almost identical REFP patterns which differed by a single restriction fragment and were clearly clonal variants. The largest fragment of strain A* was slightly smaller (9.32 kilobases) than strain A (9.59 kilobases). The two main strains involved in this study, strain A and strain B, were unrelated. Following digestion of strain A, 25 fragments ranging from 2.92-9.59 kilobases in size were identified. Strain B had 30 fragments ranging in size from 2.92-9.59 kilobases. Seven additional unrelated strains were identified following REFP and designated strains R_1 to R_7. These additional strains ranged in fragment number from 20-36 with fragment sizes ranging from 2.86-15.88 kilobases. These findings were similar to those of Jayarao *et al.* (1991) who used REFP analysis to identify and differentiate closely related and unrelated strains of *S. uberis* isolated from bovine mammary secretions. Jayarao *et al.* (1991) analysed 42 strains of *S. uberis* from 17 cows throughout lactation and

identified 17 REF patterns. Analysis of these isolates by bacteriocin-like inhibitory substance (BLIS) patterns resulted in only 12 patterns being identified (Jayarao *et al.*, 1991). Hill and Leigh (1989) also used DNA fingerprinting to analyse *S. uberis* isolates and concluded that DNA fingerprinting was a simple and reproducible typing

system based on the restriction fragment size of chromosomal DNA. They reported that the endonuclease *Hind* III was the most successful in the differentiation of *S. uberis* isolates. Matthews *et al.* (1992) used DNA REFP to analyse 51 staphylococcal isolates from the mammary secretions of cows with subclinical mastitis and identified 7 *S. aureus* isolates with 2 distinct REF patterns. Isolates were evaluated using a computer integrated scanning laser densitometer which allowed the determination of fragment sizes. Differences in REF patterns were identified as variation in the number and size of fragments, with each REFP designated as a distinct pattern. Visual comparison of REFP photographs was also undertaken to verify results obtained by densitometric scanning. In the present study, strain A* was shown to be 96% similar to strain A after digitisation and 24 of the 25 fragments matched. Strain A and strain B showed 83.6% similarity and 23 of the 30 fragments matched. Matthews *et al.* (1992), Hill and Leigh (1989) and Jarayao *et al.* (1991) all used densitometric scans and graphic displays of restriction patterns in the analysis of results. Forty-two REF patterns were compared to each other by Jarayao *et al.* (1991) and this resulted in the identification of related and unrelated strains. These methods allowed comparison of strains by fragment size and fragment numbers, distinguishing similar and unrelated strains on this basis. The accurate comparison of different strains over a period of time could also be accomplished using this method of data retrieval and storage.

In this study, a cow with two almost identical strains of *S. aureus*, which differed by only one fragment following REFP analysis was recognised. Cow 186 was thought to be naturally infected with strain A in all four quarters on initial screening. On the basis of REFP analysis, the RH quarter was shown to be naturally infected with strain

A*. It seems likely that the strain A* was derived originally from strain A. Multiple colony analysis in this project showed that, on several occasions, more than one strain of *S. aureus* was isolated from a single quarter. Both related and unrelated strains of *S. aureus* were isolated from the same quarter on the same sampling day in an individual animal. One example of the isolation of two closely related strains from the

same quarter on the same sampling day was the identification of strain A and strain A* in the RH quarter of cow 186 on day 17 post-challenge. The identification of unrelated strains of *S. aureus* from the same quarter on the same sampling day occurred in other cows in the study. The RF quarter of cow 520 on day 17 post-challenge was shown to contain a 'mixed' population of strain A and strain B, the two main strains in this study, following REFP analysis. Another example of the identification of two unrelated strains from the same quarter on the same sampling day was in the LF quarter of cow 125 on day 17 post-challenge when strain A and strain R_2 and in the RF quarter of the same cow on the same day post-challenge when strain A and R_5 were identified following REFP analysis. These findings can be compared to a study by Jayarao *et al.* (1991) which analysed the genotypic and phenotypic variation of *S. uberis* isolates from bovine mammary secretions. Jayarao *et al.* (1991) concluded that related strains of *S. uberis* were isolated in different quarters of the same gland on the same sample day, isolated in the same quarter of the same gland over a period of time and also that related strains were detected in different quarter of the same gland on the same day. Unrelated strains were identified by Jayarao *et al.* (1991) from different quarters of the same gland on the same day and also from the same quarter of the gland over a period of time. Therefore in the present study, multiple colony analysis by REFP resulted in a more accurate identification of *S. aureus* strain diversity within a quarter than single colony analysis by routine bacteriological methods. Multiple colony analysis is, therefore, a useful technique in studying bovine mastitis caused by *S. aureus*.

DNA fingerprinting is another technique used in studying the epidemiology of clinical and subclinical mastitis (Lipman *et al.*, 1995; 1996). By comparing isolates between and within herds, it is possible to draw conclusions about the source and spread of

bacteria. For example, a study by Lam *et al.* (1996) showed that different *E. coli* genotypes were identified from different quarters, suggesting that *E. coli* is an environmental pathogen and does not spread from quarter to quarter. In contrast, Lam *et al.* (1996) showed that a limited number of *S. aureus* genotypes (11) were identified among 63 *S. aureus* isolates from five herds, supporting the theory that contagious pathogens transfer between cows and quarters within a herd. *S. aureus*

strain identification using the DNA RAPD assay also showed that the majority of recurrent cases of *S. aureus* mastitis were the same genotype, suggesting either intermittent shedding from a chronically infected quarter or repeated infection with the same pathogen (Lam *et al.*, 1996). Results from the present study, indicate the existence of several strains of *S. aureus* identified from the four cows studied. REFP analysis identified eight additional strains other than the two main strains in the study, strain A and strain B, and demonstrated that mixed infection occurs and that the investigation of a single colony may not provide a complete understanding of infection patterns.

One disadvantage of REFP analysis is the lengthy process, extending to 2-3 days, required to obtain bacterial DNA of sufficient purity for subsequent digestion with restriction endonucleases. This isolation method is similar to the technique used by Matthews *et al.* (1992) who used restriction endonuclease fingerprinting of staphylococcal species of bovine origin. Other methods such as ribotyping performed by Aarestrup *et al.* (1995(c)) and MLEE by Kapur *et al.* (1995) which have been used in the identification of staphylococci are also lengthy processes. In this study REP-PCR was developed as a rapid method for the identification of strain diversity in the bovine *S. aureus*. REP-PCR was shown to clearly distinguish unrelated strains of *S. aureus* whereas closely related strains were not distinguished using REP-PCR. This technique was shown to discriminate between strain A, and strain B and also among strains R_1 - R_7. REP-PCR however, did not discriminate between strain A and strain A* and was therefore less sensitive than REFP.

Similar techniques have been employed to study variation in other mastitis pathogens. Jayarao *et al.* (1992) compared REF and DNA amplification fingerprinting (DAF) in the subtyping of *S. uberis* in cattle. REF grouped 22 strains into 12 distinct patterns whereas DAF grouped them into 15 distinct patterns, indicating that DAF is an effective typing method and more discriminatory then REF in the typing of *S. uberis* strains. Jayarao *et al.* (1991) suggested that possible variation between typing methods (REF and BLIS) could be due to the recent identification of *S. parauberis*

which is biochemically, physiologically and serologically identical to *S. uberis*, with only a slight genotypic variation differentiating the two strains.

Lam *et al.* (1996), Lipman *et al.* (1996) and Matthews *et al.* (1994) all used variations of the PCR to differentiate strains of *S. aureus* isolated from the mammary glands of cows. Lam *et al.* (1996) used ERIC primers for differentiation of *E. coli* and an arbitrary primer for *S. aureus* strain differentiation (RAPD assay). Lipman *et al.* (1996) used RAPD, ERIC1R and ERIC primers in the differentiation of *S. aureus* isolates, identifying and subtyping 71 *S. aureus* strains. The different primer sets used by Lipman *et al.* (1996) gave no variation in the ability to differentiate between strains under the conditions specified. PCR was shown to be a preferable method to biotyping as it is a much more specific typing method van Belkum *et al.* (1993), Lipman *et al.* (1995) and Wang *et al.* (1993). Matthews *et al.* (1994) used a synthetic oligonucleotide primer which provided a high degree of resolution in the differentiation of *S. aureus* strains with seventy-five *S. aureus* isolates grouped into 19 distinct profiles.

In the present study, restriction endonuclease digestion of REP-PCR amplification products was carried out to try to differentiate between strain A and strain A* which were not discriminated by REP-PCR alone. Of a number of restriction endonucleases tried, only *Stu* I gave a variation in amplification products between strain A and strain A* but this approach in conjunction with standard REP-PCR for the other strains enabled discrimination of all strains identified from the cows in this project.

The double-crossover design of intramammary infusion with *S. aureus* allowed study of the effects of challenge of an infected quarter with the indigenous or non-indigenous strain of *S. aureus.* Two cows were challenged with the indigenous strain of *Staphylococcus aureus.*

Cow 125 was naturally infected with strain A (LF, LH) and subsequently challenged with the indigenous strain A in the LF, and LH quarters. The intramammary infection persisted throughout the study in quarters which were previously subclinically infected

or uninfected. This implied that challenge with a high dose of the indigenous strain did not restimulate the immune system which might have resulted in subsequent clearance of the infection within the quarter. The identification of additional isolates both pre-challenge (LH) and post-challenge (day 17, LF: R_2; day 17, RF: R_5) indicates the presence of more than one strain within an individual quarter. However, in this cow, the predominant strain isolated post-challenge remained strain A.

Cow 703 was naturally infected with strain B (LF) and subsequently challenged with the indigenous strain in the LF and LH quarters. Strain B was isolated post-challenge from the LH quarter (days 3 and 8) and LF quarter (day 3), however, by day 8 post-challenge, additional isolates R_3, R_4, R_6 and R_7 were identified in the LF quarter. Failure to isolate strain B on day 8 post-challenge may be have resulted from an enhanced immune response to the original bacterial strain B eliminating or reducing the numbers of this strain, thus allowing different strains to become the predominant isolates from this quarter.

Two other cows were challenged with the non-indigenous strain of *S. aureus*. Cow 186, was naturally infected with strain A in three of the four quarters and strain A* in one quarter and was challenged with the non-indigenous strain B in all four quarters. In the quarter with the original infection caused by strain A*, by day 17 post-challenge a mixed population of strain A, strain A* and strain R_1 was detected. Strain B was not isolated at any point post-challenge throughout the study.

Cow 520, which was naturally infected with strain B and subsequently challenged with the non-indigenous strain A, had the non-indigenous strain isolated as a homogenous population on day 3 post-challenge when all isolates tested were shown to be strain A. This suggests a transient period of infection by the challenge strain A. By day 8 post-challenge, only the indigenous strain was identified in all isolates and this was also the case on day 17 post-challenge. The presence of strain B may be a result of cross-contamination of strains between cows or quarters. The cows were tethered separately to reduce environmental spread and the milking machine clusters

were disinfected between cows. It is possible that transfer from the RH quarter to the RF quarter may have occurred during milking in this cow.

This part of the study showed that when infected with the indigenous strain (either strain A or strain B) and subsequently challenged with the non-indigenous strain, the indigenous strain persisted as the dominant strain within the quarter and was the only strain identified on day 17 post-challenge in most quarters in both cases.

