Characterisation of inflammation and pain thresholds to mechanical stimulation in dairy cows with clinical mastitis and the effect of treatment with meloxicam.

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Abstract

Recognition, alleviation and control of pain are central to ensuring good welfare in food producing animals. An intervention trial was conducted on 117 dairy cows with naturally-occurring, mild or moderate, clinical mastitis in a single quarter in order to investigate the effect of the administration of the non-steroidal anti-inflammatory drug, meloxicam (Metacam, Boehringer Ingelheim Animal Health GmbH) used in conjunction with an intramammary antibiotic infusion of cefquinome (Cephaguard LC Intramammary, Intervet UK Limited, Milton Keynes). Cows with clinical mastitis were allocated randomly to one of 3 groups: Group 1: antibiotics only; Group 2: antibiotics and one dose of meloxicam; Group 3: antibiotics and three doses of meloxicam on day of diagnosis, day 0, and on days 3 and 6. Healthy animals were recruited as controls.

Clinical and laboratory parameters were investigated to assess their usefulness as objective markers of pain. Measuring the cows' response to a mechanical stimulus assessed altered pain processing. All cows were examined clinically on 6-8 occasions over a 42 day period.

Seventy out of the 117 cases of clinical mastitis were followed for the full period; 47 cases dropped out due to re-occurrence of mastitis, development of mastitis in more than one quarter or the development of another inflammatory focus. Of the 117 mastitis cases 56 were classed as mild in severity and 61 were classed as moderate. In the 70 complete mastitis cases 41 were mild and 29 were moderate. More moderate cases of mastitis dropped out compared to mild cases ($p=0.005$). Heart rates, respiratory rates and rectal temperatures of cows were higher in moderate cases compared to mild cases of clinical mastitis ($p<0.001$). The hindleg stance of cows, as measured by hock-to-hock distance, with clinical mastitis was greater than in normal cows ($p<0.001$). Alterations in the response to mechanical stimulation were recorded in cows with both mild and moderate clinical mastitis and treatment with meloxicam was shown to attenuate the mechanical alterations ($p=0.04$). There was however, no difference in cows that received one, compared to three doses of meloxicam ($p>0.05$). On bacteriological examination of the recruited cases, 30% of isolates yielded *S uberis*, 24% yielded no growth, 22% *E coli*, 11% *Staph aureus*, six per cent other microbiological species, four per cent *A pyogenes* and three per cent *S dysgalactiae*. When compared with mild cases of mastitis the moderate cases had a greater proportion of isolations of *S uberis* ($p<0.01$) and a smaller proportion of no growths ($p<0.01$). The median log individual quarter somatic cell count were greater in the quarter from the affected gland of cows with mild or moderate mastitis than the diagonally opposite quarter of these cows and normal cows ($p<0.001$), however, there was no difference between the median log IQSCC of the affected quarter from mild and moderate cases of mastitis ($p>0.05$). There was no effect of mastitis on the fat percentage of milk, as judged by the comparison of affected quarters to diagonally opposite quarters and to normal cows. The protein content of milk increased with mastitis and the lactose content decreased compared to diagonally opposite quarters and normal cows ($p<0.001$). The most common somatic cell type in quarters affected with mild and moderate mastitis on the day of recruitment to the study were polymorphonuclear cells, whereas large mononuclear cells were the predominant cell type in normal animals. In both mild and moderate mastitis, the milk from the affected quarter had greater haptoglobin concentrations than in milk from normal cows ($p<0.001$), and in each group the concentration was higher in the affected quarter than in the diagonally opposite quarter ($p<0.001$). The concentration of haptoglobin in milk from the affected quarters of cows with moderate mastitis was greater ($p<0.01$) than that in milk from affected quarter of cows with mild mastitis. Mammary-associated serum amyloid A concentrations were higher in the affected quarter of cases of mild and moderate clinical mastitis than in normal cows ($p<0.001$), and within each group the concentration was greater in the affected quarter than the diagonally opposite quarter ($p<0.05$ for mild and $p<0.01$ for the moderate mastitis group). Concentrations of the inflammatory mediator, prostaglandin E_2 in milk were lower in mild cases of clinical mastitis compared to moderate cases ($p<0.05$).

In conclusion, physiological and laboratory parameters may serve as indicators of clinical mastitis, help differentiate between different severities of clinical mastitis and, therefore, aid in appropriate targeting of therapy. Alterations in the pain pathways are present in animals with clinical mastitis of both mild and moderate severity, and treatment with meloxicam was shown to restore normal threshold responses to mechanical stimuli. These results indicate that a single dose of meloxicam may provide beneficial analgesic therapy to dairy cows with clinical mastitis.

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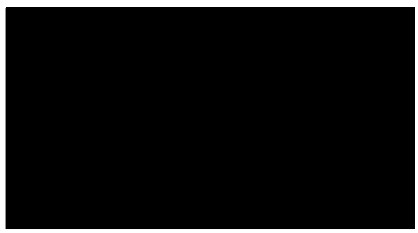
Author's Declaration

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



Maureen H. Milne, December 2004

Publications and Presentations

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

Conference proceedings

2005	**International Dairy Federation,** 'Assessing disease severity and pain in dairy cows with clinical mastitis and investigating the effects of meloxicam (Metacam®)', *Masstraicht, The Netherlands*
2004	**European meeting of Societé Française de Buiatrie,** 'Predicting the causative organisms and severity of clinical mastitis using subjective and objective measures of clinical information', *Nice, France*
2004	**XXIV World Buiatrics Congress,** 'Assessment of pain in dairy cows with clinical mastitis', *Quebec, Canada*
2004	**XXIV World Buiatrics Congress,** 'Preliminary results on the effects of meloxicam (Metacam®) on hypersensitivity in dairy cows with clinical mastitis', *Quebec, Canada*
2004	**Association Veterinary Teachers Research Workers (AVTRW),** 'The effect of meloxicam in mild and moderate cases of clinical mastitis in dairy cows' *Scarborough, UK*
2003	**10th International meeting of the International Society of Veterinary Epidemiologisits and Economists (ISVEE),** 'Objective measures of pain in mild and moderate cases of clinical mastitis in dairy cows', *Vina del Mar,Chile*
2003	**British Mastitis conference,** 'Preliminary results of a study in pain assessment in clinical mastitits in dairy cows' *Forton, Lancaster,UK*
2003	**Association Veterinary Teachers Research Workers (AVTRW), regional meeting 'Pain colloquium'** 'Quantitative measures of pain in commercial dairy cows with clinical mastitis' *Glasgow,UK*
2004	**XXIII World Buiatrics Congress** 'The acute phase response in bovine mastitis', *Hanover, Germany*
2002	**The Rank Prize Funds. Mini- symposium on Lactation and Disease** 'Assessing and manamging pain in dairy cows ith mastitis' *Grasmere, Lake District, UK*

List of Abbreviations

%	Percent
£	Pound sterling
χ^2	Chi-square
δ	delta
κ	kappa
μ	mu
μl	Microlitre
α-LA	Alpha lactalbumin
° C	Degrees Celsius
5-HETE	5-hydroperoxyeicosatetraenoic acid
ACN	Acetonitrile
APP	Acute phase protein
A pyogenes	*Arcanobacterium pyogenes*
B0	Acute phase response
BMSCC	Bulk milk somatic cell count
BRSV	Bovine respiratory syncytial virus
BSA	Bovine serum albumin
BVA	British veterinary association
CMT	California mastitis test
CNS	Central nervous system
CI	Confidence interval
C bovis	*Corynebacterium bovis*
DAISY	Dairy information system
DMSO	Dimethyl sulphoxide
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
COX-3	Cyclooxygenase 3
E coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
g	Gramme
Hp	Haptoglobin
HPLC	High performance liquid chromatography
ICSCC	Individual cow somatic cell count
IQSCC	Individual quarter somatic cell count
ILs	Interleukins
IL-1	Interleukin 1
IL-1$_\beta$	Interleukin 1$_\beta$
IL-6	Interleukin 6
IQR	Inter-quartile range
kPa	kilo Pascal
LAL	Limulus amebocyte lysate
LRS	Likelihood ratio test statistics
LOD	Limit of detection
LOQ	Limit of quantification
ml	Millilitre

mm	Millimetre
M-SAA	Mammary-associated serum amyloid A
n	Number
N	Newton
NAGase	N-acetyl beta-D-glucosaminidase
ng	Nano gram
NRS	Numerical rating scale
NSAID	Non-steroidal anti-inflammatory drug
NSB	Non-specific binding
OR	Odds ratio
PBS	Phosphate buffered saline
PGs	Prostaglandins
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGH_2	Prostaglandin H_2
$PG I_2$	Prostaglandin I_2
PMNs	Polymorphonuclear neutrophils
pg	Pico gram
r	Correlation coefficient
RIA	Radio-immunoassay
S agalactiae	*Streptococcus agalactiae*
S dysgalactiae	*Streptococcus dysgalactiae*
S uberis	*Streptococcus uberis*
spp.	Species
Strep	*Streptococcus*
Staph aureus	*Staphylococcus aureus*
SAA	Serum amyloid A
SCC	Somatic cell count
SD	Standard deviation
SDS	Simple descriptive scale
SE	Standard error
TBC	Total bacterial count
Total count	TC
TFA	Trifluoroacetic acid
TNF-α	Tumour necrosis factor alpha
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
UV	Ultraviolet
VAS	Visual analogue scale
WBC	White blood cells

Dedication

To Rich and Leah

Chapter 1

Introduction

1.1. Mastitis

Mastitis is inflammation of the mammary gland and is characterised by physical and chemical changes in the milk, and an increased number of somatic cells, especially leucocytes (Tolle 1975). Milk somatic cells consist of leucocytes and epithelial cells (O'Rourke and Blowey 1992; Blowey and Edmondson 2000). Somatic cell counts (SCC) provide a good estimate of the degree of inflammation present in the udder at the time of sampling (Sears and others 1993; Logue 1997). Mastitis is a complex disease syndrome and is a major disease problem for the dairy industry (Booth 1988; Sischo and others 1990; Bramley 1992; O'Rourke and Blowey 1992; Smith and Hogan 1996; Whitaker and others 2000; Morin 2004; Whitaker and others 2004), causing significant economic losses (Wilson and Kingwill 1975; Esslemont and Peeler 1993; Hortet and Seegers 1998; Yalcin and others 1999; Kossaibati and Esslemont 2000). Economic losses associated with mastitis are due to loss of production, the cost of treatment and prevention (De Graves and Fetrow 1993; Reneau 1993; Radostits and others 2000), discarded milk, early culling, drug costs, veterinary expenses, increased labour (De Graves and Fetrow 1993; Reneau 1993) and reduced milk yields (Janzen 1970; Nickerson 1985). Clinical mastitis increased the risk of culling (Erb and others 1985). The greatest loss caused by clinical mastitis is in milk production; there is a reduction in milk yield and milk needs to be discarded due to the use of antibiotics (Kossaibati and Esslemont 1995). Kossaibati and Esslemont (2000) calculated that the total cost per affected cow per year, using the market values in February 2000, for mild, severe and fatal cases of mastitis was £145, £436 and £1,418, respectively. The variation in costs is affected by milk price and the value of replacement heifers, where prices have fluctuated widely in the last few years. Based on the proportions of mild, severe and fatal cases recorded in DAISY (the Dairy Information System) monitoring, the authors calculated an average total cost of £176 per

affected cow per year, and that clinical mastitis caused losses of £4,389 per year for an average 100 cow herd using data from 2000 (Kossaibati and Esslemont 2000). These figures are comparable to the costs of mastitis calculated by others. Esslemont and Peeler (1993) reported an average cost of £187 per cow affected by clinical mastitis, while Yalcin and others (1999) in a study using multiple regression analysis of field data, estimated the minimum total cost of mastitis as £65.50 per cow in the herd per year. In a study by Bartlett and others (1991) using daily milk production data, temporal patterns of milk loss as a consequence of clinical mastitis were followed. These authors reported that cows that developed clinical mastitis suffered an immediate drop in production and did not regain production levels during the 60 days following clinical onset. Multiparous cows lost approximately twice as much milk as first lactation cows, and cows with mastitis occurring before 150 days in lactation lost 1.4 times as much milk compared with other cows (Bartlett and others 1991). Hortet and others (1998) estimated that for an average Holstein cow the milk losses as a result of clinical mastitis would be 4-6% of lactational yield, with 40% of cases having negligible loss, 30% losing 150-250 kg/lactation, and 30% losing 950-1,050 kg/lactation. Using this data and according to the current milk price, 18 pence per litre (Dairy Farmer July 2004), an average Holstein cow producing 8,500 litres/lactation could lose £61.20-£91.80 per lactation due to yield losses as a consequence of clinical mastitis.