Chapter 3

3 Inflammation in *Staphylococcus aureus* Mastitis

3.1 Introduction

One of the main aims of this part of the project was to develop a monoclonal antibody raised specifically against bovine SAA. In the initial stages of the project, SAA concentration in bovine serum samples was assessed by ELISA using a polyclonal rabbit anti-human SAA antibody. In order to purify SAA from bovine serum an attempt was made to identify specific diseases of cattle which might be associated with increased level of this protein. The cattle referred to Glasgow University Veterinary School included those affected by a variety of infectious, inflammatory or neoplastic conditions. The aim was to measure serum haptoglobin levels which had been shown to correlate with SAA levels in diseased cattle and to screen sera from a number of these cases using the polyclonal rabbit anti-human SAA antibody. In order to prepare antigen for monoclonal antibody production, improved methods for isolation and purification of SAA had to be developed during this project.

3.2 Materials and Methods

3.2.1 Identification and Quantification of Acute Phase Proteins from Sera of Diseased Cattle

3.2.1.1 Determination of Haptoglobin Concentration

Haptoglobin is one of several proteins associated with the acute phase response and is used as an indicator of inflammation and infection. Increased haptoglobin levels were detectable in serum within hours of experimental infection of calves with *Pasteurella haemolytica* and were correlated with elevated SAA levels (Horadogoda, 1994). The haptoglobin assay was, therefore, used in this project to rapidly identify diseased cattle likely to have a high serum amyloid A concentration.

Haptoglobin Assay. The haptoglobin assay was a semi-automated method based on that of Conner *et al.* (1988). Stock haemoglobin solution was diluted in 0.9% saline to a standard 30mg/ml. All samples, standards and controls were diluted 1:75 in standard haemoglobin solution and left for 10 minutes at room temperature to allow the formation of the haptoglobin-haemoglobin complex. Two hundred microlitres of assay reagent (3,3',5,5'-tetramethylbenzidine (TMB) stock of 6mg/ml in DMSO diluted 1:100 in chromogen buffer, 0.5g disodium ethylene diamine tetra acetic acid (di-EDTA), 15.6g sodium dihydrogen orthophosphate ((NaH_2PO_4), pH3.8)) was mixed with 6µl of diluted sample in the reaction cuvette. Fifty microlitres of start reagent (0.12% w/v hydrogen peroxide) was added and the absorbance measured at 600nm every 25 seconds until the reaction end point was reached using a MIRA discrete biochemistry analyser (Roche Diagnostics, Welwyn Garden City, Herts). The assay was calibrated using a bovine acute phase serum standardised against purified haptoglobin.

3.2.1.2 Determination of Serum Amyloid A Concentration

To obtain sufficient quantities of serum amyloid A (SAA) to use as antigen in monoclonal antibody production, serum was collected from diseased cattle admitted to the University of Glasgow Veterinary School. Seventeen samples with SAA levels greater than 50mg/l were identified by ELISA using a rabbit anti-human serum amyloid A antibody (Calbiochem-Novabiochem (UK) Limited, Nottingham) (Table 3.1). High levels of SAA (>50mg/l) were measured in cattle with a wide variety of clinical diseases (Table 3.1). The conditions that the cattle were affected by included viral, bacterial and parasitic infections, tumours and metabolic disease.

The sera were pooled and high density lipoprotein (HDL) with which SAA is associated was isolated by ultracentrifugation thus removing the majority of serum proteins. The SAA was further purified by electro-elution of the high density lipoprotein fraction following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) while western blot analysis was used to confirm the isolation and purification of the SAA.

3.2.2 Isolation and Purification of Serum Amyloid A

3.2.2.1 Separation of Serum from Blood

Blood samples from diseased cattle described in Table 3.1 were obtained by venepuncture of the jugular vein. The sample was incubated at 37^0C for 30 minutes to improve clot retraction and then at 4^0C for a further 30 minutes. The sample was then filtered to remove the clot and centrifuged at 1900g at room temperature for 20 minutes. After centrifugation the serum fraction was carefully removed, heat inactivated at 56^0C for 30 minutes and stored at -20^0C.

Case number	Diagnosis	SAA (mg/l)
120942	Cellulitis	195.0
120898	Laryngeal abscess	52.6
120917	Mastitis and hepatitis	50.0
121121	Necrotising pneumonia	85.0
121194	Peritonitis and polyarthritis	92.3
121374	Intestinal carcinoma	68.1
121991	Meningitis and encephalitis	64.0
122058	Oral and interdigital ulceration	72.0
122105	Nephrosclerosis	77.5
122247	Hepatic lipidosis	76.1
122754	Lungworm	78.0
123302	Mucosal disease	82.0
123437	Mucosal disease	81.2
123589	Endocarditis	52.4
123669	Pyelonephritis	58.5
123745	Endocarditis and pulmonary thromboembolism	58.6
123811	Pulmonary thromboembolism/abomasal ulceration	72.3

Table 3.1: Serum amyloid A concentrations in diseased cattle.

3.2.2.2 Removal of Non-Lipoprotein Serum Proteins

To obtain serum amyloid A for use in the monoclonal antibody production, several purification stages were carried out initially to isolate the high density lipoprotein fraction and then the SAA from the other apolipoproteins.

To raise the non-protein density of the serum to 1.21g/ml, 10mls of serum was mixed with 3.56g of potassium bromide and 6.5ml aliquots were placed in thermoplastic ultracentrifuge tubes (Beckman Instruments UK Limited, High Wycombe, Buckinghamshire). The tubes were capped, ensuring that air bubbles were kept to a minimum, and centrifuged at 190,000g, at 4^0C for 48 hours in a Beckman L-70 Ultracentrifuge (fixed angle rotor type 50.4 Ti). Following centrifugation, the top 1ml fraction of the samples containing the HDL was carefully removed by pipette.

Potassium bromide was removed from the SAA fraction by dialysis. Dialysis tubing D-9277 (Sigma) was soaked in distilled water for at least 60 minutes before use. Fractions from the ultracentrifugation were dialysed against distilled water at 4^0C for 48 hours. Following dialysis, samples were removed from the tubing and placed in sterile universals for lyophilisation. Samples were frozen horizontally to achieve the largest surface area possible and then freeze-dried overnight.

3.2.2.3 Determination of Total Protein Concentration of Serum

The total protein assay based on bicinchoninic acid reagent (Sigma) was used for the determination of total serum protein and also for quantification of SAA after purification. Bovine serum albumin (BSA, 2mg/ml in 0.15M NaCl with 0.05% sodium azide) was used as a standard at a range of concentrations from 0-2mg/ml. Dilutions were performed in distilled water. Ten microlitres of the standard and

the samples and 200µl of assay reagent (1 part copper (II) sulphate (pentahydrate 4% (w/v) solution) mixed with 50 parts bicinchoninic acid solution) were incubated in a 96-well mictotitre plate (Microtitre plates, Greiner) at 37^{0}C for 30 minutes. Following incubation the absorbance was read at 562nm using a MR5000 ELISA reader (Dynatech) and protein concentration calculated from the BSA calibration curve.

3.2.2.4 Separation of Serum Amyloid A from High Density Lipoproteins

Further purification of SAA was required for monoclonal antibody production. Following ultracentrifugation, dialysis and lyophilisation the semi-purified SAA was still associated with HDL. Serum Amyloid A was separated from other HDL apolipoproteins by SDS-PAGE followed by electro-elution in order to increase the immunogenicity of the SAA, as fewer antigenic determinants of the SAA are exposed in the native state (Godenir *et al.*, 1985; Sipe *et al.*, 1989).

SDS-PAGE gel and samples were prepared as described previously in the Large Gel System. Electro-elution was performed using the Bio-Rad Model 422 Electro-eluter. Prior to electro-elution, membrane caps were placed in elution buffer (25mM Tris-base, 192mM glycine and 0.1% SDS) at 60^{0}C for at least 60 minutes. After heating, membrane caps were stored in elution buffer, which contained 0.05% sodium azide as a preservative, at 4^{0}C. After electrophoresis, the gel was washed three times in deionised water and then stained for 10 minutes using 0.1% coomassie brilliant blue in 25% methanol, 10% acetic acid and 1% glycerol. The stained gel was washed in distilled/deionised water for two hours, or until the protein bands became clearly visible. After washing the SAA band was identified on the basis of molecular weight and a narrow strip of gel incorporating the band was removed. The gel segment was then cut into 5mm x 5mm squares and stored in elution buffer at 4^{0}C until electro-elution. The gel squares were placed in the glass chambers within the electro-eluter module and eluted at 8-10mA per chamber in protein elution buffer for approximately five hours on ice, after which the sample was dislodged from the membrane caps by

rinsing with 200µl of fresh elution buffer and stored a 4^0C until the concentration stage. Alternatively, a narrow strip of stained gel was used as a template for locating the SAA region from the unstained portion of a gel and samples were electro-eluted as before.

3.2.2.5 Concentration of Serum Amyloid A

Concentration of the eluate from electro-elution was carried out using Centricon 3 Microconcentrators (Amicon Limited, Stonehouse, Gloucestershire) and stored at -20^0C. Eluate was tested using SDS-PAGE gel electrophoresis to determine purity and the protein concentration was assessed using the BCA protein assay.

3.2.3 Identification of Serum Amyloid A

3.2.3.1 Enzyme Linked Immunosorbent Assay

To determine the SAA concentration in serum samples an ELISA was used (Horadogoda *et al.*, 1994). The standard used for this assay was a bovine serum sample which had previously been quantified for SAA and haptoglobin levels. The standard contained 200µg/ml of SAA and was diluted with fetal calf serum (FCS) to give a range of SAA concentrations between 0-200µg/ml to establish a calibration curve.

Standards, test samples and a control sample which was a 'normal' serum sample containing negligible SAA were all diluted 1:400 in coating buffer (10mM sodium bicarbonate ($NaHCO_3$)/sodium carbonate (Na_2 CO_3), pH 9.6). One hundred microlitres was dispensed in duplicate into wells of a 96 well flat bottom microtitre plate (Greiner Labortechnik Limited, Stonehouse, Gloucestershire). The microtitre plate was incubated at room temperature (20-22^0C) for eighteen hours to allow the samples to bind to the plate. The fluid was then decanted and the plates rinsed three times with phosphate buffered saline-Tween 20 (PBS-T,

20mM NaH_2PO_4, 20 mM disodium hydrogen monophosphate (Na_2PO_4), 154mM sodium chloride (NaCl) and 0.05% (v/v) Tween-20), at pH 7.4.

To reduce non specific protein binding, 300µl of 10% (w/v) dried skimmed milk in PBS-T was added to each well and incubated at room temperature for sixty minutes. Following incubation each well was washed three times with PBS-T.

Rabbit anti-human serum amyloid A (Calbiochem-Novabiochem (UK) Limited, Nottingham) was prepared at a 1:10,000 dilution in PBS-T and 100µl was added to each well. The plate was then incubated at room temperature for ninety minutes, and washed three times in PBS-T to ensure complete removal of any excess primary antibody solution.

Horseradish peroxidase-conjugated donkey anti-rabbit IgG (Scottish Antibody Production Unit, Law Hospital, Carluke) was prepared at a 1:2500 dilution in PBS-T and 100µl was added to each well, incubated at room temperature for ninety minutes, and washed thoroughly three times in PBS-T.

Peroxidase substrate (0.01M acetate buffer, (pH5.5), 0.04% hydrogen peroxide, and 0.1% TMB in dimethyl sulfoxide) was freshly prepared and 150µl was added to each well and incubated at 37^0C for 15 minutes. The substrate reaction was terminated by the addition of 50µl of 2M sulphuric acid to each well.