Mastitis has until recently been classified as either contagious mastitis or environmental in origin (Rebhun 1995). Contagious mastitis was defined as being caused by organisms that colonised the mammary gland and were spread by the milking procedure, contaminated machinery (Tolle 1975; Cullor and Tyler 1996; Logan and Gillespie 1996), the hands of milkers' (Tolle 1975; Fox and Gay 1993; Rebhun 1995; Cullor and Tyler 1996; Smith and Hogan 1996) and nursing calves (Cullor and Tyler 1996). The main contagious pathogens were *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and many *Mycoplasma* spp. (Bramley and Dodd 1984). In contrast, environmental mastitis occurred due to opportunistic invasion of environmental pathogens that gained entry to the udder when contamination of the cow's teats, udder or milking machine occurred (Rebhun 1995). Gram-negative enterobacteriaceae and environmental streptococci, i.e. coliforms and *Streptococcus uberis* were the primary environmental pathogens (Bramley and others 1979). Coliforms included *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytocia*, *Enterobacter aerogenes*, *Citrobacter*

spp., *Serratia* spp. and *Proteus* spp. (Smith and others 1985). It is no longer considered appropriate to class mastitis-causing organisms as contagious or environmental, due to increasing evidence that *Streptococcus uberis*, which is an organism found ubiquitously in the environment can also behave in a contagious manner (Peeler and others 2000; Phuektes and others 2001; Zadoks and others 2001). Furthermore there is recently documented evidence that *E coli*, which was historically classed as an environmental organism that was quickly eliminated from the gland, can persist in the mammary gland (Bradley and Green 2001).

Mastitis may also be classified as subclinical, where the mammary gland and milk appear normal, or clinical, which is characterised by observable inflammatory changes in the mammary gland, such as heat or swelling, alterations in the appearance of the milk (Philpot 1984; Sears and others 1993; Cullor and Tyler 1996) and systemic effects in some cases.

Clinical mastitis can be further classified as peracute, acute, subacute or chronic (Tolle 1975; Radostits and others 1994; Cullor and Tyler 1996) and is often also defined as mild, moderate or severe. Authors often use different grades to aid in the classification of severity of clinical mastitis. Guterbock and others (1993) described mild mastitis as the occurrence of abnormal milk, flakes or chunks in normal or watery milk, with or without swelling of the affected quarter. Eckersall and others (2001) used a similar classification system, cases were classed as mild when there was clots in milk and classed as moderate when there was clots and observable inflammation in the infected quarter such as heat, redness or swelling. Hogan and others (1989a) and Bradley and Green (2001) ascribed numbers to the severity coding. Hogan and others (1989a) ascribed 3 to abnormal milk with no apparent swelling of the quarter; 4 to normal or abnormal milk with swelling of the quarter; 5 to abnormal milk with swelling of the quarter and systemic signs. Bradley and Green (2001) used the following severity grades, grade 1 milk changes only (e.g. clots); grade 2 milk and udder changes (e.g. swelling and/or heat); grade 3 systemic signs (e.g. depression and/or pyrexia).

1.1.1. Clinical mastitis

The European Union Health and Hygiene Directive 92/46/EEC requires bulk milk to have a total bacterial count (TBC) of < 100,000 bacteria/ml and SCC < 400,000 cells/ml. In addition to reducing SCC, pressures to reduce the incidence of clinical cases of mastitis, to produce milk free of antibiotics, other chemical residues and potential human pathogens are increasing (Smith and Hogan 1993). There is very limited information on the incidence of clinical mastitis at a national level. Studies in the 1980's estimated the incidence of clinical mastitis to be 37 cases per 100 cows per year (Booth and Rowlands 1990). From DAISY recorded herds, the incidence for 1997 was given as 40 cases per 100 cows per year (Kossaibati and others 1998), while Booth (1997) estimated the national incidence in 1997 to be 35-40 cases per 100 cows per year. Whitaker and others (2000) used data collected as part of health assurance schemes to calculate the incidence of important diseases in 340 dairy farms. They reported that clinical mastitis was recorded in 36.6% of cows in the year of the study with the top quartile having clinical mastitis in 13% of cows, and the bottom quartile having 70% of cows with clinical mastitis. A later study by Whitaker and others (2004) calculated the overall incidence of clinical mastitis on 244 dairy farms in 2001/2002 as 43.4 cases per 100 cows per year. On six Somerset farms with three-month geometric mean bulk milk somatic cell count (BMSCC) of < 250,000 cells/ml the mean annual incidence of mastitis was 41.6 cases per 100 cows per year (Bradley and Green 2001). In a recent retrospective study of low BMSCC herds, Peeler and others (2000) reported a mean incidence of clinical mastitis of 23 cases per 100 cows per year. In a study in Devon, where herds were not selected on level of BMSCC, the mean number of cases was 17 cases/100 cows/year in herds with a median BMSCC of 142,000 cells/ml (Milne and others 2002).

The widespread application of control measures has been successful in reducing the incidence of contagious mastitis but has had limited effect on environmental pathogens (Dodd and Neave 1970; Bramley and Dodd 1984; Hill 1992). Thus, environmental organisms have been reported increasingly as a cause of clinical mastitis in cattle (Bramley 1984; Jayarao and others 1999; Leigh 1999).

1.1.2. Subclinical mastitis

Subclinical mastitis occurs when the mammary gland is infected and the number of leucocytes (somatic cells) is increased, but the milk appears to be grossly normal and there is no clinically detectable sign of inflammation in the mammary gland (Sears and others 1993; Cullor and Tyler 1996). Mastitis is most often subclinical (Radostits and others 1994) and indeed subclinical mastitis was reported to account for 70% of all infected quarters (Newbould 1984). Over time, repeated or persistent bouts of subclinical mastitis may lead to fibrosis of mammary tissue producing firmer glands and a reduction in milk production (Cullor and Tyler 1996). Contagious pathogens are more likely to produce subclinical infections, and hence higher SCC, than environmental pathogens (Eberhart and others 1982; Blowey and Edmondson 2000). This is due to the rapid elimination of environmental organisms in cases of environmental mastitis, when high SCC usually occurs only during the period of clinical mastitis (Blowey and Edmondson 2000). Chronically infected glands can however have a low SCC because of the cyclic shedding of organisms (Sears and others 1993; Lawrence 1997). The main cause of high BMSCC in Scottish dairy herds was subclinical infection with *S agalactiae* infection, which was recorded in 83% of the herds sampled; the bacteriological examinations were assessed from individual cow or quarter sampling (Logue 1997). *Staphylococcus aureus* infection was reported as the most prevalent pathogen in herds where the BMSCC remained above 400,000 cells/ml for at least one year after the first sampling (Logue 1997). The average reduction in milk output per quarter per lactation resulting from subclinical mastitis has been reported to be 15-40% depending on the type, severity and duration of infection (Hale and others 1956; Beck and others 1992).

1.1.3. Diagnostic tests

Diagnostic tests are used to identify the nature and extent of a mastitis problem in a dairy herd and/or to identify an individual animal for segregation, treatment or culling (Bramley 1992). Clinical mastitis is detected by the presence of abnormal milk. Clinical signs alone are considered to be insufficient to differentiate between infections caused by Gram-positive and Gram-negative bacteria with certainty (White 1987), but recent work indicates that some clinical signs, e.g. demeanour of cow and milk yield changes, are more likely to be associated with certain types of pathogens than others (Milne and

others 2003). Optimal treatment of mastitis relies on the identifcation of the causal organism and its antibiotic sensitivity (Waage and others 1994).

1.1.3.1. Bacteriology

In general, pathogenic microorganisms, usually bacteria (Wilson and Richards 1980; Sandholm and others 1990; Watts 1990; Sears and others 1993; Cullor and Tyler 1996) cause mastitis, although the organism may be undetectable by the time of examination (Sears and others 1993). Bacteriological culture of milk samples is therefore useful to aid in the identification of the cause of mastitis.

The prognosis and treatment of a cow with clinical mastitis depends on the causal agent, with antibiotic selection often being different for Gram-positive and Gram-negative organisms. Following concerns about antimicrobial resistance, the British Veterinary Association (BVA) produced guidelines regarding good clinical practice, recommending that narrow spectrum antimicrobials be used in preference to broad-spectrum antimicrobials (Anon 2000). The need to rapidly instigate treatment prior to bacteriological investigation, however, often results in the use of a broad-spectrum treatment. The ability to differentiate between mastitis caused by Gram-negative agents and mastitis caused by Gram-positive agents using clinical parameters would, therefore, be beneficial, as it would allow more appropriate antimicrobial selection. In order to diagnose mastitis accurately by culture techniques, milk samples need to be taken carefully (Watts 1990). Poor sampling technique often leads to contamination of milk samples and confusion over the significance of the organisms isolated (Buswell 1995). A negative bacterial result will be obtained in 10-30% of clinical samples, which may be due to the infection being controlled by natural defence mechanisms with rapid elimination of the bacteria (Radostits and others 2000), continued activity of natural inhibitors in the milk between sampling and culturing, the presence of non-viable bacteria, or the presence of exogenous inhibitory substances. Zorah and others (1993) showed that 51% of 84 samples that showed no growth on bacteriological culture were identified as containing *E coli* antigen.

1.1.3.2. Somatic cell counting

Intramammary infections are the most important factor influencing the concentration of somatic cells in milk (Reneau 1986). Normal milk from uninfected dairy cows contains approximately 200 x 10^3 cells/ml of milk for the majority of the lactation (Hillerton 1997). A healthy quarter can be considered as one that has a SCC <100,000 cells/ml and is free of mastitis pathogens (Dohoo and Meek 1982; Harmon 1994; Hamann 1996). Other factors influencing SCC include breed, age, stage of lactation and physiological stresses such as excitement, high temperatures or trauma (Reichmuth 1975; Tolle 1975; Philpot 1984; Rebhun 1995). A massive recruitment of additional leucocytes, mainly neutrophils, is elicited into the gland in response to bacterial invasion of the udder (Ziv 1992). White blood cells enter the milk from the blood in response to inflammation and epithelial cells are shed from the lining of the udder tissue (Philpot 1984; Nickerson 1985; Blowey and Edmondson 2000).

Somatic cell counting is a practical and effective method of evaluating a herds' mastitis status and gives a good indication of the level of infection in a herd (Booth 1985; Dohoo and Meek 1982; Sears and others 1993). Somatic cell counting can be done (i) at the quarter level when individual milk samples are collected from each quarter i.e. individual quarter somatic cell count (IQSCC), or (ii) at the cow level, when a composite sample of equal volumes of milk is collected from all quarters of the cow into one container i.e. individual cow somatic cell count (ICSCC), or (iii) at the bulk tank level i.e. bulk milk somatic cell count (BMSCC) (Jackson 1980; Newbould 1984; Sears and others 1993; Biggs 1996). Somatic cell couting may help monitor response to treatment.

The SCC changes dramatically during the early stages of acute infection reaching a peak within hours or days, depending on the challenge organism. An experimental study by Sears and others (1990) reported a rapid increase in the IQSCC, which peaked, at the approximately 2,000 $\times 10^3$ cells/ml, by two to three days post-infusion. An ICSCC of 200 – 5,000 $\times 10^3$ cells/ml can be seen in cases of clinical mastitis, the magnitude depending on the severity of the infection and the type of organism (Cullen 1966). Clumps of bacteria can interfere with the counting methods. A preservative, such as potassium dichromate, is used to try to reduce further clumping of bacteria during transport to the laboratory.

1.1.3.3. Miscellaneous tests

Clinical mastitis is detected by the presence of abnormal milk; in-line filters, foremilk stripping, udder palpation, and electronic sensors can be used to detect clinical disease (Morresey 1999). Milner and others (1997) assessed the impact of electrical conductivity detection of clinical mastitis compared to conventional diagnosis, such as the recognition of clots in the milk. They found that the time required for SCC to reduce to less than 400,000 cells/ml was significantly shorter, and that the bacteriological and clinical response to treatment was more complete in cases detected by conductivity rather than conventional means. The usefulness of the conductivity test was confirmed by studies assessing the ability of automated detection systems to detect clinical mastitis. These studies showed that the use of milk conductivity and milk yield in automated systems were suitable methods to detect mastitis at a farm level (Mol and Ouweltjes 2000).

N-acetyl-β-D-glucosaminidase (NAGase) is a cell-associated enzyme in milk and a high level indicates cellular damage (Mattila and Sandholm 1985) and correlates with high cell count (Ball and others 1989). The NAGase test is suited to rapid handling of large numbers of samples, as it is an automated test and it can be performed on fresh milk with the results available on the same day (Ball and others 1989). It is considered to be the most accurate of the indirect tests, being equivalent to SCC in predicting the infection status of a quarter (Radostits and others 2000) and is related to both the presence and types of pathogen, in addition to the SCC (Kitchen and others 1984).

The ability to differentiate between Gram-positive and Gram-negative pathogens to enable optimal, targeted therapy has been the focus of many new diagnostic tests. The Limulus amebocyte lysate (LAL) cow side test to determine endotoxin was trialled by Waage and others (1994) against standard laboratory bacteriology identification. These researchers achieved a 93% agreement between the test and culture result, with the positive predictive value (PPV) of the LAL test result being 70% and the negative predictive value (NPV) being 95%. These results suggested that the LAL test might be a valuable cow-side test to aid in the selection of initial anti-microbial therapy. Yazdankhah and others (2001) described another cow-side test making use of selective decolourisation of Gram-negative bacteria following the dilution, filtration and staining of a milk sample from a mastitic quarter. This test had an overall specificity of 86%

when compared to bacteriological culture. The HyMast test also claimed to differentiate Gram-negative bacteria from Gram-positive bacteria. Jansen and others (1999) reported that this test, which involved the incubation of milk, had reasonable sensitivity when read after 36 hours but resulted in several false positives. The ColiMast test consists of a selective growth medium in a vial that detects coliform organisms after inoculation with milk and an incubation period of 12 hours by virtue of a colour change from blue to pink. This test has a sensitivity of 74% and specificity of 78% (Leslie and Dingwell 2002).

The acute phase proteins in milk show considerable potential for use as biological markers of mastitis. Eckersall and others (2001) assessed the value of acute phase proteins in the diagnosis of mastitis by comparing the concentrations in mild and moderate mastitis with concentrations from quarters of healthy cows. The sensitivity, specificity and accuracy of the haptoglobin test in milk for differentiating between mastitic and normal cows were 86%, 100% and 93%, respectively; and for milk-associated serum amyloid A they were 93%, 100% and 97%, respectively.

The California Mastitis Test (CMT) is an indirect method of estimating SCC and is based on a gelling reaction between the nucleic acid of the somatic cells and an anionic surface-active agent with bromocresol-violet as an indicator (Schalm and Noolander 1957). Although it is not very sensivie or specific, the CMT is a practical and useful on-farm test to identify individual cows and quarters with subclinical mastitis (Jaartsveld 1975).

1.1.4. Genetics and resistance to mastitis

Genetic variation associated with disease resistance in dairy cattle with direct relevance to mastitis was reviewed as early as the 1950s (Legates and Grinnells 1952). Direct selection of animals which may possess enhanced disease resistance with regard to mastitis, is limited due to the lack of accurate and standard recording of clinical incidence, mainly due to the difficulty and cost of collecting such information (Mrode and others 1998). The heritability of clinical mastitis was estimated to vary from two to eight percent (Emmanuelson and others 1988). Udder health, in the past, has been overlooked in preference for improvement in production traits. A negative gentic correlation between milk production and resistance to mastitis has been shown (Shook 1989), indicating that the incidence of mastitis will continue to increase if the selection

criteria of improved production traits are maintained. There is, however, increasing pressure to include mastitis resistance in breeding programs for diary cattle for welfare and economic reasons.

1.2. Inflammation

Inflammation was recognised by Celsius around 35 A.D. as being characterised by 'redness, swelling, heat and pain', and in the 19th century, Virchow added 'loss of function'. The complex set of reactions that follow injury, trauma or infection of a tissue serve to prevent further tissue damage, isolate and destroy the infective organism and activate repair processes to return the tissue to normal function (Higgins and Lees 1984; Baumann and Gauldie 1994). At the site of injury, a number of responses by the tissue itself and the vascular system involving platelets, clot formation, leakage and inflammatory cells are elicited (Gruys and others 1994). This homeostatic process is known as inflammation and the early and immediate sets of reaction are known as the acute phase response (Baumann and Gauldie 1994). These responses in turn are associated with the production of cytokines and other mediators, which activate receptors on different target cells leading to a systemic reaction (Gruys and others 1994; Moshage 1997). The tissue macrophage or blood monocyte are most commonly associated with the initiation of the acute phase response as they subsequently release inflammatory mediators (Baumann and Gauldie 1994; Kushner 1982; Koj 1996).

1.2.1. Inflammatory mediators

Pathophysiological changes underlying acute and chronic inflammatory responses are brought about by chemical mediators acting locally within tissues (Cunningham and Lees 1994). Many chemical mediators have been implicated in the acute inflammatory response. To date, the inflammatory mediators identified in milk include bradykinin (Eshraghi and others 1999), prostaglandins (PGs) (Anderson and others 1985), leukotrienes (Rose and others 1989), histamine and serotonin (Schalm and others 1971), complement components (Kehrli and others 1991), interleukins (ILs) (Shuster and others 1993; Daley and others 1991), tumour necrosis factor α (TNFα) (Babiuk and others 1991; Shuster and others 1993), interferon (Babiuk and others 1991) and leukotriene B4 (Babiuk and others 1991; Daley and others 1991; Kehrli and others 1991). In addition to

acting on the tissues locally, circulating cells and the microcirculation, these mediators interact with one another (Cunningham and Lees 1994).

The activation of the kinin-forming system is mediated via injury, trauma, infection and immune complexes and once activated causes release of other powerful mediators, such as PGs, histamine and cytokines (Eshraghi and others 1999). The kinins result in vasodilation, increased vascular permeability, stimulation of ion transport and fluid secretion, contraction of intestinal and uterine smooth muscle and sensitisation of pain nerve endings. Eshraghi and others (1999) reported kinins, such as bradykinin, to be the most potent mediators of pain and oedema in bovine clinical mastitis.

Prostaglandins are potent biological mediators derived from arachidonic acid and are produced in many tissues where they induce a wide variety of responses (Xie and others 1992). The PGs are divided into subgroups using the letters A-J, and the presence and number of double bonds in the alkyl side chains is denoted by a numerical subscript. Vasodilator PGs enhance both pain inducing and permeability increasing activities of other mediators (Moncada and others 1975). The production of PGs begins with the liberation of arachidonic acid from membrane phospholipids by phospholipase A_2 in response to inflammatory stimuli, following which arachidonic acid is then converted to PGH_2 by cyclooxygenase enzymes, COX-1 and COX-2 (Harris and others 2002). Cell specific prostaglandin synthases convert PGH_2 into series of PGs including PGI_2, $PGF_{2\alpha}$, PGD_2 and PGE_2.

Thromboxanes are also derived from arachidonic acid and cause vasoconstriction and platelet aggregation. Thromboxane A_2 (TXA_2) is a highly unstable bicyclic compound with strong biological effects while thromboxane B_2 (TXB_2) is its stable derivative (Samuelsson 1983).

Cytokines are peptides released from inflammatory tissue, connective tissue and immune system cells. There at least 50 known cytokines (Rang and others 1999) that have been implicated in modulating immune responses (Babiuk and others 1991). Cytokines are extremely potent and each one can exert multiple functions and they often act synergistically. An example of the synergistic action is the induction of acute phase proteins, such as serum amyloid A (SAA) and haptoglobin (Hp), by IL-6-like cytokines

syngerising with IL-1-like cytokines (Moshage 1997). The cytokine superfamily includes interleukins, chemokines, colony-stimulating factors, growth factors, interferons, transforming growth factor and tumour necrosis families.

Studies comparing the inflammatory response following intramammary inoculation with *Aerobacter aerogenes* in reversibly induced neutropaenic cows with normal cows, demonstrated that the degree of inflammatory response was decreased in neutropaenic cows. These animals had minimal changes in the swelling of the udder, reduced degree of leukocyte infiltration to udder, normal appearance of milk, minimal changes in the concentration of albumin in milk compared to non-neutropaenic cows (Jain and others 1968). These results indicate that neutrophils play a significant role in producing changes in vascular permeability, which are necessary for the initiation of the inflammatory reaction and the production of the cardinal signs of inflammation. Inoculation of histamine into the bovine mammary gland produced an increase in vascular permeability leading to oedema and hardening of the gland accompanied by an increase in serum albumin concentrations, but not leukocytes numbers in the milk (Jain and others 1972). Inoculation of serotonin produced slight oedema and marked tenderness of the gland and inoculation of washed leukocytes, disrupted leukocytes and lyososomal preparations from leukocytes all produced signs of acute inflammation; swelling and hardening of the gland, increased concentrations of blood serum albumin in the milk and diapedesis of neutrophil leukocytes from blood to milk (Jain and others 1972). The results of these studies suggest that histamine and serotonin are mediators of inflammation, and leukocyte products are more potent mediators of inflammation in the bovine mammary gland.

In experimental studies, thromoxane and PGI_2 concentrations were increased in equine endotoxic shock (Bottoms and others 1982). Thomas and others (1997) demonstrated increased concentrations of PGE_2 in experimental models of bovine inflammation using *Mannnheimia (Pasteurella) haemolytica* and *S uberis*-inoculated dialysis sacs. Endotoxin was instilled into the teat of four cows at differing doses and resulted in increased concentrations of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ but no increase in TXB_2 or PGE_2 (Giri and others 1984). Atroshi and others (1987) reported that PGE_2, $PGF_{2\alpha}$ and TXB_2 concentrations in samples from the affected quarter of cows with mastitis were approximately 40%, 15% and 44% higher, respectively, than the concentrations of

normal control cows. Anderson and others (1985) reported an increase $PGF_{2\alpha}$ and TXB_2 in bovine mastitis and that the increases in the concentrations corresponded to the clinical severity of the mastitis. Bradykinin levels in the milk increase in bovine mastitis and increasing levels were correlated with increasing severity of mastitis (Eshraghi and others 1999).

1.2.2. Acute phase proteins

An acute phase protein (APP) has been defined as one whose plasma concentration increases (positive APP) or decreases (negative APP) (Kushner and MacKiewicz 1987) by at least 25% during inflammatory disorders (Kushner and others 1981). Negative APP are pre-albumin (retinal binding protein and transthyretin), transferrin and albumin (Gruys and others 1994). The positive APPs, including Hp, SAA, alpha-1-anti-trypsin, ceruloplasmin and fibrinogen, are mainly glycoproteins released by hepatocytes upon specific stimulation by cytokines, IL-6, IL-1 and TNFα that are produced by leucocytes and macrophages during infection and inflammation (Bornstein 1982; Laurell 1985; Marinkovic and others 1989; Gruys and others 1994; Eckersall and others 1999). As a result of the total changes in blood protein composition during the acute phase response, the plasma viscosity and erythrocyte sedimentation rate increases (Gruys and others 1994). The functions of APP include scavenging for haemoglobin, free radicals and cell nests, binding bacterial components, activating complement and having a role in the cholesterol distribution, and promotion of immunoglobulin production (Kushner and others 1981). The serum profile of APP varies between species and depends on the type of inflammatory stimulus (Eckersall and Conner 1998; Conner and others 1988; Eckersall and others 1999).

In cattle, Hp, ceruloplasmin, α-1 proteinase inhibitor, fibrinogen and α-1 acid glycoprotein have all been shown to increase during the acute phase response (Conner and others 1986; Conner and others 1988; Conner and others 1989; Itoh and others 1990; Skinner and others 1991; Morimatsu and others 1992; Motoi and others 1992). In cattle the most useful acute phase proteins to distinguish healthy cows from those with inflammatory diseases were Hp and SAA (Alsemgeest and others 1994; Horadagoda and others 1999). Haptoglobin and SAA are defined as major APPs, as their concentrations

can increase 100-fold during an infection (Eckersall and others 2001) and have low or undetectable concentrations in normal animals.