The absorbance was measured at 450nm using a plate reader (Dynatech MR5000, Dynatech Laboratories Limited, Billinghurst, West Sussex) and converted to SAA concentration by the immuno assay software.

3.2.3.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.

Serum amyloid A was identified by molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) stained with coomassie brilliant blue. The identification of SAA was confirmed by immuno blot analysis using antibodies specific for human SAA previously shown to cross react with bovine SAA (Horadogoda, 1994).

Mini-Gel System. Standards, controls and test samples were diluted 1:20 in distilled/deionised water. The wide range molecular weight marker (M.W. 6,500-205,000, Sigma-Aldrich Company Limited, Poole, Dorset) and samples were mixed 1:1 with treatment buffer (0.125M Tris-Cl, (pH 6.8), 4% sodium dodecyl sulphate (SDS), 20% glycerol and 10% 2-mercaptoethanol) and boiled at 100^0C for 2-3 minutes to denature the protein structure by reduction of disulphide bonds. Samples were then centrifuged at 13800g using a Eppendorf 5415 centrifuge (Eppendorf limited, Hamburg) for 30 seconds.

SDS-PAGE resolving gel solution (0.375M Tris-HCl, (pH8.8) 0.1% SDS; 0.1% (w/v) ammonium persulphate (APS); 0.07% N, N, N', N'-tetremethyl-ethylenediamine (TEMED) and 15% bis acrylamide) was prepared and added to the gel apparatus (8.0cm x 7.3cm x 0.75cm, Biorad Minigel System, Biorad Laboratories Limited, Hemel Hempstead) adding a barrier layer of 70% propanol to aid the formation of the gel matrix. When the resolving gel had solidified at room temperature, the gel was washed with distilled water to remove the propanol. The stacking gel (0.125M Tris-HCl, (pH6.8) 0.1% SDS; 0.05% (w/v) APS, 0.05% TEMED, 4% bis acrylamide) was added, inserting the combs carefully. The combs were removed, gel apparatus was placed in the gel tank (Biorad - Mini Protean II cell) and the central well filled with tank buffer (0.025M Tris, (pH8.3), 0.192M glycine and 0.1% SDS). Tank buffer was also added into the buffer tank. Fifteen microlitres of molecular weight marker and 20µl of each sample was loaded into each gel and electrophoresed at 200V for 60 minutes.

After electrophoresis, one gel was removed and placed in coomassie stain (0.125% coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid) for 5-10 minutes, followed by destaining in destain (5% (v/v) methanol, 7% (v/v) acetic acid) for 20 minutes, while a duplicate gel was processed by immunoblotting.

Large Gel System. The method used was similar to the mini-gel system except that the gel dimensions used were 16cm x 20cm x 1.5mm and larger sample volumes (60µl per well) were loaded. The gel was electrophoresed for three hours at 200 Volts.

3.2.3.3 Immuno Blotting

The duplicate SDS-PAGE gel (unstained) was used for the Immuno Blotting technique. Nitrocellulose membrane (Transblot Transfer medium, 0.45µm, Biorad) (1 piece) and filter paper (1 piece) were prepared before use by soaking in transfer buffer (25mM tris, 192mM glycine and 20% (v/v) methanol).

The SDS-PAGE gel was placed between the filter paper and nitrocellulose membrane in the transfer plates. The plates were covered with transfer buffer and electrophoresed at 100V for 30 minutes. After transfer, the nitrocellulose membrane was blocked overnight with 5% (w/v) dried skimmed milk solution in Tris buffered saline-Tween 20 (TBS-T, 10mM Tris-Cl (pH8.2), 150mM NaCl and 0.5% Tween 20) on a rotatest shaker (Luckham R100, Luckham Limited, Sussex) at 4^0C. After blocking, the nitrocellulose membrane was rinsed three times in approximately 50mls of TBS-T. Fifty millilitres of antibody solution (rabbit anti-human SAA, Calbiochem) was added at 1:1000 in TBS-T, and incubated on a rotatest shaker for 60 minutes at room temperature. The antibody solution was then removed and the nitrocellulose membrane was rinsed three times with TBS-T. To ensure complete removal of excess antibody solution a further two, 10 minute, washes in 50mls of TBS-T were carried out. Fifty millilitres of secondary

antibody (donkey-anti-rabbit IgG labelled with horseradish peroxidase) was added at 1:1000 in TBS-T and incubated for 60 minutes on the shaker. The nitrocellulose membrane was then rinsed and washed in TBS-T as above. Amino ethyl carbazole (AEC) substrate (20mg AEC dissolved in 2.5ml dimethyl formamide and added to 0.05M acetate buffer (pH5) to give a final volume of 50ml, plus 100µl of 30% hydrogen peroxide solution) was freshly prepared and poured onto the blot and incubated for 5-15 minutes. The blot was then washed in distilled water to terminate the reaction and dried.

3.2.3.4 Immuno Dot-Blot

The immuno-dot blot was used as a rapid, semi-quantitative method for identifying samples with elevated SAA. Ten microlitre aliquots of the samples and the standards were added in duplicate to nitrocellulose membrane and allowed to air dry at room temperature. The nitrocellulose membrane was blocked with 5% (w/v) dried skimmed milk in TBS-T for two hours on a rotatest shaker at room temperature. Following incubation the method was as described previously for the immunoblotting technique (after the blocking procedure stage).

3.2.4 Identification and Quantification of Acute Phase Proteins in Milk Samples from Mastitic Cows

3.2.4.1 Determination of Haptoglobin Concentration

To determine if acute phase proteins could be detected in milk, the concentration of haptoglobin was determined using a modification of the semi-automated haptoglobin assay. Stock solutions were used at concentrations described as for detection of haptoglobin from bovine sera unless otherwise stated. The haptoglobin standard (bovine acute phase serum of known haptoglobin concentration) was diluted in normal serum (negligible haptoglobin) to give a range of concentrations from 0.3mg/ml to 3.0mg/ml. Twenty microlitres of the

standards, controls and samples were then placed in test tubes, 100µl of standard haemoglobin solution was added and the samples were mixed thoroughly and incubated at room temperature for 10 minutes. Five millilitres of 0.9% saline was added to the tubes, mixed, and then 20µl of the sample was pipetted in duplicate into a 96-well microtitre plate (Greiner). Two hundred microlitres of chromogen solution was added and the plate incubated at 37^{0}C for 60 minutes. Fifty microlitres of substrate was added and the samples were then incubated at room temperature until adequate colour development had occurred in the standard samples. The reaction was terminated by adding 50µl of 20% sulphuric acid and the absorbance was read at 450nm using an MR5000 plate reader (Dynatech). The haptoglobin concentrations were calculated by comparison to the standard haptoglobin solution.

3.2.4.2 Determination of Serum Amyloid A Concentration

SAA concentrations in milk samples were determined by ELISA as described previously for SAA in bovine sera. The concentrations of samples, primary antibody and secondary antibody were varied to determine the optimum assay conditions. Due to the original antibody being unavailable due to an alteration in the manufacturing procedures, other possible antibodies had to be assessed to attempt to and find a suitable replacement for detecting bovine SAA. The alternative antibodies were assessed initially with a serum standard with a known SAA concentration by ELISA, SDS-PAGE and immunoblotting, using the method described for measurement of SAA in sera. Primary antibody concentrations varying from 1:1000 to 1:20,000 were incubated with samples diluted in coating buffer from 1:100 to 1:1,200 to try to optimise the assay. The antibodies assessed by ELISA, SDS-PAGE and immunoblotting were: rabbit anti-human serum amyloid A component, non-precipitating, (Calbiochem) and a rat monoclonal anti human SAA, (2^0 antibody-rat anti IgG HRP labelled, (Biosource International, Serotec, Kidlington, Oxford). A rabbit anti-bovine AA and a rabbit anti-bovine SAA kindly provided by Professor Gruys, Utrecht University, The Netherlands were analysed by SDS-PAGE and immunoblotting.

3.2.5 Development of a Monoclonal Antibody to Serum Amyloid A

The techniques attempting to develop a monoclonal antibody to purified SAA were carried out by Rhone Poulenc Diagnostics Limited, West of Scotland Science Park, Maryhill Road, Glasgow. Collaboration with colleagues from Rhone Poulenc took place throughout the purification stages and the immunisation procedure for antibody production.

3.2.5.1 Immunisation of Mice with Bovine Serum Amyloid A

The initial immunisation procedure used purified SAA at a concentration of 180μg/ml in elution buffer (24mM tris, 0.19M glycine and 0.02% SDS). The SAA was obtained by the electro-elution method described previously where the SDS-PAGE gel was partially stained with coomassie to identify the SAA rich portion. This resulted in the coomassie stain binding to the SAA which remained even following elution.

A group of 3 BALB/c mice were given a subcutaneous injection of 100μl of immunogen which contained 50μg of antigen in an emulsion prepared with complete Freund's adjuvant. Twenty one days later, 100μl of immunogen containing 20μg of antigen in an emulsion of incomplete Freund's adjuvant was injected subcutaneously and repeated 14 days later. Approximately 7 days after the third subcutaneous immunisation a test bleed was carried out to determine if an antibody response had occurred. A sample of blood was removed from the tail vein and allowed to clot, serum was removed after centrifugation and the serum antibody titre tested by ELISA with SAA bound to the wells of the microtitre plates.

In monoclonal antibody production if a good antibody response occurs the animals are rested and then boosted by a intravenous injection of antigen in saline, with the removal of the spleen and blood occurring 4 days later, followed by

fusion of spleen cells. However if a poor antibody response occurs then re-immunisation with antigen is generally performed.

Due to the poor antibody response of the mice after the initial immunisation with SAA (coomassie bound) a second immunisation with purified SAA (184μg/ml in elution buffer) which had been isolated using the alternative electro-elution method, and therefore, free of the coomassie stain, was carried out.

3.2.5.2 Immunisation of Mice with Bovine Serum Amyloid A-Apoferritin Complex

SAA is a relatively small molecular weight protein (approximately 12.5kDa). A good immune response to the antigen may not occur due to poor antigenicity of a small protein. To improve the immune response of small immunogens a carrier protein may be attached which will increase the size of the immunogen and may provide the binding sites essential for an immune reaction. An additional immunisation technique used SAA bound to apoferritin, a carrier protein isolated from horse spleen, to try to improve the immune response of the animals to the SAA by increasing the molecular mass of the immunogen.

Purified SAA was desalted and concentrated using centricon 3 microconcentrators (Amicon Limited) with 50mM triethanolamine, 0.15M sodium chloride, 2mM EDTA, (pH 8.0) to a final volume of 0.3ml. Freshly prepared 2 iminothiolane, a protein modification reagent, was mixed with bovine SAA under nitrogen at room temperature for 45 minutes and desalted through a precalibrated PD10 column of G25 sephadex (Pharmacia Limited, Milton Keynes) with 0.083M sodium phosphate, 0.9M sodium chloride, 0.1M EDTA, (pH7.2) and the eluted peak of labelled SAA collected.

Two milligrams of the carrier protein apoferritin was mixed with 300μl of PBS/EDTA. Four-(N maleimidomethyl) cyclohexane carboxylic acid N-hydroxy succinimide ester (SMCC) was mixed with dimethylsulfoxide (DMSO) and

combined with the apoferritin solution at room temperature for 30 minutes and desalted again through a PD10 column and the purified peak of apoferritin collected. The desalted fractions of modified SAA and apoferritin were mixed and allowed to react overnight at 4^0C. All traces of EDTA were removed from the protein conjugate by centrifugation in centricon 3 microconcentrators and buffer exchange with PBS until a final volume of 1ml was obtained.

The immunisation procedure was similar to the initial protocol described above, with the exception that 4 BALB/c mice were immunised with bovine SAA-apoferritin complex. The first and second immunisations involved the subcutaneous injection of 100µl of immunogen containing 100µg of apoferritin and 20µg SAA prepared as an emulsion in Freund's adjuvant (1st immunisation - complete adjuvant, 2nd - incomplete adjuvant). A third subcutaneous injection containing 50µg of apoferritin and 10µg of SAA in Freund's incomplete adjuvant was carried out. A test bleed to determine the antibody titre was taken before the animals were boosted, with a 200µl intravenous injection containing 25µg of apoferritin and 5µg of SAA in saline.

3.2.5.3 Measurement of Antisera Response to Immunisation of Mice with Bovine Serum Amyloid A

An ELISA assay was developed to measure the antisera response to immunisation of mice with bovine serum amyloid A. Microtitre plates were coated with purified bovine SAA and the serum response measured from the immunised mice using non-immunised mouse serum as a control. The bound antibody was detected using an anti-mouse IgG-horseradish peroxidase labelled antibody (Sigma) with TMB as a substrate.

The immuno dot blot technique was used as a more sensitive method of determining the antibody response of the mice. The streptavidin/biotin system was used to determine if any significant response had occurred as this system is more sensitive than other detection procedures and can be used accurately for the

detection and localisation of antigens, glycoconjugates and nucleic acids. Dot blot analysis was carried out using 2µl sample volumes with serial dilutions of the purified standard SAA, high and low serum SAA standards and 1:250 dilutions of the mouse control serum and test samples. Anti-mouse IgG biotin labelled antibody (Sigma) was used followed by streptavidin labelled HRP. Bound peroxidase was detected after washing with the substrate 4 chloro, 1 naphthol.

3.3 Results

3.3.1 Identification and Quantification of Acute Phase Proteins from Sera of Diseased Cattle

A bovine serum sample of known SAA concentration was used as a standard allowing construction of a calibration curve and determination of the SAA concentration in the unknown serum samples using a polyclonal rabbit anti-human SAA antibody. Cattle affected by conditions which the farm animal clinicians at Glasgow Veterinary School considered to involve a significant inflammatory response failed to consistently show elevated levels of SAA in ELISA using the polyclonal rabbit anti-human SAA antibody. It was, therefore, not possible to select animals with high SAA on clinical criteria. Previous work by Horadogoda *et al.* (1994) had shown that SAA levels were related to haptoglobin levels in cattle, but that the SAA was raised in a number of samples where haptoglobin was normal. However, haptoglobin levels in these selected cases varied from normal (less than 0.1mg/ml) to increased (3.06mg/ml) indicating that haptoglobin levels were not consistently elevated in all animals with high SAA. Blood samples from all cattle cases were subsequently screened for SAA and cases were then selected on the basis of having a SAA concentration of > 50mg/l and these included a variety of infectious and inflammatory conditions (Table 3.2).

3.3.2 Determination of Serum Amyloid A Concentration

A calibration curve for bovine SAA measured by ELISA was constructed by dilution of an acute phase serum standard with a SAA concentration of 192μg/ml in FCS to give a range of concentrations from approximately 0-192μg/ml (Table 3.3; Figure 3.1).

3.3.2.1 Isolation and Purification of Serum Amyloid A

In order to obtain SAA for use in monoclonal antibody production, the SAA had to be purified, which involved a number of isolation stages and confirmation of the presence of the SAA after each purification step.

The initial purification of SAA from serum involved ultracentrifugation of the samples which separated the serum into density determined fractions containing very low density proteins, low density proteins, medium density proteins and high density proteins which were designated fractions 1-4 respectively. The BCA total protein assay gave typical results as shown in table 3.4 and in figure 3.2 and was used for determination of total protein content of fractions 1-4 (Table 3.5).

3.3.2.2 Identification of Serum Amyloid A

To assess the presence of SAA in serum, SDS-PAGE was used to identify the SAA, which is a 12.5 kilodalton protein (Betts *et* al., 1991) and can be identified by comparison with molecular weight (MW) standards which ranged from M.W. 6,500 to 205,000. The presence of SAA was also confirmed by comparison with serum samples known to contain negligible, or high levels of SAA, which were used as negative and positive controls respectively.

Following identification of SAA by molecular weight, the presence of SAA was confirmed by immunoblotting using the polyclonal rabbit anti-human SAA

Case number	Diagnosis	SAA (mg/l)	Hp (g/l)
120942	Cellulitis	195.0	1.4
120898	Laryngeal abscess	52.6	< 0.06
120917	Mastitis and hepatitis	50.0	<0.06
121121	Necrotising pneumonia	85.0	0.8
121194	Peritonitis and polyarthritis	92.3	< 0.06
121374	Intestinal carcinoma	68.1	0.06
121991	Meningitis and encephalitis	64.0	0.24
122058	Oral and interdigital ulceration	72.0	<0.06
122105	Nephrosclerosis	77.5	0.36
122247	Hepatic lipidosis	76.1	<0.06
122754	Lungworm	78.0	2.34
123302	Mucosal disease	82.0	1.68
123437	Mucosal disease	81.2	2.34
123589	Endocarditis	52.4	1.92
123669	Pyelonephritis	58.5	3.06
123745	Endocarditis and pulmonary thromboembolism	58.6	<0.06
123811	Pulmonary thromboembolism/abomasal ulceration	72.3	1.62

Table 3.2: Serum haptoglobin concentrations in diseased cattle with SAA levels >50mg/l referred to Glasgow University Veterinary School. SAA = serum amyloid A. Hp = haptoglobin, haptoglobin reference range < 0.3mg/ml for normal animals.

SAA Concentration (μg/ml)	Mean Absorbance (450nm)
192	0.951
96	0.691
48	0.536
19.2	0.402
9.6	0.230
0	0.047

Table 3.3: Standard SAA (concentration 192μg/ml) used for the construction of a calibration curve for bovine SAA measured by ELISA. The average absorbances of 10 curves at 450nm are shown. SAA = serum amyloid A.

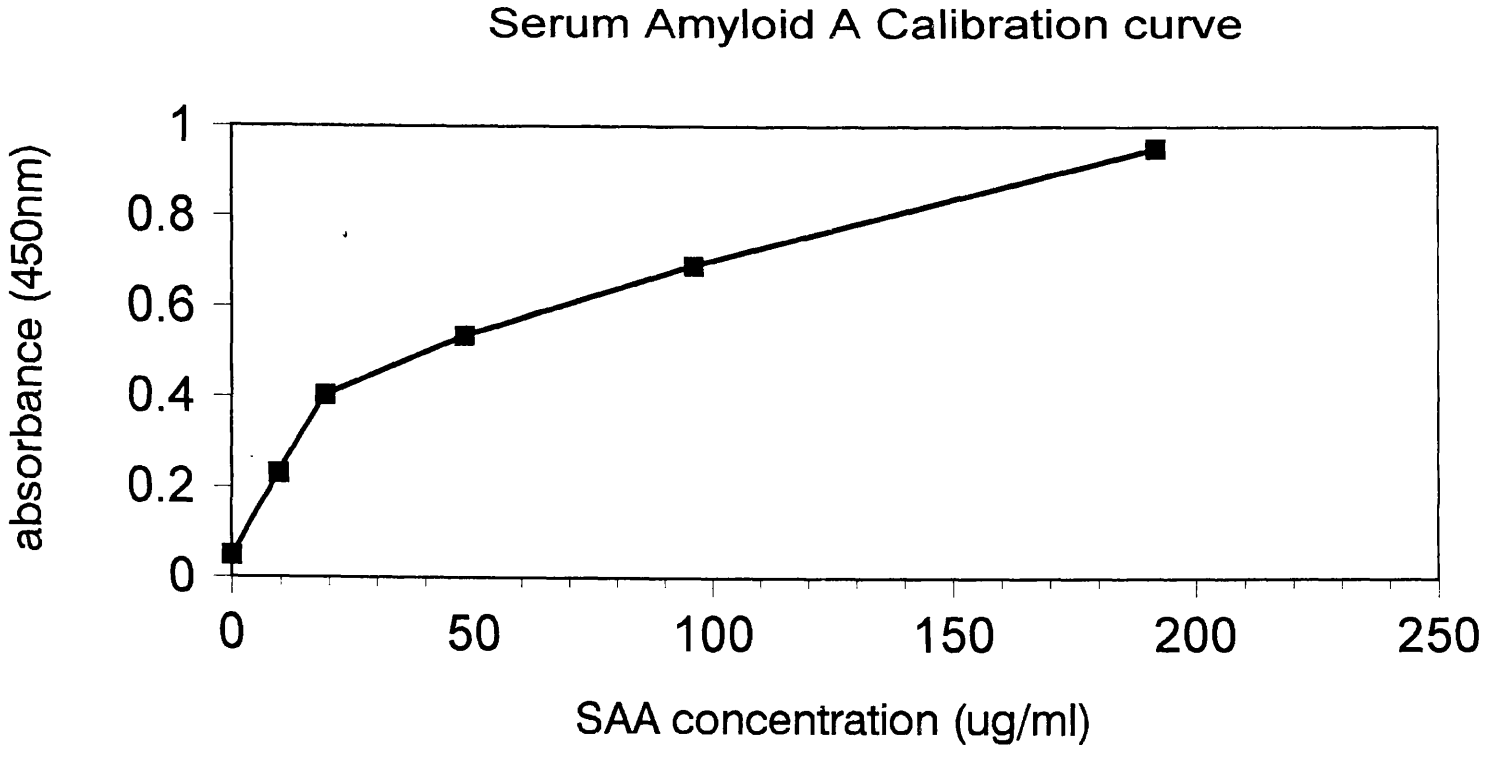

Figure 3.1: Calibration curve for Bovine SAA

BSA Concentration (mg/ml)	Mean Absorbance (562nm)
2.0mg/ml	0.997
1.5mg/ml	0.830
1.0mg/ml	0.606
0.5mg/ml	0.352
0.0	0.050

Table 3.4: Standard BSA (concentration 2.0mg/ml) used for the construction of a calibration curve for total protein measured by BCA total protein assay. The average absorbances of 10 curves at 562nm are shown. BCA = bicinchoninic acid