1.2.2.1. Haptoglobin

Haptoglobin is a macromolecular protein complex of >1,000 kilodaltons (kD), consisting of subunits of 16, 40 and 67 kD (Gruys and others 1993). Haptoglobin is an anti-oxidant and stimulates angiogenesis, thus aiding in wound repair (Gabay and Kushner 1999). Haptoglobin was detected in bovine plasma in the late 1950s (Liang 1957), but it was not until the mid-1960s that it was discovered that healthy cows had low to zero concentrations of plasma Hp (Bremner 1964). Bovine Hp has been identified in cattle with a variety of inflammatory conditions; mastitis (Spooner and Miller 1971; Conner and others 1986), liver abscesses and *Corynebacterium* spp. infected abscesses (Spooner and Miller 1971), pyometra, traumatic reticulitis, traumatic pericarditis and abomasal displacement (Panndorf and others 1976; Makimura and Suzuki 1982). It was previously thought that haptoglobin was not stimulated by viral disease. Spooner and Miller (1971) reported no increase in Hp concentrations in samples from cattle naturally infected with the viral condition rinderpest, and Godson and other (1996) reported no increase in Hp after experimental infection with bovine herpes virus. Heegaard and others (2000), however, did report significantly increased Hp concentrations following experimental infection of calves with bovine respiratory syncytial virus (BRSV). Haptoglobin concentrations were reported to be normal in non-infectious conditions, such as milk fever and ketosis, and in chronic endometritis, but were significantly increased in infectious conditions (Skinner and others 1991). It should be noted that Hp cannot be accurately measured in the presence of haemolysis, as haemoglobin binds to Hp and the complex is then removed by the liver (Bremner 1964). Haptoglobin disappeared from the blood when free haemoglobin was injected and was also absent during haemolytic crises due to babesisois (Bremner 1964).

1.2.2.2. Serum Amyloid A

Serum amyloid A is a non-glycosylated 14kD apolipoprotein (Gruys and others 1993). Serum amyloid A has been identified in cattle with a variety of inflammatory conditions; mastitis (Gruys and others 1993; Horadagoda and others 1999; Eckersall and others

2001; Nielsen and others 2004), abomasal ulceration, traumatic reticulitis, traumatic pericarditis (Gruys and others 1993; Horadagoda and others 1999), mucosal disease, pulmonary thromboembolism, meningitis and acute respiratory disease (Horadagoda and others 1999) including BRSV (Heegaard and others 2000). Serum amyloid A concentrations also increased following parturition (Alsemgeest and others 1993), suggesting SAA concentrations increase in the presence of acute inflammation, whereas Hp increase following infectious disease processes (Alsemgeest and others 1994; Gruys and others 1994).

Serum amyloid A gene expression has been described at several extra-hepatic sites, and several cell types have been found that can synthesise the protein (Vreugdenhill and others 1999). This, together with recent identification of a unique isoform of the protein in normal bovine colostrum (McDonald and others 2001), suggests that SAA might be synthesised locally during episodes of mastitis. Indeed Eckersall and others (2001) and Winter and others (2003) reported no correlation between serum and mammary-associated SAA (M-SAA) in cows and sheep, respectively, with clinical mastitis. It was proposed that M-SAA might prove to be more sensitive than bacteriological examination and less influenced by the physiological state of the cow than either the SCC or electrical conductivity of the milk (Biggadike and others 2002) and thus may be valuable as a rapid test for the presence of mastitis. A recent study, by Nielsen and others (2004), comparing the levels of M-SAA in healthy cows, cows with clinical mastitis and cows with extra-mammary inflammatory disease, such as interdigital phlegmon, purulent metritis and/or periarthritis/bursitis, reported that M-SAA levels were within normal ranges in the group with extra-mammary inflammation despite serum SAA being increased in 36% (4/11) of cases. Thus intramammary stimulus is required for the concentration of M-SAA to increase (Nielsen and others 2004).

1.2.2.3. Diagnostic value of acute phase proteins

Haematological examination has been perceived as the recommended diagnostic method for assessing inflammation. Ruminants, however, rapidly develop neutrophilia and lymphopaenia during severe, acute bacterial infection to give an increased white blood cell count and thereafter the rate of destruction of neutrophils exceeds their rate of production and there is a reduction in the white blood cells (WBC), despite a marked

positive neutrophil response (Jain and others 1978; Skinner and Roberts 1994). Studies comparing the use of haematological screening and the measurement of APP reported that APP were more sensitive indicators of inflammation than haematological screening, in dogs (Solter and others 1991), in sheep (Skinner and Roberts 1994), and in cattle (Horadagoda and others 1999). The APP concentrations remain increased in the circulation for at least 24 hours and often for several days (Hol and others 1987). Heegaard and others (2000) reported peak levels of SAA and Hp on day seven after experimental infection with BRSV. Acute phase proteins are stable and can be measured in previously frozen samples (Horadagoda and others 1999), whereas haematological examination requires fresh blood samples.

Concentrations of APP have been used to differentiate between diseased and healthy animals (Spooner and others 1971; Conner and others 1986; Skinner and others 1991; Solter and others 1991; Gruys and others 1993; Skinner and others 1994; Eckersall and others 2001; Nielson and others 2004). The concentrations of APP can also provide an indication of the severity of the inflammatory disease (Horadagoda and others 1999). An experimental study by Conner and others (1988) used turpentine as a model for inflammation and they demonstrated that Hp concentrations increased incrementally when the amount of turpentine injected subcutaneously was increased; their results suggest that Hp concentrations increase with increasing inflammation. Concentrations of Hp correlated with severity of clinical signs in bovine respiratory cases (Godson and others 1996). Sheldon and others (2001) reported that the concentrations of APP were increased relative to severity of bacterial contamination of uterus post-calving. The concentrations of APP in serum and milk correlated with the severity of clinical signs of mastitis (Eckersall and others 2001) and with an increase in CMT score (Nielsen and others 2004).

In cattle, SAA is reported to be the more sensitive indicator of inflammation but Hp is the more specific indicator. Eckersall and others (2001) reported the sensitivity, specificity and accuracy of Hp in milk for differentiating between mastitic and normal cows were 86%, 100% and 93%, respectively; and for SAA they were 93%, 100% and 97%. Gruys and others (1993) reported sensitivities and specificities for Hp and SAA in distinguishing between cattle with diseases, such as abomasal ulceration, mastitis, endocarditis, peritonitis, and normal animals of 80% and 100% and 100% and 50%,

respectively. The ratio of Hp/SAA in the blood could aid in distinguishing between acute and chronic inflammation, as SAA is an indicator of more acute inflammation and Hp of more chronic inflammation (Alsemgeest and others 1994), a higher ratio indicates chronicity.

In addition to differentiating between healthy and diseased animals, APP can be used to monitor disease progression and thus act as a prognostic aid. In humans, APPs are used to monitor the progression of disease in patients with chronic illness and used to differentiate between types of disease, e.g. rheumatoid arthritis can be differentiated from osteoarthritis using APP profiles (Kushner and MacKiewicz 1987).

1.3. Assessment of pain

The Farm Animal Welfare Council (FAWC), the independent advisory body established by the British Government in 1979, has provided a framework to define ideal states that are applicable to all animal production systems termed the 'Five Freedoms'. The third freedom states "Freedom from pain, injury or disease". Pain assessment in animals and man is inherently difficult, and it has been recognised from work in the human field that pain refers to an endless variety of qualities that are categorised under a single linguistic label and not a specific sensation that varies only in intensity (Melzack and Torgerson 1971; Melzack 1975). Beecher (1975) reviewed the literature from over 106 years of experimental work and concluded that pain could not satisfactorily be defined, and that the pain threshold could not be demonstrated as being constant from subject to subject or from one time to another in the same subject. He concluded that many factors, such as sex, age, anxiety and fear, circulatory change, distraction, inattention, lethargy, and training in the protocol (human) produced variations in pain thresholds.

Nociceptors are specialised sensory receptors that are activated by high intensity noxious insults to peripheral tissues and pain is mediated by physiological pathways, however, nociception does not necessarily lead to the experience of pain (Kandel and others 2002). Instead the perception of pain is the product of the brain's abstraction and elaboration of sensory input, thus pain is highly individual and subjective making it difficult to define and treat clinically (Kandel and others 2002). Beecher (1975) suggested that in animals,

neonates and non-communicative humans it might be the reaction to pain we are assessing, whereas in communicative man it is the perception of pain.

Pain has been defined by the International Association for the Study of Pain (IASP) as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage' (Merskey and others 1979). Building on this Molony (1997) described animal pain as 'an aversive sensory and emotional experience representing an awareness by the animal of damage or threat to the integrity of its tissues; it changes the animal's physiology and behaviour to reduce or avoid the damage, to reduce the likelihood of recurrence and to promote recovery; non-functional pain occurs when the intensity or duration of the experience is not appropriate for the damage sustained and when physiological and behavioural responses are unsuccessful in alleviating it'. Kitchell (1987) had previously suggested that the protective motor actions can lead to learned avoidance, and may modify species-specific traits of behaviour, including social behaviour. Species-specific guidelines for the assessment of pain were suggested by a working party for the Association of Veterinary Teachers and Research Workers to be used in the regulation of animal experiments (Sanford and others 1986). The judgement of the severity of pain was based on the recognition and assessment of a wide range of parameters by trained and experienced observers (Sanford and others 1986). They suggested that cattle in pain often appear dull and depressed with little interest in the surroundings, can be inappetant with subsequent weight loss and in milking cows, a sudden drop in milk yield. In severe pain, they noted that rapid shallow respirations were observed and that on handling cattle may react violently or adopt a rigid posture possibly designed to immobilise the painful region. The rigid posture might lead to a lack of grooming due to unwillingness to turn the neck. Grunting and grinding of the teeth may be heard and in abdominal pain cattle may look at, bite or kick abdomen, get up and lie down frequently, walk in circles, or roll.

In assessing pain physiological parameters, such as heart rate, respiratory rate, blood pressure, sweating, piloerection, gut motility, have been suggested as useful indicators, however, they should not be used alone, as they have been reported to be inaccurate due to the interaction with other variables, e.g. drug administration (Carroll 1996; Holton and others 1998a; Livingston and Chambers 2000). Endocrine changes and the release of hormones are again unreliable indicators of pain when used alone, but are often used in

combination with other parameters in animal studies (Livingston and Chambers 2000). In a study assessing post-operative pain in horses some correlation between pain scores and β-endorphin concentration was found, but not during the immediate post-operative period (Raekallio and others 1997). Any change in the behaviour or temperament from the animal's normal state may be important (Morton and Griffiths 1985; Haskins 1987), and this has historically been the basis for the pain scoring systems.

1.3.1. Pain classification

Pain can be divided into two distinct and qualitatively different categories. The simplest form is physiological pain, which represents the transient response of the system to potentially damaging noxious stimuli but is not associated with extensive inflammatory response or damage to the nervous system (Woolf 1989). Pathological pain, or clinical pain, is the sensation that arises as a result of either the inflammatory response that is associated with severe injury, or damage to the nervous system (Woolf 1989). Physiological pain can be induced by mechanical, thermal or chemical stimulation and clearly defined thresholds can be established experimentally at which the sensation stops being one of pressure, hot or cold and becomes painful (LaMotte and others 1983). Pathological pain differs from physiological pain in that there is no measurable threshold at which stimulation will induce pain, as pathological pain can occur in the absence of an apparent stimulus. In response to the mildest stimulation, the sensation can be excessive or more prolonged than would be expected the response for the nature of the stimulation (Woolf 1989). Physiological pain is a sensation that reflects certain specific types of peripheral stimuli, whilst pathological pain is a sensation that is a consequence of changes within the nervous system that result in an alteration in the way information from the periphery is handled (Woolf 1989). Pathological pain can be subdivided into inflammatory pain and neuropathic pain. Theories have evolved to suggest that inflammatory pain retains some resemblance to physiological pain in that it has a protective role e.g. tenderness helps avoid further damage whilst the injured area heals (Woolf 1989), whereas neuropathic pain represents the pathological product of a disturbed nervous system (Woolf 1989). In inflammatory reactions there are (1) peripheral sensitisation of primary afferents, (2) central sensitisation of dorsal horn neurones and (3) abnormal properties in the central circuits of the central nervous system (CNS). In neuropathic pain there are (1) central sensitisation of dorsal horn neurones, (2)

abnormal properties of the central circuits in the CNS and (3) permanent changes in the nervous system (Woolf 1989).

1.3.2. Pathophysiology of pain

The specificity and gate-control theories were considered to be the basis for pain transmission (Jones 1992). Descartes devised the concept of the specificity theory in 1664; he described nerves as tubes composed of large numbers of fine threads that connect the brain with end organs in the skin and in other tissues. This theory was developed, by use of experimental work stimulating localised points and histological examination of skin sections, to find that each area is associated with a specific sensory end organ and that pain is a distinct sensation in itself with its own sensory apparatus. The gate-control theory was proposed by Melzack and Wall in 1965 and attempted to explain mechanisms by which cutaneous and cognitive factors can modulate pain transmission. It was hypothesized that in the superficial laminae of the dorsal horn of the spinal cord, the afferent input was modulated by neuronal activity in large and small afferent fibres before being conveyed to transmission neurons located in the deep dorsal horn laminae (Melzack and Wall 1965). The aspect of the gating system is not clear, as not all neurons in the superficial laminae receive input from both small and large fibres. The current theory of pain (Jones 1992) is that pain is considered to be an afferent sensory experience with the transmission of pain information being modulated by afferent input from the periphery, descending inhibitory systems and cognitive and emotional factors. Pain perception reflects activation of the nociceptors (either direct noxious stimulation or by mediators released from injured tissue), afferent transmission to the spinal cord, and relay via dorsal horn to higher centres (Jones 1992; Yaksh 1999). Pain perception can be divided into two major components; the sensory discriminative component, which describes the location and quality of the stimulus and is transmitted via the myelinated fibres and relayed to the higher centres, which activates the motor component to result in the withdrawal from the noxious stimulus and the tonic input to the system from higher levels of the central nervous system (CNS) exerts inhibitory effects on the sensory input. The classic injury response was termed the 'triple response' and is characterised by increased blood flow, tissue oedema and sensitisation of nociceptors (Lewis 1936). Most inflammatory processes lead to the development of hyperalgesia (Lewis 1936; LaMotte and others 1982; Nolan and others 1987; Ley and

others 1989; Chambers and others 1990; Coderre and others 1993; Whay and others 1996; Fitzpatrick and others 1998; Lascelles and others 1998; Yaksh 1999). Hyperalgesia defines the altered state of sensibility in which the intensity of pain sensation induced by noxious stimulation is greatly increased (Merskey and others 1979; LaMotte and others 1982; Raja and others 1984; Raja and others 1988). Two classes of hyperalgesia have been defined; primary hyperalgesia, which is the enhanced sensitivity at the injury site and occurs within minutes of the injury, and secondary hyperalgesia, which refers to changes in the non-traumatised regions surrounding the injured tissue and has a delayed onset (Lewis 1936). Secondary hyperalgesia involves both peripheral sensitisation and changes in the CNS (Hardy and others 1950; Woolf 1983; Woolf 1989). The changes in the CNS (central sensitisation) are considered to arise from inputs from the periphery overwhelming the inhibitory mechanisms and triggering long lasting changes in the dorsal receptive fields, which may outlast the changes observed peripherally (Woolf 1983; Coderre and Melzack 1987; Cook and others 1987; Woolf 1989; Yaksh 1999). Clinical manifestations of central sensitisation include increased pain intensity, allodynia, prolonged facilitation of ipsi-lateral and contra-lateral flexion reflexes and alteration in regional sympathetic tone (Woolf 1989). Allodynia refers to the state where ordinarily non-noxious stimulation (pressure, light touch) is perceived as being exquisitely painful (Merskey and others 1979; Raja and others 1988) and is a feature of some clinical pain states, e.g. trigeminal neuralgia. Hypoalgesia is the state of having reduced sensivity to noxious stimulation (Merskey and others 1979). Dairy cows subjected to acute stressors, which involved alterations in their housing environment, displayed hypoalgesia towards nociceptive laser stimulation (Herskin and others 2004).

1.3.2.1. Innervation to the udder

There is a diffuse arrangement of innervation to the udder, with multiple innervations from lumbar and sacral spinal nerves. The skin covering the udder is supplied from three directions. The ventral branches of the first and second lumbar nerves pass caudoventrally to supply the cranial parts of the forequarters; the genitofemoral nerve (comprising branches from second, third and fourth lumbar spinal nerves) passes throught the inguinal canal and sends superficical branches to the skin over the middle section of the udder; the mammary brances of the pudendal nerve descend through the perineum to supply the skin over the caudal aspect of the hindquarters (Dyce and others

1987). The gland substance and the deeper parts of the teat walls are served byt the genitofemoral nerve only (Dyce and others 1987).

1.3.3. Measurement of pain

Reflexive behaviours are useful for certain pharmacological tests, but they have serious limitations as methods of quantifying animal pain, as changes in reflex activity can result from alterations in motor as well as sensory processing (Chapman and others 1985). Combinations of behaviour and physiological measures are generally more comprehensive than either alone (Sanford and others 1986). Pain scoring systems have been used in the medical field since the 1920's (Freyd 1923). Scoring systems include the simple descriptive scale (SDS) (Huskisson 1974; Taylor and Houlton 1983; Waterman and Kalthum 1986; Holton and others 1998b), visual analogue scale (VAS) (Huskisson 1974; Reid and Nolan 1991; Nolan and Reid 1993; Welsh and others 1993; Holton and others 1998b) and the numerical rating scale (NRS) (Taylor and Houlton 1983; Taylor and Herrtage 1986; Welsh and others 1993; Holton and others 1998b) and have been reviewed by Dobromylskyj and others (2000). Keele originally described the SDS in 1948. The simple descriptive scale is a categorical scale comprising four or five expressions used to describe various levels of pain intensity (no pain, mild pain, moderate pain and severe pain) (Keele 1948). Each expression is assigned an index value, which becomes the pain score for the animal. The visual analogue scale (VAS) consists of a 100mm line anchored at one end 'no pain' and at the other end 'worst pain imaginable'. The clinician assessing the patient places a mark on the line to indicate the amount of pain they believe the patient to be suffering. The distance in mm from the 'no pain' end of the line to the mark is the pain score (Freyd 1923; Welsh and others 1993). The numerical rating scale (NRS) is similar but the observer assigns a numerical score for the pain intensity (Ley and others 1989; Welsh and others 1993) rather than placing a mark on the line, typically a scale from zero is used. In animals the most commonly used methods to assess pain rely on subjective observation of animal's behaviour and the use of SDS, VAS or NRS systems. Holton and others (1998a) investigated the association between physiological parameters, heart rate, respiratory rate and pupil dilation, and a subjective pain score using the NRS in groups of dogs which had undergone orthopaedic surgery, undergone soft tissue surgery, had non-painful medical conditions or were medically normal. They found that none of these variables were likely to be useful

indicators of pain in hospitalised dogs. Welsh and others (1993) used the VAS and NRS when assessing lameness in sheep and found good agreement between the two different systems and between two observers, but the VAS was more sensitive. Holton and others (1998b) compared the SDS, VAS and NRS as means of assessing pain in dogs post-operatively and found that while there was significant inter-observer variation, agreement amongst the different methods was reasonable. They suggested that the NRS system provided a suitable compromise between the over interpretation that can be a feature of the VAS and the lack of sensitivity that can be associated with the SDS. Morton and Griffiths (1985) suggested the use of a scoring system using a combination of appearance, appetite and water intake, behaviour, clinical signs and behavioural response to external stimuli to assess the presence of pain in animals. A similar approach was taken by Holton and others (2001) who constructed a composite scale based on observations of behaviour and the use of words and expressions similar to the approach described by Melzack (1975) for quantification of pain in humans. Words and expressions were allocated into seven behavioural categories (demeanour and response to people, posture, mobility, activity, response to touch, attention to painful area and vocalisation) to produce, in the authors' opinion, a more detailed scale than previously reported scales for assessing pain in dogs. In some instances animals, particularly prey animals may have evolved ways of disguising responses to pain or suppressing both nociception and pain, and this can confound pain assessment (Rutherford 2002), leading to requirement for a more qualitative assessment of pain.

1.3.3. Pain threshold testing

The phenomenon of hyperalgesia accompanying tissue and/or nerve damage has led to the use of pain threshold measurements in an attempt to quantify pain in various disease conditions. Pain threshold testing is the induction of physiological pain, with the level of response in normal animals compared to that in animals with tissue damage/inflammation. Various stimuli, including thermal, electrical, mechanical and chemical, can be used in pain threshold measurements (Beecher 1975). The skin with its greater accessibility of receptor organs is used more commonly than the viscera in experimental studies of pain (Beecher 1975). Threshold response testing using thermal stimulation involves the application of a heat stimulus, which could be direct contact applied to the skin by hot or cold objects or by radiant heat using projection lamps or

conducted heat using hot plate methods (Beecher 1975). An example of thermal stimulation from conducted heat was used in the studies by Nolan and others (1987) and Robertson and others (2003). Nolan and others (1987) used a resistor housed in a fine copper tube which was attached to the pinna of the ear in sheep to produce a clear flicking motion when the pain threshold was reached and Robertson and others (2003) used a heater element attached to the thorax of cats with twitching, flinching, turning to bite the stimulus and jumping forward indicating the pain threshold. Methods of mechanical stimulation includes the von Frey technique of producing pain by bending the epithelium, applying gross pressure to areas of the body, applying tourniquets to induce muscle ischaemia and causing distension of viscera by inflation of a balloon (Beecher 1975). An example of threshold response testing using gross pressure as the mechanical stimulation has been described by Nolan and others (1987) and Chambers and others (1990). Briefly, it involves a gas-driven moveable blunt-ended pin applying pressure to the limb and once the pain threshold is reached the response is the lifting of the limb or shifting of weight from that limb.

For a method to be useful in the assessment of pain and monitoring of analgesics the technique used must be reproducible and reliable with clear identification of the pain threshold and no damage to the tissue integrity. It might be expected that the responses to thermal stimulation may vary with the ambient temperature. The subject must be accustomed to application of the device to deliver the thermal stimulation to avoid pressure and touch affecting the threshold results. In both mechanical and thermal stimulation adaptation to the stimulation may be a problem, therefore sufficient time must be allowed between repeat measurements to allow the tissue to return to the state it was prior to the stimulation.

Hyperalgesia has been documented in many species with inflammatory disease (Nolan and others 1987; Ley and others 1989; Chambers and others 1990; Whay and others 1996; Fitzpatrick and others 1998; Lascelles and others 1998). A study assessing footrot in sheep measured the thermal and mechanical thresholds in the presence of the lesion after temporary alleviation of pain using local anaesthesia and following resolution of the lesion and compared the results to normal healthy sheep (Ley and others 1989). There was no significant difference in the thermal mechanical threshold between the normal sheep and the sheep with footrot at any of the time points. The sheep with footrot had a

significantly lower mechanical threshold response (mean 2.5Newtons (N) SE±0.6 low severity footrot and mean 2.7N SE±0.4 high severity footrot) than normal sheep (mean 4.4N SE±0.2). The application of the local anaesthetic resulted in the mechanical threshold of the sheep with footrot rising to levels that were not significantly different to the control sheep. Approximately three weeks after treatment and apparent resolution of the lesions the sheep with high severity footrot still had significantly lower mechanical threshold (mean 2.8N SE±0.3) than normal sheep. A larger study monitoring the pain thresholds in lame sheep compared to sound sheep used the mean threshold values on a flock basis and subtracted the measurements obtained from the lame sheep from the measurements from the sound sheep on the same flock to give a difference in threshold (Ley and others 1995). They found that 44% (12/27) of the flocks had a significant difference between the thresholds of sound sheep and lame sheep (mean difference 2.0N SE±0.4). In cattle, Ley and others (1996) reported significantly lower thresholds on the lame limb (mean 5.84N SE±0.3) compared to the thresholds of normal (mean 6.82N SE±0.1) cows. However, they found no correlation with qualitative severity using lameness scoring. A study by Welsh and Nolan (1995) monitored the effects of abdominal surgery on the response to thermal and mechanical stimulation in sheep every 30 minutes for a two hour period. They showed that thermal but not mechanical hyperalgesia was induced. These studies suggest that central changes in the processing of noxious information may not be detected by mechanical stimulation in the same time course as thermal stimulation. A group of heifers were monitored for a four month period, which spanned parturition, to assess the occurrence of lameness and monitor the pain threshold measurements (Whay and others 1997). These authors reported a significant decrease in pain threshold as the lameness severity increased, as judged by a NRS, indicating increased sensitisation to noxious stimulus with increasing severity of lameness The mean pain threshold for sound heifers was approximately 12N and for severely lame heifers 8N. A case-control study on pain induced by lameness in dairy cattle assessed using the response to noxious mechanical stimulation, found a significant reduction in the pain threshold on lame limb (mean 7.9N SE±0.3) compared to control cows (mean 13.3N SE±0.3), however, in cows that were bilaterally lame the pain threshold was only slightly decreased (mean 11.3N SE±0.8) (Whay and others 1998). The unilaterally lame cows were re-examined one month later and the pain threshold was still significantly reduced in cows with solar ulcers (mean 8.8N SE±0.5) and white line

disease (mean 9.7N SE±0.5) but not in cows with acute digital tissue infection, which included 'foul-in-the-foot' and digital dermatitis (mean 12.7N SE±0.6) compared to normal cows (mean 13.3N SE±0.3) assessed on day of recruitment to the study (Whay and others 1998), indicating that the persistence of hyperalgesia differed between lesion type.