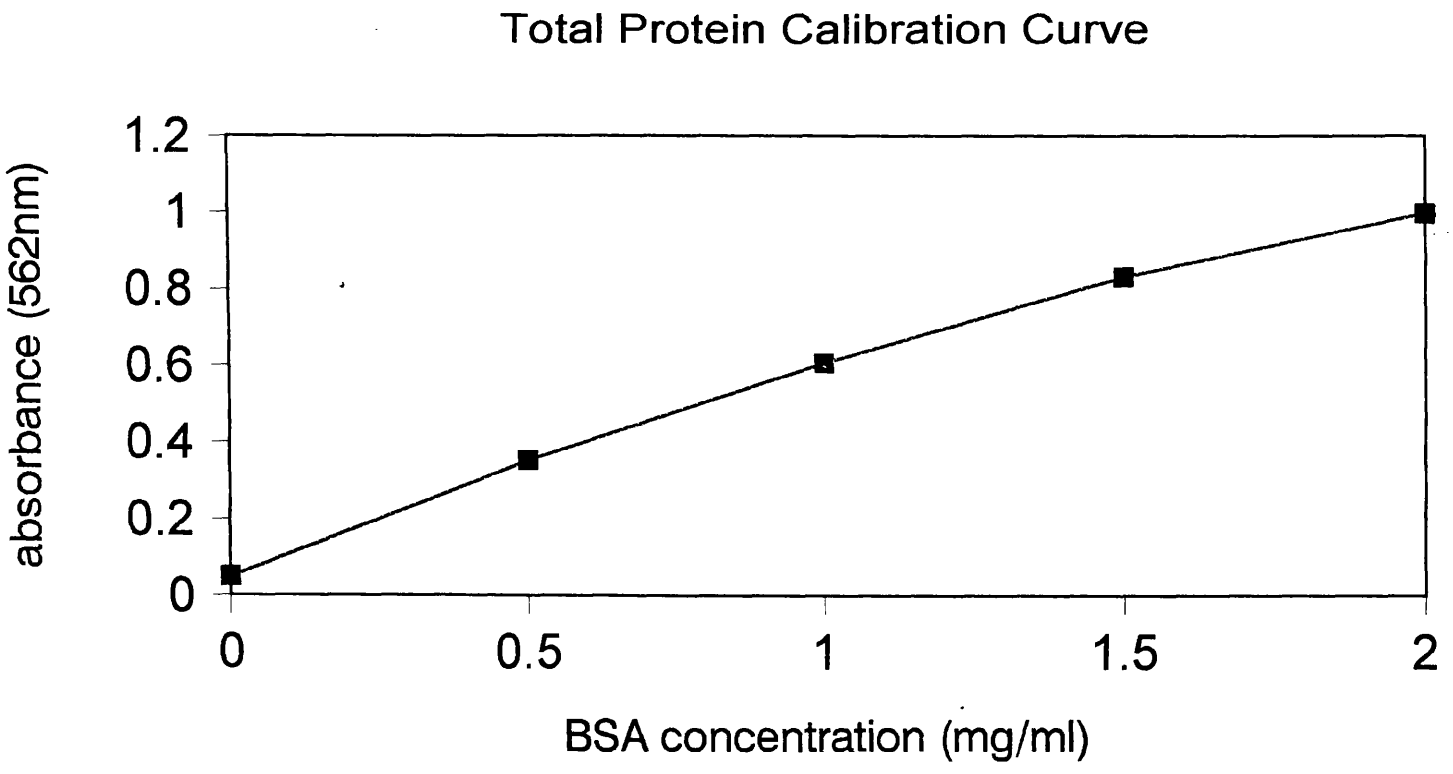

Figure 3.2: Calibration curve for BCA total protein assay

Sample	Dilution	Average Absorbance (562nm)	Total Protein Concentration (g/l)
Fraction 1	1:10	0.354	5.08
Fraction 2	UNDIL	0.690	1.17
Fraction 3	1:100	0.445	67.10
Fraction 4	1:1000	0.299	412.00

Table 3.5: The average absorbances for each fraction following ultracentrifugation are shown. Total protein concentration calculated from BCA total protein assay using BSA calibration data. BSA = bovine serum albumin. UNDIL = undiluted sample. BCA = bicinchoninic acid

antibody. Positive and negative control sera were also immunoblotted to confirm the specificity of the rabbit anti-human SAA for bovine SAA.

The SAA with a molecular weight of approximately 12,500 Daltons was identified in fraction 1 (high density lipoprotein fraction (HDL)) after SDS-PAGE (Figure 3.3) and immunoblotting (Figure 3.4). SAA was also identified in fraction 2 (medium density proteins) at a lower concentration. Fractions 3 and 4 (low and very low density proteins respectively) contained no SAA.

The SAA fraction was further purified by dialysis to remove any remaining K-Br and then freeze dried. The presence of SAA was again confirmed by SDS-PAGE (Figure 3.5) and immunoblotting (immunoblots not shown).

Electro-elution of the SAA containing fraction which had been semi-purified by ultracentrifugation, dialysis and lyophilisation was used to further purify the SAA. The electro-elution was carried out on three separate occasions to further purify three batches of semi-purified SAA. Following electro-elution the SAA was concentrated to approximately 200μg/ml, determined using the BCA total protein assay (Table 3.6). The purified SAA was then checked for purity by SDS-PAGE and immunoblotting where the band isolated showed positive staining as in Figure 3.4 (results not shown).

3.3.3 Development of a Monoclonal Antibody to Bovine Serum Amyloid A

3.3.3.1 Immunisation of Mice with Bovine Serum Amyloid A

The sera from mice immunised with SAA were analysed using an immuno dot blot and the streptavidin/biotin system. No colour development was detected in serum samples from mice immunised with the purified bovine SAA, at any of the dilutions of sera assessed.

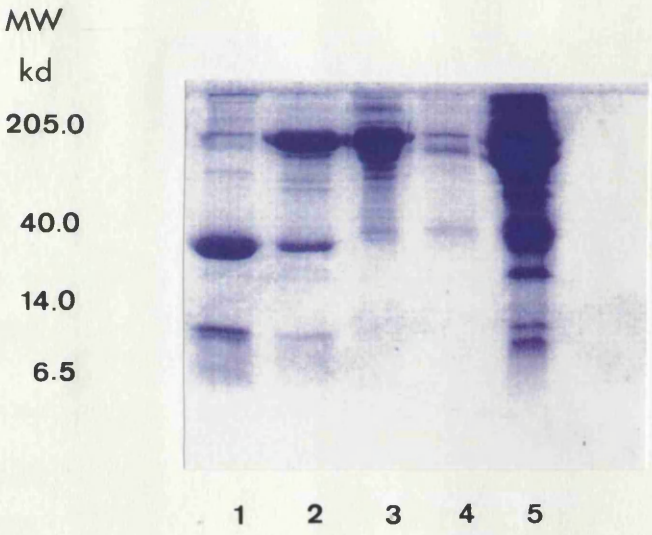

Figure 3.3: SDS-polyacrylamide gel (15%) electrophoresis of acute phase bovine serum fractions following ultracentrifugation. Lane 1 shows fraction 1 (HDL fraction containing SAA). Lane 2 shows fractions 2 (medium density proteins) which contains SAA at a lower concentration than fraction 1. Lanes 3 and 4 show fractions 3 and 4 respectively which contain no SAA. Lane 5 shows the standard serum which contained SAA at a concentration of 192μg/ml.

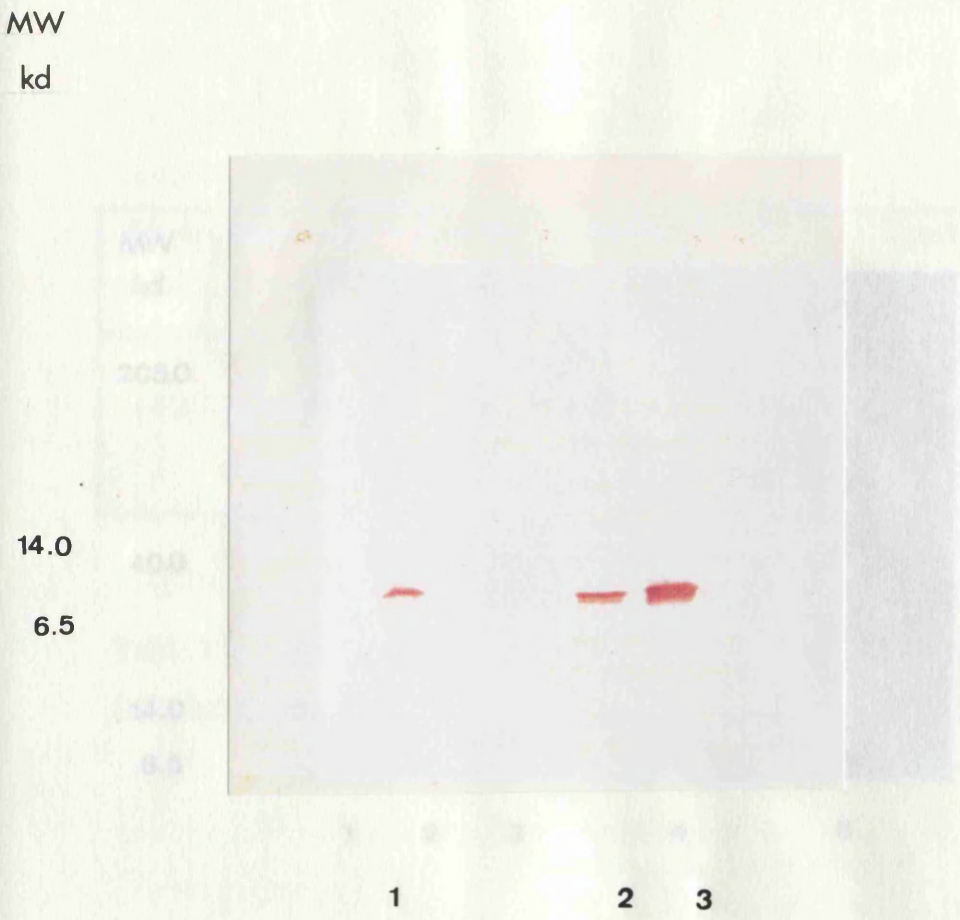

Figure 3.4: Immunoblot after SDS-PAGE of acute phase bovine serum fractions following ultracentrifugation. Polyclonal rabbit anti-human SAA was used as the primary antibody. Lane 1 shows the standard serum which contained SAA (concentraion 192μg/ml). Lane 2 shows fraction 2 (medium density protein fraction) which contains SAA. Lane 3 shows fraction 1 (HDL fraction) with the SAA region clearly identified by the immunoblot.

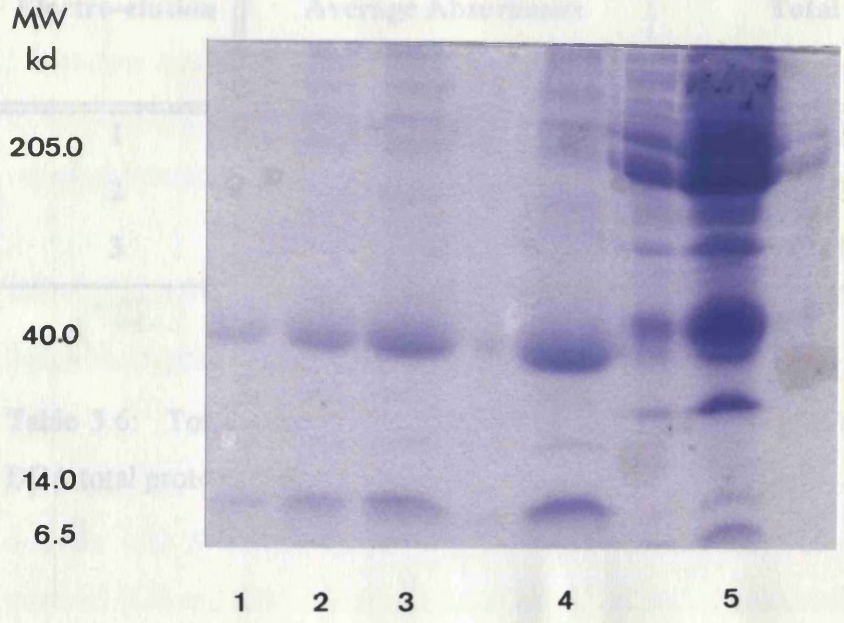

Figure 3.5: Coomassie stained SDS-polyacrylamide gel (15%) electrophoresis of the HDL portion of acute phase serum following ultracentrifugation, dialysis and lyophilisation at a range of dilutions. Lanes 1-4, shows the fraction 1 with SAA identified by comparison with the MW marker at dilutions of fraction 1 of 1:16, 1:8, 1:4 and 1:2 respectively. Lane 5 shows the standard SAA (concentration 192μg/ml).