Pain threshold measurements can be used to assess the efficacy of analgesics (Ley and others 1989; Chambers and others 1990; Welsh and Nolan 1994; Lascelles and others 1998). Mean mechanical thresholds for sheep with mild and severe footrot were increased from 2.5N (SE±0.6) and 2.7N (SE±0.4) to 4.0N 9SE±1.0) and 3.6N (SE±0.5), respectively, following administration of a local anaesthetic block and local infiltration above the affected foot and below the mechanical testing device using 0.25% bupivacaine HCl (Ley and others 1989). Welsh and Nolan (1994) conducted a study involving the application of a tourniquet to induce localised ischaemia in sheep to allow the investigation of the efficacy of analgesic drugs. Noxious mechanical stimulation was used to assess the presence of pain. It was found that the application of the tourniquet decreased the pain thresholds on the ipsilateral leg (mean 1.7N SE±0.3) but not on the contra-lateral leg (mean 3.1N SE±0.3), thereby indicating that the mechanical hyperalgesia observed was a local peripheral effect. They reported that intravenous administration of flunixin meglumine, at 1.0 mg/kg, and carprofen, at 0.7 mg/kg had a beneficial pain relieving affect, in that the administration of these drugs lessened the degree of reduction in pain thresholds brought about by the application of the tourniquet. Lascelles and others (1998) used combination of a VAS scoring system and mechanical pain threshold testing to assess the efficacy of the non-steroidal anti-inflammatory drug (NSAID), carprofen, in bitches undergoing elective ovariohysterectomy. They reported that both pre- and post-operative carprofen administration significantly reduced the degree of pain exhibited, however, pre-operative carprofen had greater analgesic properties in the early post-operative period compared to post-operative carprofen.

1.4. Pathogenesis of clinical mastitis

The primary defence mechanism of the mammary gland is the teat canal, as this represents the portal of entry for nearly all mastitis-causing micro-organisms (Bramley

and Dodd 1984; Craven and Williams 1985; Nickerson 1985; Sandholm and others 1990; Bramley 1992; Vestweber and Leipold 1993). Injury to the teat end and teat canal almost always leads to intramammary infection (Benedixen 1935; Ferguson 1944; Smith and Hogan 1996). The development of intramammary infections occurs by the contamination of the teat end with pathogenic organisms (Tolle 1975; Bramley and Dodd 1984), followed by penetration into the teat canal and the colonisation by the organisms in the sinuses, ducts or alveolar tissue (Kingwill 1980; Bramley and Dodd 1984). Any infection gaining access to the gland must remain adherent to the inner surfaces, despite the out-flow of milk, and escape the action of the defence mechanisms (Reichmuth 1975). The division of the udder into four independent quarters helps to restrict the spread of infection (Reichmuth 1975). The transfer of some pathogens between quarters, however, is inevitable at milking time, even under the best hygienic conditions (Philpot 1975). Most infections which occur in the dry period develop in the first two or three weeks after drying off and are thought to be due to pathogens remaining in the udder from the lactation (Philpot 1975).

1.5. Treatment of clinical mastitis

Elimination of infection from infected quarters within a herd can be achieved by (1) the defence mechanisms of the udder eliminating the pathogen i.e. spontaneous cure, (2) by treatment during lactation, (3) by treatment during the dry period, or (4) by culling infected animals (Bramley and Dodd 1984). Spontaneous recoveries often equal the number of infections eliminated by antibiotic therapy in lactating cows (Griffin and others 1982), but spontaneous recoveries often take longer i.e. there may be a prolonged period of infection before recovery occurs (Bramley and Dodd 1984). The proportion of spontaneous elimination is low for staphylococcal infections at < 20%, high for *E coli* at > 70%, and intermediate for streptococcal infections 40-60% (Bramley 1992). Treatment of mastitis is carried out for many reasons: (1) to treat clinical infections in order to regain normal milk secretion, (2) prevent progression of the disease, (3) improve the welfare of the cow, (4) eliminate clinical or subclinical infections, or at least reduce their number and frequency, so that the milk yield from the herd is improved, (5) prevent the spread of infection, by eliminating the main source of bacteria, in the case of infectious organisms, (6) prevent development of infection in an animal or herd exposed to a

particular or temporary risk, and (7) avoid penalties imposed by milk purchasers for milk with high SCC (Plommet and le Louedec 1975; Blowey and Edmondson 2000).

1.5.1. Antimicrobials

Antimicrobials have been used in the treatment of mastitis for over 50 years. Mastitis is now the most frequent reason for the use of antimicrobials in dairy herds (Meek and others 1986; Gardner and others 1990; Grave and others 1999). With intramammary treatment, the antimicrobial drug tends to remain within the treated quarter, but some will diffuse into the blood, circulate systemically and diffuse into untreated quarters, therefore, milk from all quarters should be withheld from the bulk tank for the prescribed withdrawal period for that particular preparation (Blowey and Edmondson 2000). The extent of drug crossing into the circulation will depend on the physico-chemical properties of the drug and the degree of inflammation.

The therapeutic success of antimicrobial therapy in mastitis depends on; (1) the sensitivity of the organism and its' ability to evade the effects of the antimicrobial, (2) the concentration and persistence of the drug in the environment of the pathogen, and (3) the contribution made to exclusion of the organism by the host immunity, which may be influenced by concurrent therapy, disease or age (McKellar 1996). Treatment efficacies for intramammary infections during lactation reported by Smith and Hogan (1996) were: 80-90% for *S agalactiae*, 50-60% for coagulase negative staphylococcal infections, 50% for environmental streptococcal infections, 30-50% for *Staph aureus*, < 10% for Gram-negative infections and no response was seen in infections caused by yeasts, moulds, *Mycoplasma* spp. and *Prototheca* spp., using the antimicrobials benzathine penicillin and cephalosporins. Similar cure rates were recorded by Huber (1977) using a benzathine penicillin, novobiocin and penicillin product or a spiramycin product: 100% for *S dysgalactiae*, 98% for *S agalactiae*, 82% for *S uberis* and only 35% for *Staph aureus*.

Antimicrobial therapy is more effective in the dry period than during lactation, and is more effective after the first clinical episode than after further repeated episodes of infection, i.e. younger cows respond better than older cows (Sandholm and others 1990; Meaney 1992; Lawrence 1997). The intramammary route is a practical, inexpensive and convenient route for the treatment of bovine mastitis (McKellar 1991; Vestweber 1993).

In acute severe disease, however, the distribution of intramammary antibiotics may be impaired by inflammation within the udder (Sandholm and others 1990; Prescott and Baggot 1993) and the presence of systemic signs due to bacteraemia may, therefore, necesitate parenteral antibiotics. A study by Wenz and others (2001) reported that 32% (46/144) of cows with naturally occurring acute coliform mastitis were bacteraemic, assessed by the identification of bacteria in blood. The choice of antibiotic will depend on the *in vitro* sensitivity of the organism, and it should be remembered that the sensitivity may change over a period of time, especially following intensive antibiotic usage, and also on different farms (Owens and Watts 1988; McKellar 1991). The antibiotic for mastitis therapy should be chosen after considering the activity, pharmacodynamics and pharmocokinetics of the drug, the probable cause of mastitis, the milk withdrawal period (McKellar 1991) and finally after all other considerations, the cost, though the cost of discarded milk may be more significant than the cost of the antimicrobial drug (Prescott and Baggot 1993).

Penicillin G is probably the most effective antibiotic against penicillin-sensitive organisms and where *Streptococcus* spp, *Arcanobacterium pyogenes* or sensitive *Staph aureus* are the aetiological agents (McKellar 1991). Penethamate is a basic ester of penicillin G that has similar but enhanced physicochemical characteristics and therefore has similar overall activity to penicillin G. Penicillin is less effective against many *Staph aureus* strains due to the production of β-lactamase which renders the organism resistant to penicillin (Sandholm and others 1990). Clavulanic acid is a synthetic drug that results in the irreversible inhibition of β-lactamase (Sandholm and others 1990; McKellar 1991). It is combined in commercial preparations with amoxycillin to produce a drug with a broad spectrum of activity and good action against β-lactamase producing *Staph aureus* (Sandholm and others 1990; McKellar 1991). Cloxacillin and nafcillin are potentiated penicillins with better activity than amoxycillin against *Staph aureus* and are also effective against β-lactamase producing strains (McKellar 1991). Cephalosporins are semi-synthetic, bactericidal antibiotics with good activity against Gram-positive bacteria and are resistant to β-lactamase (McKellar 1991). Macrolides are bacteriostatic antibiotics with good tissue distribution, high intracellular concentrations and activity against Gram-positive bacteria, including *Staph aureus*, and *Mycoplasma* spp. (McKellar 1991). The Gram-negative activities of aminoglycosides are useful to combat coliform

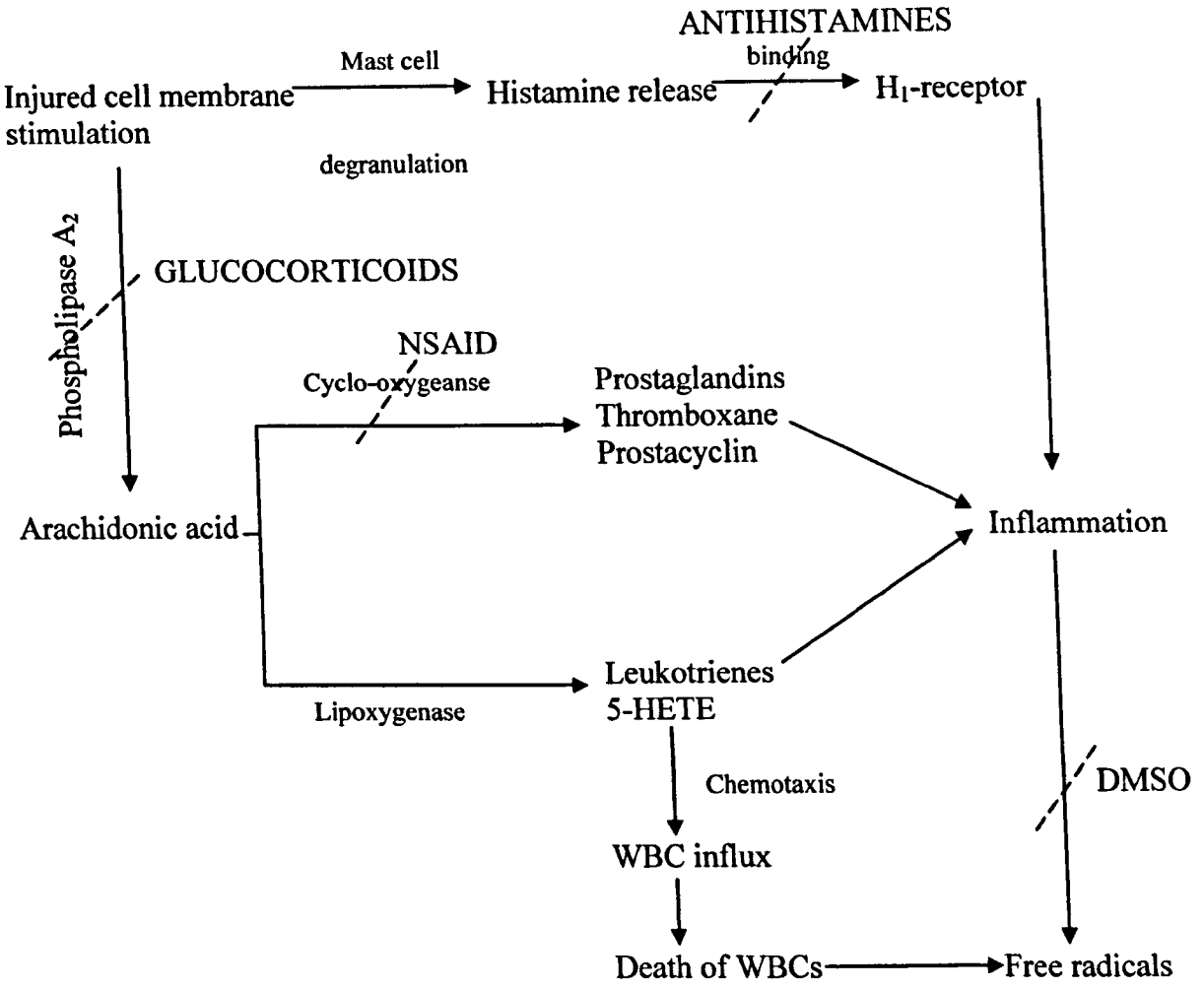
organisms. Many products contain penicillin and aminoglycoside combinations. Tetracyclines are active against Gram-negative and Gram-positive organisms with some activity against β-lactamase producing *Staph aureus* (McKellar 1991).

1.5.2. Anti-inflammatory drugs and analgesics

Anti-inflammatory drugs are widely used in veterinary practice in order to provide symptomatic relief in the treatment of both acute and chronic inflammatory conditions (Cunningham and Lees 1994). The principal value of these drugs is to relieve pain and reduce swelling. The most commonly used drugs are the corticosteroids (glucocorticoids) and NSAID. These drugs act by inhibiting the formation or release of mediators with inflammatory properties, Figure 1.1 shows the site of action of the common anti-inflammatory agents. Common uses in veterinary medicine include the management of musculoskeletal and joint disorders and in endotoxaemic conditions such as *E coli* mastitis.

1.5.2.1. Corticosteroids

Glucocorticoid agents are reported to be the most effective anti-inflammatory agents (Langston 1999). These drugs inhibit the activity of the enzyme phospholiase A_2 (Figure 1.1), thus inhibiting the formation of prostaglandins, thromboxane, leukotrienes and platelet activating factor (Cunningham and Lees 1994; Langston 1999). There are several different glucocorticoid agents with differing formulations causing the onset and duration of action and routes of administration to vary. As a group, these drugs are associated with a wide range of side-effects and in general they tend to delay healing and the immunosuppressive effects and modification of the inflammatory reactions may facilitate the progression of concurrent infectious disease. Of particular importance in the therapy of food animals is the abortifacient activity of the glucocorticoids; the non-methylated compounds given parenterally, such as prednisolone, have a lower risk of inducing abortion (Lauderdale 1972). Corticosteroids are often added to antibiotics in intramammary preparations to reduce inflammation in the udder tissue. Prednisolone has not been shown to adversely affect the function of white blood cells in the udder, despite being proven to be generally immunosuppressive (McKellar 1991).



NSAID Non-steroidal anti-inflammatory drug; 5-HETE 5-Hydroperoxyeicosatetraenoic acid; DMSO Dimethyl sulphoxide; WBC White blood cells

Figure 1.1. Sites of action of common anti-inflammatory agents (Langston 1999)

In experimental models of mastitis, when used in combination with antibiotics 10mg of prednisolone infused by the intramammary route significantly increased the resolution of swelling compared to antibiotics only (McKellar 1991). Experiments by Bywater and others (1988) showed that prednisolone effectively increased the rate of reduction of the swelling seen in mastitis, with no effect on SCC in milk.

1.5.2.2. Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs are relatively weak acids and are well absorbed from the upper gastrointestinal tract (Jenkins 1987). As a group, NSAIDs have anti-inflammatory, analgesic (peripheral and central sites of action) and anti-pyretic properties (central site of action) (Nolan 2000). They inhibit the transformation of arachidonic acid to stable PGs (i.e. PGE_2 and prostacyclin), by inhibition of cyclo-oxygenase (COX) enzymes, whose products elicit many signs of inflammation. There is, however, a lack of evidence to generalise this hypothesis to all products of the arachidonic acid cascade and all NSAIDs at all dosages (Abramson and Weissmann 1989). There are three general types of COX inhibitors: reversible competitive inhibitors, irreversible inhibitors and anti-oxidant radical trapping inhibitors (Jenkins 1987). It was discovered in the 1990's that there were two isoforms of the COX enzyme inhibitors (Xie and others 1991) and a third isoform was identified in the 2000's (Willoughby and others 2000). Cyclo-oxygenase oxidises arachidonic acid to PGG_2 through a COX activity and then peroxidises PGG_2 to PGH_2. Following this cell specific prostaglandin synthases convert PGH_2 into a series of PGs including PGI_2, $PGF_{2\alpha}$, PGD_2 and PGE_2.

Cyclo-oxygenase-1 (COX-1) is a constitutive enzyme concerned with such roles as gastroprotection, renoprotection and, through the release of TXA_2, platelet aggregation in the process of blood clotting (Monacada and others 1976; Whittle and others 1980; Vane 1994; Lees and others 1998). Cyclo-oxygenase-2 (COX-2) is normally absent from tissues but may be induced by mitogens, lipoploysaccharide and inflammatory mediators including interleukin-1_β (IL-1_β) and TNFα, resulting in the synthesis and release of mediators such as PGE_2 at sites of inflammation (Xie and others 1992; Lees and others 1998). Synthesis of TXB_2 and exudate PGE_2 is mediated by COX-1 and COX-2 respectively (Landoni and Lees 1997), thus the degree of inhibition of serum TXB_2 is used as an indictor of COX-1 and the degree of inhibition of PGE_2 in inflammatory

tissue/fluid is used as an indicator of COX-2 (Lees and others 1998). The actions of COX-1 and COX-2 are summarised (Vane 1994) in Figure 1.2. The activation of COX-1 leads to the production of prostacyclin which, when released by the endothelium, is anti-thrombogenic (Monacada and others 1976) and when secreted by the gastric mucosa, is cytoprotective (Whittle and others 1980).

The second isoform, inducible enzyme COX-2 is unregulated in damaged tissue and is believed to be primarily responsible for synthesis of PGs that increase the sensitivity of the nociceptive fibres to pain stimuli and pain mediators after injury (Ricketts and others 1998). Cycloxygenase-2 is found at sites of inflammation, whereas COX-1 is considered to be the 'housekeeping' enzyme serving physiological roles (Landoni and Lees 1997). Vane (1994) reported that the undesirable side-effects of NSAIDs are due to their ability to inhibit COX-1, whereas their anti-inflammatory (therapeutic) effects are due to inhibition of COX-2. The roles of COX-1 and COX-2 may not be as clear as previously reported. Wallace (1999) reported that there are examples where COX-1 derived PGs contribute to the generation of inflammation and COX-2 derived PGs play a crucial role in the maintenance of gastro-intestinal integrity. Therefore, suggesting that selective inhibition of COX-2 might not produce anti-inflammatory effects comparable to those achieved by combined COX-1 and COX-2 inhibition (Wallace 1999). A comparative *in vitro* study demonstrated that meloxicam inhibited the synthesis of PGE_2 (Ricketts and others 1998). The identification of selective inhibitors of each isoform will not only provide an opportunity to test this hypothesis but also lead to advances in the therapy of inflammation (Vane 1994). Prostaglandin E_2 is directly involved in the maintenance of mucosal blood flow and involved in the production of gastric mucus macromolecular glycoproteins that protect the gastric mucosa, thus drugs with significant efficacy and potency against COX-1 are highly ulcerogenic (Nolan 2000). Thus, COX-2 is thought to be the optimal therapeutic target. Nephropathy is another important toxic effect of NSAIDs, however, in healthy animals NSAIDs have very little effect on the renal function (Jenkins 1987).

The third COX isoform was identified by Chandrasekharan and others (2002), but its presence was postulated earlier by Willoughby and others (2000). Willoughby and others (2000) detected the presence of a second substantial increase in COX-2 like protein expression by Western blotting when investigating the duration of selective and dual

COX inhibitors. Cyclooxygenase-3 is unlike COX-1 and COX-2 in that it does not produce pro-inflammatory prostanoids instead it produces anti-inflammatory members of the prostanoid family (Willoughby and others 2000). Chandrasekharan and others (2002) demonstrated using insect cells that COX-3 was more sensitive to acetaminophen (paracetamol) than COX-1 or -2. The inhibition of COX-3 might be the long sought after mechanism of action for aceteminophen and could represent a primary central mechanism by which the NSAIDs could decrease pain and possibly pyrexia (Chandrasekharan and others 2002) and may prove to be the new therapeutic target (Willoughby and others 2000).

Non-steroidal anti-inflammatory drugs inhibit the isoenzymes to various degrees (Kay-Mugford and others 2000), thereby resulting in a wide variation in the profiles of the available NSAIDs. It was thought that NSAIDs only inhibit prostanoid function and thus have no effect on other inflammatory mediators involved in mastitis, and therefore will have no direct effect on milk SCC or bovine serum albumin (Persson 1990). McCormack and Brune (1991) and Twomey and Dale (1992) suggest that there is evidence that NSAIDs may have anti-inflammatory and other effects, which are independent of COX inhibition. Landoni and others (1995a, 1995b, 1996) reported that flunixin, ketoprofen and tolfenamic acid effectively inhibited β-glucuronidase activity, and bradykinin induced swelling in calves, in addition, to inhibition of COX. Other actions of NSAIDs have been reported but not assessed for therapeutic effects (Abramson and Weissman 1989; Vane 1994). At high doses aspirin, sodium salicylate and newer NSAIDs (at anti-inflammatory doses) inhibit non-PG dependent processes, such as the activity of enzymes, proteoglycan synthesis by chondrocytes, transmembrane ion fluxes, chemoattractant binding (Abramson and Weissmann 1989). The ability of NSAIDs to inhibit the activation of inflammatory cells such as the neutrophil may contribute to the anti-inflammatory properties of this class of drug (Abramson and Weissmann 1989).

Non-steroidal anti-inflammatory drugs are highly protein bound, > 99% in some cases. The extent of protein binding explains the extended therapeutic activity in inflamed tissues that these drugs display relative to their plasma elimination half-lives (Nolan 2000). Many of the NSAIDs are available as solutions for parenteral administration, but most preparations are highly alkaline and are thus tissue irritant (Jenkins 1987).

A number of studies have shown that there is a marked species difference in the pharmacokinetic parameters such that data cannot be transposed between species (McKellar and others 1990; Welsh and others 1993; McKellar and others 1994; Cunnigham and Lees 1994; Landoni and others 1995a; Landoni and others 1995b; Landoni and others 1996). Jenkins (1987) suggested that the elimination kinetics of NSAIDs showed the greatest differences between species of all the drugs used in animals. The elimination rate of NSAIDs in ruminants tends to be slower than in monogastric species such as the horse and dog, e.g. the half-life of phenylbutazone in plasma was 3 to 6 hours in the horse, but 36 to 72 hours in cattle (Jenkins 1987) and the elimination half-life for meloxicam was 13 hours in cattle, four hours in the mini-pig and two hours in the horse, but 12-36 hours in the dog (Lees and others 1991). The impact of the anti-pyretic, anti-inflammatory and analgesic effects produced by these drugs is largely dependent on the stage of the inflammatory process at which treatment is commenced (Baggot 2001), e.g. early diagnosis of coliform mastitis and prompt initiation of treatment with flunixin allowed maximal anti-pyretic and anti-inflammatory actions to be exhibited.

Non-steroidal anti-inflammatory drugs have been advocated for the treatment of coliform mastitis (Lohuis 1991; Ziv 1992). DeGraves and Anderson (1990) observed beneficial effects on various clinical, haematological, biochemical and pathological parameters, however, they suggested that there was no evidence that these drugs actually improved cow survival or milk production. In field cases of clinical mastitis, Shpigel and others (1994) reported that 2 g of ketoprofen administered intramuscularly once daily for a maximum of five days significantly improved recovery from clinical mastitis in dairy cows; recovery was categorised as a return to at least 75% of the pre-mastitis daily milk production. Many studies have been conducted over the last decade investigating the use of NSAIDs in castration and dehorning procedures. Ketoprofen was found to reduce behavioural signs of pain in calves after hot-iron dehorning (McMeekan and others 1999; Faulkner and Weary 2000) and was found to attenuate the increase in plasma cortisol associated with calf castration (Earley and Crowe 2002). In lambs, the use of carprofen did not have any effect on the behavioural aspects of discomfort when the rubber ring method was used to castrate and/or dock tails (Price and Nolan 2001). In bovine respiratory disease there was found to be no difference clinically in the degree of depression, illness score, dyspnoea or coughing between groups of calves treated with

antibiotics (ceftiofur) alone, antibiotics plus carprofen, antibiotics plus ketoprofen and antibiotics plus flunixin (Lockwood and others 2003). There was, however, less lung consolidation in the three groups treated with NSAIDs, but this was only significant for the group that were treated with flunixin (Lockwood and others 2003).