Electro-elution	Average Absorbance (562nm)	Total Protein (µg/ml)
1	0.228	227
2	0.297	229
3	0.231	184

Table 3.6: Total protein concentration of electro-eluates 1, 2 and 3 using the BCA total protein assay.

3.3.3.2 Immunisation with Bovine Serum Amyloid A-Apoferritin Complex

The sera from mice immunised with serum SAA-apoferritin complex was analysed using ELISA. No colour development was detected in serum samples from mice immunised with the purified bovine SAA-apoferritin complex, at any of the dilutions of sera assessed.

These two approaches to produce a monoclonal antibody to bovine SAA were unsuccessful and it was not possible to attempt further immunisation regimes within the duration of the project.

3.3.4 Identification and Quantification of Acute Phase Proteins in Milk Samples From Mastitic Cows

Milk samples were taken from the four cows challenged by intramammary infusion with *S. aureus* (cows 125, 186, 520 and 703), but only from the hind quarters (LH and RH) on four separate dates, i.e. days 0 (pre-challenge), 4, 8 and 17 post-challenge.

3.3.4.1 Quantification of Total Protein and Haptoglobin

The skimmed milk fraction was analysed to determine the total protein concentration using the total protein assay described for serum. The same samples used for total protein measurements were also used to assess haptoglobin levels (Tables 3.7 - 3.10). Normal serum haptoglobin levels are <0.10g/l and a serum haptoglobin level of >0.40g/l is indicative of infection (Makimura and Suzuki, 1982; Skinner *et al.*, 1991). The haptoglobin assay detection limit was between 0.10g/l and 0.14g/l.

All quarters sampled in three of the four cows on all sample dates, had either very low concentrations of haptoglobin or levels of haptoglobin which were below the limit of detection of the assay (tables 3.7-3.9). A haptoglobin concentration of

0.50g/l was measured on day 8 post-challenge in the LH quarter of cow 703 which was non-infected and which had been challenged with strain B. The concentration of haptoglobin in this quarter was not elevated on day 4 and day 17 post-challenge (table 3.10).

3.3.4.2 Detection of Serum Amyloid A

Alternative antibodies were assessed for their ability to detect bovine SAA due to the unavailability of the original rabbit anti-human SAA from Calbiochem. The polyclonal rabbit anti-human serum amyloid A component (non-precipitating) (Calbiochem) and monoclonal rat anti-human SAA (Biosource International) antibodies were assessed by ELISA, SDS-PAGE and immunoblotting using serum standards with known concentrations of SAA. Both antibodies gave a disappointing result with the ELISA showing no significant increase in absorbance in the positive control serum compared to the negative control serum. High background also occurred and this was reduced by changing the blocking agent to 2% BSA in PBS-T, and diluting the primary antibody in 0.1% BSA in PBS-T. Following SDS-PAGE and immunoblotting, the monoclonal rat anti-human SAA gave a weak positive result for all the proteins in the serum sample and was not specific for bovine SAA. The polyclonal rabbit anti-human SAA component, (non-precipitating) antibody was also shown to be non-specific for bovine SAA. The monoclonal rabbit anti-bovine AA and the monoclonal rabbit anti-bovine SAA antibody (kindly donated by Professor Gruys, Utrecht, Netherlands) both gave very poor responses to bovine SAA on immunoblotting, however a weak but specific response to bovine SAA on immunoblotting was just detectable using the rabbit anti-bovine SAA.

Due to the lack of a reliable antibody for detection of bovine SAA, SDS-PAGE was performed to determine whether SAA was present in milk. As SAA has a characteristic MW of approximately 12,500 and can be observed in SDS-PAGE gels of acute phase serum, identification of a protein of identical size to SAA not present in normal milk, but detectable in milk after infection or inflammation could suggest the presence of SAA in milk. Initially, cow 703 was studied as she had

Cow Number	Sample day	LH Quarter Infected Strain A Challenged Strain A		RH Quarter Control	
		Haptoglobin (mg/ml)	Total protein (mg/ml)	**Haptoglobin (mg/ml)**	Total protein (mg/ml)
125	00	**< LD**	8.40	**0.16**	12.20
125	04	**< LD**	12.40	**< LD**	11.50
125	08	**0.20**	9.10	**0.17**	9.80
125	17	**< LD**	7.30	**0.15**	9.70

Table 3.7: Haptoglobin and total protein concentrations identified in mammary secretions from the hind quarters of cow 125 days 0 (pre-challenge), 4, 8 and 17 post-challenge. LD indicates limit of assay detection which is <0.10g/l. LH = left hind. RH = right hind.

Cow Number	Sample Day	LH Quarter Infected Strain A Challenged Strain B		RH Quarter Control	
		Haptoglobin (mg/ml)	Total protein (mg/ml)	**Haptoglobin (mg/ml)**	Total protein (mg/ml)
186	00	**0.12**	5.00	**0.15**	13.50
186	04	**0.15**	11.90	**0.18**	13.90
186	08	**0.18**	11.70	**0.15**	6.2
186	17	**0.19**	17.30	**0.21**	16.00

Table 3.8: Haptoglobin and total protein concentrations identified in mammary secretions from the hind quarters of cow 186, days 0 (pre-challenge), 4, 8 and 17 post-challenge. LH = left hind. RH = right hind.

Cow Number	Sample day	LH Quarter Control		RH Quarter Infected Strain B Challenged Strain A	
		Haptoglobin (mg/ml)	Total protein (mg/ml)	**Haptoglobin (mg/ml)**	Total protein (mg/ml)
520	0	**0.14**	14.00	**0.18**	12.70
520	4	**0.14**	11.70	**0.19**	14.20
520	8	**0.15**	14.50	**NS**	NS
520	17	**0.13**	13.70	**0.19**	20.60

Table 3.9: Haptoglobin and total protein concentrations in mammary secretions from the hind quarters of cow 520 days 0 (pre-challenge), 4, 8 and 17 post-challenge. LH = left hind. RH = right hind. NS = no sample.

Cow Number	Sample day	LH Quarter Challenged Strain B		RH Quarter Control	
		Haptoglobin (mg/ml)	Total protein (mg/ml)	**Haptoglobin (mg/ml)**	Total protein (mg/ml)
703	0	**NS**	NS	**0.16**	20.05
703	4	**0.22**	15.50	**0.22**	14.25
703	8	**0.50**	26.80	**NS**	NS
703	17	**0.17**	14.2	**0.13**	11.5

Table 3.10: Haptoglobin and total protein concentrations in mammary secretions from the hind quarters of cow 703 days 0 (pre-challenge), 4, 8 and 17 post-challenge. LH = left hind. RH = right hind. NS = no sample.

shown an increase in haptoglobin levels in the LH quarter on day 8 post-challenge. Samples from both the LH and RH quarters were analysed by SDS-PAGE from the four sampling dates i.e., days 0 (pre-challenge), 4, 8 and 17 post-challenge.

A protein of similar molecular weight to SAA was detected in the LH quarter of cow 703 on day 8 post-challenge and this protein was not present in any other sample days (Figure 3.6; lane 5). The 'suspected' SAA was identified by molecular weight and compared to the semi-purified SAA, confirming the presence of a protein of similar MW to SAA in milk samples. This approach was thought to be as accurate as possible in the absence of a specific antibody for use in confirming SAA by immunoblotting. To confirm that this additional protein band was probably SAA a negative control milk sample was mixed with a range of concentrations of SAA purified from serum. An additional band was observed at SAA concentrations of 120mg/l and 60mg/l which was at a similar position in the SDS-PAGE milk gel as the additional protein previously identified (figure 3.7). Figure 3.8 shows a negative control milk sample from the RH quarter on day 3 post-challenge and a milk sample from the LH quarter of cow 703 on day 8 post-challenge which contains a protein of similar molecular weight to SAA. The purified SAA and the milk sample which was mixed with the serum SAA all show a protein with similar molecular weight to SAA.

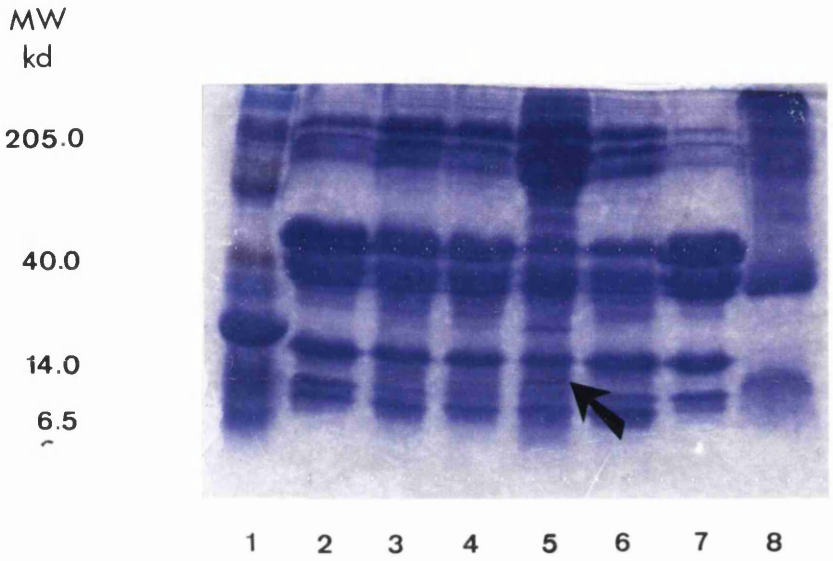

Figure 3.6: Coomassie stained SDS-polyacrylamide gel (15%) electrophoresis of milk samples from cow 703 on days 0, 4, 8 and 17 post-challenge from the LH and RH quarters. Semi-purified SAA was used as a control in the identification of similar molecular weight proteins. Lane 1 shows MW marker. Lane 2 shows RH quarter day 3 post-challenge. Lanes 3 and 4 show LH and RH quarters respectively from day 4 post-challenge. Lane 5 shows LH sample from day 8 post-challenge. Lane 6 and 7 show LH and RH samples from day 17 post-challenge. Lane 8 shows semi-purified SAA. ↑ indicates the 'suspected SAA'.

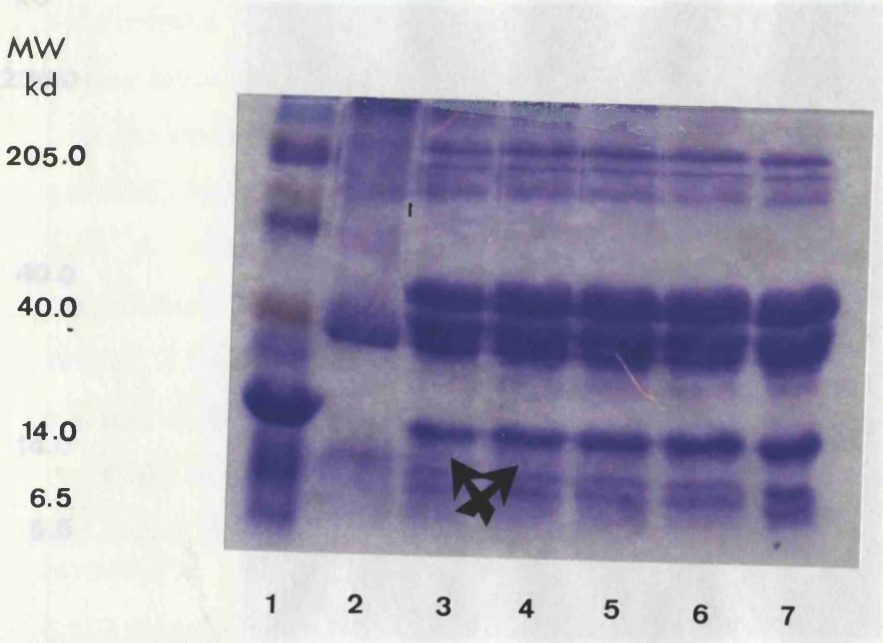

Figure 3.7: Coomassie stained SDS-polyacrylamide gel (15%) electrophoresis of a control milk sample 'spiked' with a range of concentrations of semi-purified SAA. Lane 1 shows MW marker. Lane 2 shows semi-purified SAA. Milk samples 'spiked' with SAA at concentrations of 120mg/l (lane 3) and 60mg/l (lane 4). Milk samples 'spiked' with SAA at concentrations of 30 (lane 5), 15 (lane 6) and 0mg/l (lane 7) respectively. ↑ indicates the 'suspected SAA'

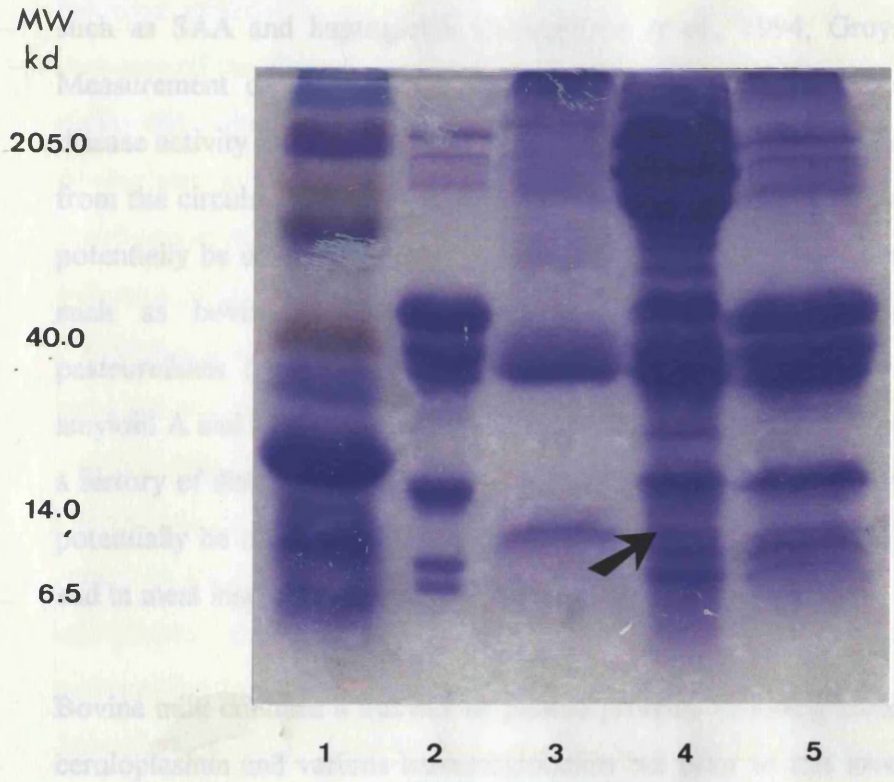

Figure 3.8: Coomassie stained SDS-polyacrylamide (15%) electrophoresis. Lane 1 shows MW marker. Lanes 2 shows a negative control milk sample (RH, day 3 post-challenge). Lane 3 shows semi-purified SAA. Lane 4 shows a suspected SAA milk sample (LH, cow 703, day 8 post-challenge). Lane 5 shows a 'spiked' milk sample with an SAA concentration of 120mg/l. ↑ indicates the 'suspected SAA'.

3.4 Discussion

Identification of the presence of pathological lesions resulting from infectious or inflammatory disease in cattle has been shown to be possible by monitoring APP such as SAA and haptoglobin (Alsemgeest *et al.*, 1994; Gruys *et al.*, 1993). Measurement of APP, which remain elevated for hours or days, to quantify disease activity is more useful than measuring cytokines which are rapidly cleared from the circulation (Hol *et al.*, 1987). Assays for APP, particularly SAA, may potentially be used to provide aid in prognosis and early diagnosis of conditions such as bovine mastitis (Conner and Eckersall, 1986) and pneumonic pasteurellosis (Horadogoda *et al.*, 1993; Horadogoda *et al.*, 1994). Serum amyloid A and haptoglobin were found to be elevated in most animals which had a history of disease at point of slaughter (Gruys *et al.*, 1993) and may, therefore, potentially be useful in future monitoring of the health status of slaughter cattle and in meat inspection (Saini and Webert, 1991).

Bovine milk contains a number of plasma proteins including albumin, transferrin, ceruloplasmin and various immunoglobulins but prior to this investigation it was not known if any SAA or haptoglobin could be detected in mammary secretions. However, it is known that haptoglobin acts as an inflammatory marker in serum in *E. coli* mastitis which is an acute, usually self-limiting mastitis (Salonen *et al.*, 1996). A 52-fold increase in serum haptoglobin was measured after the first intramammary challenge with *E. coli* and the peak concentration occurred 48-120 hours after the challenge (Salonen *et al.*, 1996). Thus, an APR was induced by *E. coli* mastitis and it was of interest to establish if an APR could be identified in milk following *S. aureus* infection in the present study.

SAA binds bacterial products including lipopolysaccharides (Tobias *et al.*, 1982), harmful molecules and debris produced after tissue damage (Baumann and Gauldie, 1994). Lipopolysaccharides are produced by Gram negative mastitis organisms especially the coliforms (Lipman *et al.*, 1995). Following intradermal or intravenous administration of endotoxin the concentration of SAA increased

100-fold (Boosman *et al.*, 1990). Hirvonen *et al.* (1996) studied the APR in heifers with experimentally induced mastitis, by measurement of serum haptoglobin, acid soluble glycoproteins, plasma fibrinogen and alpha-1-proteinase inhibitor. They concluded that haptoglobin and acid soluble glycoproteins were the most effective in indicating the severity of infection and predicting the final outcome of the disease, whereas alpha-1-proteinase inhibitor activity was of low diagnostic value in this study. The measurement of alpha-1- antitrypsin and bovine serum albumin in milk as an indicator of mastitis was carried out by Sandholm *et al.* (1984), who concluded that alpha-1-antitrypsin was an effective indicator of mastitis. Recent studies at Glasgow Veterinary School indicate that the SAA is a more sensitive marker for inflammation than haptoglobin (Eckersall, personal communication). Therefore, a major aim of this study was to identify and quantify SAA in mastitic milk and a monoclonal antibody raised specifically against bovine SAA was desirable for the development of a standard detection assay, as alternative antisera had proved to be unreliable, and ultimately to be unavailable. In order to achieve this, bovine SAA had to be accurately identified and purified before being used as the antigen in monoclonal antibody production. Identification of bovine clinical cases with elevated SAA concentrations proved to be difficult, as cattle which were considered to be affected by clinically-recognisable inflammatory conditions did not have consistently high levels of SAA. The previously reported correlation between elevated haptoglobin and SAA concentrations reported by Horodagoda *et al.* (1994) was not consistently observed in the cases studied during this project. However, bovine SAA was identified from a proportion of randomly selected clinical cases including those with infectious and inflammatory conditions, using, a commercial polyclonal rabbit anti-human antibody. The specificity of reaction with this antibody was confirmed originally by staining of a low molecular weight band of approximately 12,500 Daltons, which was consistent with the SAA band identified in previous studies. Sera with high concentrations of SAA were pooled, and SAA was then purified by ultra-centrifugation, dialysis, lyophilisation, electrophoresis and electro-elution. The purification of SAA by these methods resulted in isolation of purified bovine SAA with a concentration of approximately 200μg/ml. Development of this

method during the project provided an alternative to the hydrophobic interaction chromatography and repeated gel filtration previously performed by Horadagoda *et al.* (1993) in the purification and quantitative measurement of bovine SAA Thus, the newly developed method did not require the high ionic strength guanidium chloride, used previously to desalt the SAA and to keep it solubilised. The gel filtration step had previously led to high loss of protein and was avoided in the current method. Hydrophobic interaction chromatography was also used as a purification method for human SAA by Raynes and McAdam (1988) with affinity chromatography used in the preparation of hamster SAA (Niewold and Tooten, 1991). Rossevatn *et al.* (1992) purified SAA by isolating the HDL fraction by ultracentrifugation followed by de-lipidation and gel filtration. Purification of proteins from polyacrylamide gels (Harlow and Lane, 1988) is a standard method in parasitic antigen separation (McKeand, 1992) and purification of proteins by electro-elution for subsequent use in antibody production has been successfully carried out by many authors including Kennedy *et al.* (1986) and Tomlinson *et al.* (1989). Antigens purified from polyacrylamide gels often induce good antibody responses even though some denaturation of the antigen occurs. Indeed, many molecules become more immunogenic following denaturation, as injecting denatured antigens is more likely to produce an antibody response against epitopes that are not found on the native antigen (Harlow and Lane, 1988).

In this study, the production of a monoclonal antibody to bovine SAA was unsuccessful. No response was observed in the sera from mice immunised with bovine SAA and analysed by immunodot-blot. Unfortunately, it was not possible to attempt further immunisation required for monoclonal antibody production during this short project. Further attempts at antibody production should examine alternative strategies, including the effect of varying dose of immunising antigen, the effect of the isolation procedure on the native state of the protein which may have rendered it non-immunogenic (Zola, 1995), and the strain of mouse used for monoclonal antibody production, as not all strains of mouse are equally responsive to a particular antigen (Mouton *et al.*, 1988). As SAA is a low molecular weight antigen (Rossevatn *et al.*, 1992) which may be too small to

induce immunity, an alternative strategy was employed to stimulate a response by binding SAA to a protein carrier, apoferritin (Harlow and Lane, 1988). However, immunisation with bovine SAA-apoferritin complex again produced no antibody response in the sera from mice when analysed by ELISA.

Serum amyloid A was detected initially in this study using a polyclonal rabbit anti-human SAA antibody. Only a very small volume of this antibody was available and it was not possible to obtain sufficient quantities of it from any source in order to complete the study. An exhaustive assessment of alternative antibodies, both monoclonal and polyclonal, failed to identify a suitable replacement antibody. The lack of a suitable antibody for the detection of bovine SAA emphasises the need for the production of a successful monoclonal antibody which could be used for the accurate detection of bovine SAA in future work.

One aspect of this part of the project was to try to identify and quantify the acute phase proteins haptoglobin and SAA in milk following intramammary challenge with *S. aureus*. Haptoglobin levels were measured in the skimmed milk fraction of samples taken from two quarters in all four cows. Our results showed an increased haptoglobin concentration in only one quarter in one cow. The increased haptoglobin was detected in the LH quarter which was originally uninfected and subsequently challenged with strain B on day 8 post-challenge. The haptoglobin concentration increased from 0.22g/l on day 3 post-challenge to 0.50g/l on day 8 post-challenge and then returned to a basal level of 0.17g/l on day 17 post-challenge. In the other quarter of this cow, which was originally uninfected and received no intramammary challenge, and in all quarters sampled from the other cows, haptoglobin levels remained within the normal range despite the presence of active infection. This suggests that haptoglobin may not be a useful marker of inflammation in mammary secretions following infection with *S. aureus*. This may be due to the chronicity of the original subclinical infection or perhaps the challenge dose failed to induce a detectable acute phase response when other inflammatory mediators may predominate in the gland. Another explanation as to why elevated haptoglobin were not detected may be the choice of days on which the samples were taken. An elevation in haptoglobin due to the

acute phase response may have occurred between two of the sampling dates which were at least five days apart and returned to base levels by the time the next samples were taken.

Due to the lack of a reliable antibody for the detection of bovine SAA, SDS-PAGE was carried out to determine if SAA was present in milk by estimating the molecular weight of the suspected SAA protein and by comparing it with the semi-purified SAA. Only cow 703 was studied following analysis of haptoglobin results, as she had been the only cow to show elevated levels of haptoglobin. A protein of similar molecular weight to SAA was detected in the LH quarter on day 8 post-challenge. Identification of SAA by molecular weight in milk was not straightforward as there are many other proteins in milk of similar molecular weights to SAA, whereas serum SAA can be clearly identified by SDS-PAGE as the majority of other serum proteins are considerably larger in size. This part of the work is incomplete as no antibody was available to confirm immunologically that this protein was indeed SAA at the point when the present project finished.

Chapter 4

General Discussion

In this study, the experimental approach was to infuse large numbers of *S. aureus* bacteria into quarters which were originally infected with subclinical mastitis caused by *S. aureus,* and also into quarters which were uninfected. Although the numbers of bacteria infused during this project were much greater than in previously published work, the *S. aureus* strains were derived originally from cows with subclinical *S. aureus* mastitis and were probably less virulent than many *S. aureus* strains. In three of the four cows studied, the resulting mastitis was acute, with obvious swelling and reddening of the udder, but the cows did not show signs of being sytemically ill. The fourth cow, however, became anorexic, developed a marked pyrexia, and was subsequently treated with antibiotics.

Two main strains of *S. aureus*, strain A and strain B, were identified by REFP and used in the challenge studies. Quarters which were naturally infected with *S. aureus* and then challenged by the intramammary route with *S. aureus,* remained persistently infected three weeks following challenge, irrespective of the challenge strain. Quarters which were uninfected at the beginning of the project and were subsequently challenged with either strain, also remained persistently infected throughout the study. These results confirm the chronicity of *S. aureus* intramammary infection which is well recognised in the field and in experimental studies.

The results showed that in the cow infected subclinically with strain A, intramammary challenge with the indigenous strain of *S. aureus* into the infected quarter failed to induce an anamnestic immune response which might have

instigated clearance of the original infection from the quarter. In the cow infected subclinically with strain B, intramammary challenge with the indigenous strain of *S. aureus* into the infected quarter resulted in the original strain B being replaced by a heterologous population of distinct strains R_3, R_6 and R_7 in that quarter. It is possible that a secondary immune response to the indigenous strain B resulted in clearance of the original stimulating bacteria thus allowing a different strain to multiply and establish infection in the quarter.

This study also suggests that intramammary challenge of subclinically infected quarters with the non-indigenous strain did not result in permanent replacement of the indigenous strain with the recently infused non-indigenous strain. In the cow infected with strain A and challenged with strain B, strain A was not detected at any point following intramammary challenge. In contrast, in the cow infected with strain B and challenged with strain A, strain A was detected on day 3 post challenge, although only transiently, and by day 8 the original strain B had again become the predominant strain in that quarter.

REFP analysis also identified eight *S. aureus* strains other than the two main strains involved in the study. One additional strain was designated strain A* and was found to be very similar to strain A, with a coefficient of variation of 96% and 24 out of 25 fragments matching on REFP analysis. Seven other additional strains R1-R7 were also identified in the same way, but they showed lower coefficients of variation and reduced numbers of matching fragments when compared to strain A or strain B. It may be postulated, therefore, that while strain A* was likely to be a clonal variant derived from strain A, the strains R_1-R_7 may be unrelated to strain A and strain B. The identification of these strains from individual quarters both pre- and post-challenge indicated that a cow, or even a quarter, may be infected simultaneously by more than one strain of *S. aureus*.

REFP analysis involved multiple colony analysis from each sample, which allowed the identification of the presence of more than one *S. aureus* strain in an individual quarter, and which would have failed to have been detected on routine single colony analysis. This study showed that the examination of multiple colonies per

milk sample identified the diversity of *S. aureus* strains present and maximised the benefit of bacterial strain identification as an epidemiological tool in mastitis investigation. However, REFP analysis is a time consuming method, involving a lengthy process to obtain sufficiently purified DNA. The REP-PCR technique was developed during this project to facilitate more rapid identification of bovine *S. aureus* strains. REP-PCR analysis was shown to be a successful rapid technique for the differentiation of all but one bovine *S. aureus* strain isolated in this study. This method did not allow differentiation of strain A* from strain A. In order to overcome this problem, digestion of the PCR product by a number of restriction endonucleases was carried out. Of the endonucleases tested, only *Stu* 1 was found to produce a variation in amplification products which enabled discrimination of strain A* from strain A.

Future developments of this part of the work should involve further validation of the REP-PCR with analysis of a wider range of *S. aureus* isolates. In this study, the DNA was purified for REP-PCR using the method for REFP which is a two-day process but which results in large quantities of 'purified' DNA. Another development of this technique would be to improve the method for isolating bacterial DNA, allowing a more rapid purification method to be incorporated into the REP-PCR.

The experimental challenge model developed during this project may be more suitable for future investigations of mammary gland infections caused by *S. aureus* strains associated with subclinical mastitis, than strains derived from clinical cases or from commonly used laboratory strains. Now that a safe, relatively repeatable, method for infusion of these strains of *S. aureus* has been developed, it is necessary for further work on intramammary challenge with different strains to be carried out in greater numbers of cows. This is important to establish if either changes in, or persistence of, certain *S. aureus* strains are the result of the strain employed, or are the result of immune responsiveness of the individual cow. It would be a useful avenue for further study to examine the competitive behaviour of strains A and A* *in vitro* and also following intramammary challenge. It is

possible that strain A* is more 'fit' than strain A in terms of persistence in the mammary gland perhaps by virtue of reduced antigenicity.

To investigate the acute phase response as an indicator of inflammation in mastitis, mammary secretions from the four cows with naturally occurring subclinical mastitis caused by *S. aureus* were collected prior to, and following, intramammary challenge with *S. aureus*. Serum amyloid A was isolated and purified from the serum of bovine clinical cases by ultracentrifugation, gel electrophoresis and electro-elution. Serum amyloid A was identified by enzyme linked immunosorbent assay and immunoblotting using a polyclonal rabbit anti-human SAA antibody. The purified SAA was used to immunise mice in an attempt to produce a monoclonal antibody against bovine SAA but this proved to be unsuccessful, even when the SAA was bound to a carrier protein. Increased levels of haptoglobin were identified in a single quarter of one cow. This quarter had been originally uninfected and then challenged by the intramammary route with strain B. In the absence of a polyclonal or monoclonal antibody to bovine serum amyloid A, SDS-PAGE was used to identify proteins in mammary secretions with a similar molecular weight to serum amyloid A. A protein of similar molecular weight to serum amyloid A was identified in the same quarter in which the increased haptoglobin levels were measured. These preliminary results suggest that haptoglobin is not a suitable candidate as a marker of the acute phase response in *S. aureus* mastitis as all but one of the infected quarters has normal levels of this acute phase protein. Further investigation of the levels of SAA in infected mammary secretions will require the development of suitable antibodies or detection systems.

One possible method which could be used in the future to produce a successful monoclonal antibody to detect bovine SAA could be to sequence and express bovine SAA. The gene encoding bovine SAA (Rossevatn *et al.*, 1992) would be amplified using PCR, with the products of amplification cloned using a standard cloning vector. Purified plasmid DNA samples could then be sequenced. The amino acid sequence for bovine SAA has already been determined (Rossevatn *et al.*, 1992) and once the DNA sequence has been accurately established, a gene

fusion system would be employed to obtain high level expression of the SAA. Recombinant SAA fusion proteins following purification, would be used for monoclonal antibody production and the development of specific immunological assays. Further work is required in order to assess the usefulness of SAA and other acute phase proteins in integrated quality control of meat and dairy products.

Glossary

1^0	primary
2^0	secondary
A*	variant strain
AA	amyloid A
AEC	amino ethyl carbazole
APP	acute phase protein (s)
APR	acute phase response
B cells	B lymphocytes
BCA	bicinchoninic acid
BHI	brain heart infusion
BSA	bovine serum albumin
BMSCC	bulk milk somatic cell count (s)
^0C	centigrade (celsius)
cm	centimetre
CRP	C-reactive protein
Da	Dalton
dATP	deoxyadenosinetriphosphate
dCTP	deoxycytosinetriphosphate
dGTP	deoxyguanidinetriphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dTTP	deoxythyminetriphosphate
EDTA	ethylenediaminetetra-acetate
ELISA	enzyme linked immunosorbent assay
g	gram
FCS	fetal calf serum
HRP	horseradish peroxidase
HDL	high density lipoprotein
IL-1	Interleukin-1
IL-6	Interleukin-6

ICSCC	individual cow somatic cell count (s)
IgA	immunoglobulin A
IgG_1	immunoglobulin G type 1
IgG_2	immunoglobulin G type 2
IgM	immunoglobulin M
IQSCC	individual quarter somatic cell count (s)
Kcl	potassium chloride
kDa	kilo Dalton
λ	lambda
l	litre
LDL	low density lipoproteins
LF	left fore
LH	left hind
mg	milligrams
MgCl	magnesium chloride
ml	millilitre
MLEE	multilocus enzyme electrophoresis
mm	millimetre
mM	millimolar
NaCl	sodium chloride
NAGase	N-acetyl-β-D-glucosaminidase
NaH_2PO_4	sodium dihydrogen orthophosphate
Na_2PO_4	disodium hydrogen monophosphate
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20
PCR	polymerase chain reaction
PHA	phytohaemmagglutinin
PMNL	polymorphonuclear leucocyte (s)
REF	restriction endonuclease fingerprinting
REFP	restriction enzyme fragmentation pattern
REP-PCR	repetitive extragenic palindromic polymerase chain reaction

RF	right fore
RH	right hind
RNA	ribonucleic acid
SAA	serum amyloid A
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMCC	Four- (N maleimidomethyl) cyclohexane carboxylic acid N-hydroxy succinimde ester
TBC	total bacterial count (s)
TBE	Tris base, boric acid disodium EDTA
TBS-T	Tris buffered saline-Tween 20
T cells	T lymphocytes
TE	Tris base disodium EDTA
TE_{10}	10mM Tris base, 10mM disodium EDTA
TEMED	N', N', N', N',-tetramethylethylenediamine
TES	Tris ethylene diamine tetra acetic acid in sodium chloride buffer
TMN	3, 3', 5, 5'-tetramethylbenzidine
TNFα	tumour necrosis factor-alpha
Tris-HCL	Tris base buffered in hydrochloric acid
TSST-1	toxic shock syndrome toxin-1
µ	micro
µg	microgram
µl	microlitre
µmol	micromole
v/v	volume per volume
w/v	weight per volume

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