At present the NSAIDs that are licensed for use in lactating dairy cattle include; flunixin meglumine, ketoprofen, meloxicam and tolfenamic acid. Flunixin meglumine is a nicotinic acid derivative. Flunixin is licensed for use by the intravenous route, at a dose rate of 2.2 mg/kg once daily for up to five days and has a half-life of eight hours. Ketoprofen is a propionic acid NSAID. Ketoprofen is licensed for use by the intramuscular or intravenous routes at a dose rate of 3 mg/mkg once daily for up to three days and has a half-life of 0.4 hours. Tolfenamic acid belongs to the fenamate group. Tolfenamic acid is licensed for use by the intravascular route at a dose rate of 2 mg/kg as a single administration and has a half life of 11.3 hours. Meloxicam is an enolic acid derived NSAID of the oxicam group. Meloxicam is licensed for use by the intravenous and subcutaneous routes at dose rate of 0.5 mg/kg as a single dose and has a half-life of 13 hours by the intravenous route.

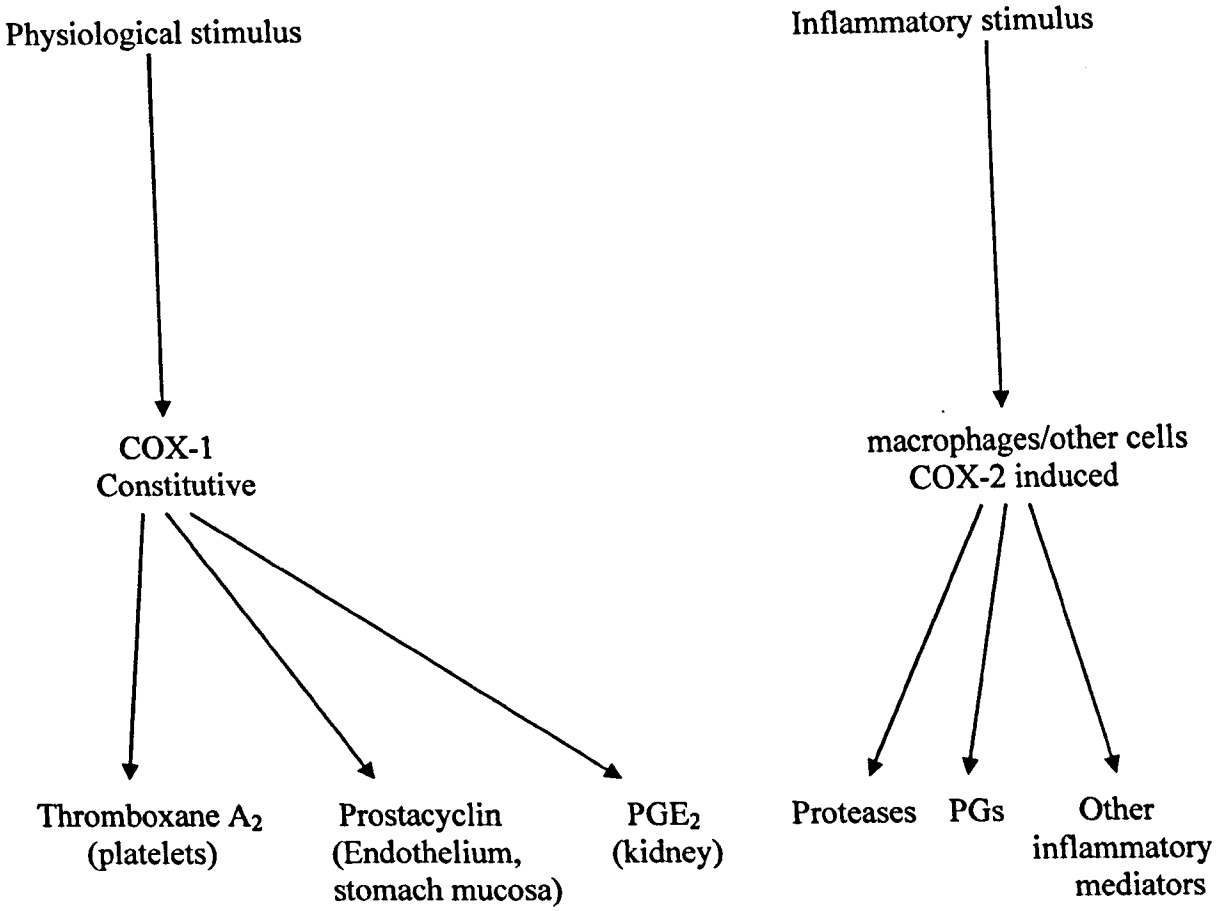
Meloxicam preferentially inhibits human COX-2, but at higher concentrations is an equipotent inhibitor of COX-1 and COX-2 in humans (Churchill and others 1996). In rats and guinea pigs, meloxicam was consistently shown to be COX-2 preferential *in vitro*, but also capable of sustained COX-2 inhibition *in vivo* (Engelhardt and others 1995). Meloxicam has a more favourable gastrointestinal profile, in rats and guinea pigs, compared to other NSAIDs and is reported to be a potent anti-inflammatory agent in acute and chronic inflammation (Engelhardt and others 1995; Churchill and others 1996). Meloxicam was licensed for use as an oral solution in dogs in 1993. In 1998 meloxicam was licensed for use in non-lactating cattle as a 5mg/ml solution for injection, and subsequently gained its licensed for use in lactating cows in 2001. This was later followed by the development and launch of a 20mg/ml solution for injection. Meloxicam can be administered by a single subcutaneous or intravenous injection at a dose rate of 0.5mg meloxicam/kg bodyweight. It is indicated for (a) use in acute respiratory infection with appropriate antibiotic therapy to reduce clinical signs in cattle (b) use in diarrhoea in combination with oral rehydration therapy, to reduce clinical signs in calves of over one week of age and young non-lactating cattle, and (c) adjunctive therapy in the treatment of

acute clinical mastitis, in combination with antibiotic therapy. The elimination half life is 26 hours and 17.5 hours after subcutaneous injection in young cattle and lactating cows, respectively (Anon 2003).

1.5.2.3. Analgesic drugs

The term analgesia means the absence of pain, but is commonly used to mean a reduction in perceived pain (Carstens 1987). In animals, analgesia is usually defined as an increase in threshold and/or latency for withdrawal reflexes (Carstens 1987). The administration of analgesic agents in association with other treatments may affect the magnitude and duration of hyperalgesia through the modulation of noxious inputs to the spinal cord (Lascelles and others 1998; Slingsby and Waterman-Pearson 1998; Whay and others 1998). Analgesic drugs can be categorised into (1) opioids, (2) NSAIDs, (3) local anaesthetics, (4) α_2-adrenoceptor agonists, (5) centrally acting non-opioid drugs, e.g. antidepressants, which appear to have an analgesic action in patients not suffering from depression, (6) drugs used for specific painful conditions e.g. ergotamine for migraine, and (7) miscellaneous e.g. ketamine, combination product containing phenylbutazone, and ramifenazone.

The main type of drug used for analgesia in severe pain is the opioids, which have been available for animal use since the 1960s (Branson and Gross 2001). The opioids act at specific sites in the central nervous system (CNS) and other tissues. Three are three main receptor types; μ (mu), κ (kappa) and δ (delta). Stimulation of μ receptors results in analgesia, mainly at supraspinal sites, respiratory depression, miosis, reduced gastrointestinal motility and euphoria. Stimulation of κ receptors produces analgesia mainly at the spinal cord level and less intense miosis and respiratory depression. Stimulation of δ receptors probably provides analgesia. The opioid analgesics act at one or more of these receptors as agonists, antagonists, or a combination of both (partial agonists). There are major species differences in the responses elicited by opioids. Cats, horses, cattle, sheep, goats and pigs often become hyperexcited at high doses. Excitement is, however, less likely in animals in pain compared to pain-free animals. Opioids are subject to the Misuse of Drugs Regulations 1985 due to the potential addiction in humans and it is recommended that they be used only when there is no non-opioid alternative for



COX Cyclooxygenase; PGE_2 Prostaglandin E_2; PGs Prostaglandins

Figure 1.2. Actions of the two known isoforms of COX (Vane 1994)

moderate to severe pain and where possible newer, less addictive drugs should be used rather than morphine or methadone. At present there are no opioids licensed for use in ruminants.

The most widely available analgesics for cattle in the UK are the non-steroidal anti-inflammatory drugs. Ketoprofen, flunixin meglumine, tolfenamic acid and meloxicam are all licensed for use in lactating cattle and carprofen is licensed for use in calves. The NSAIDs interfere with the initiation of peripheral pain impulses (Einstein and others 1994).

Xylazine, medetomidine, romifidine and detomidine are α_2-adrenergic agonists, they are used as dose-dependent sedatives. They are classed as having analgesic and skeletal muscle relaxant properties, in addition to being a sedative (Gross 2001). They are generally used for restraint, to facilitate handling and transport and to modify behaviour and not as a primary analgesic agent. Xylazine can be used by the epidural route to provide sensory anaesthesia with minimal ataxia. Xylazine is licensed for use by the intramuscular route in cattle.

Ketamine is a dissociative anaesthesic. It interrupts the association between the limbic and cortical sytems such that an animal may appear to be under a light plane of anaesthesia but is insensitive to surgical stimulation. It is often combined with an α_2-adrenergic agonist to improve muscle relaxation. Ketamine is not licensed for use in ruminants.

Tomanol® is a combination product that contains the NSAIDs phenylbutazone, and ramifenazone. It is used for analgesia and inflammation in soft tissue injury and lameness in the horse.

Local anaesthetics are drugs that when applied locally to nerve tissue cause reversible blockade of nerve impulse conduction, thus they prevent the perception of pain (Einstein and others 1994). The blockade of conduction along pain fibres is useful for diagnostic purposes and to permit minor surgery. The speed of onset of the nerve blockade depends on the drug used, its concentration, the accuracy of injection and the diameter of the nerve. The duration of activity depends on the type of drug, the amount used, the site of

injection and whether or not a vasoconstrictor, such as adrenaline, has been used. The only licensed local anaesthetic product for ruminants is procaine hydrochlroide, which has a short duration of activity as it is absorbed rapidly and destroyed quickly (Mama and Steffey 2001).

Carroll (1996) suggested that if analgesics were successfully used in the peri-operative period, then post-operatively the animal should be eating, drinking, urinating and grooming as normal and its heart rate, respiratory rate and mucous membranes colour should be within the normal ranges. Many studies have been conducted over the last decade looking into the use of NSAIDs in castration and dehorning procedures. Ketoprofen was reported to reduce behavioural signs of pain in calves after hot-iron dehorning (McMeekan and others 1999; Faulkner and Weary 2000) and it attenuated the increase in plasma cortisol associated with calf castration (Earley and Crowe 2002). It is believed that pre-operative administration of analgesics has the greatest effect by reducing or eliminating the 'wind-up' mechanism generated by the barrage of stimulation of the nociceptors created as a result of surgical procedures. This was exhibited in a study testing the efficacy of carprofen in bitches undergoing elective ovariohysterectomy. Both pre- and post-operative carprofen administration significantly reduced the degree of pain exhibited, however, pre-operative carprofen had greater analgesic properties in the early post-operative period compared to post-operative carprofen (Lascelles and others 1998). In lambs the use of carprofen before castration or docking, however, did not have any effect on the behavioural aspects of discomfort when the rubber ring method was used to castrate and/or dock tails (Price and Nolan 2001).

The administration of NSAIDs intravenously or intramuscularly in cattle, in addition to the normal treatment for both mastitis and lameness, has been found to result in significant reductions in the levels or duration of hyperalgesia (Whay and others 1998; Fitzpatrick and others 1999).

A small study to investigate and control pain in cows with natural mastitis was conducted by Fitzpatrick and others (1998). These authors reported that on application of a mechanical stimulus cows with clinical mastitis showed an increased sensitivity to pain compared to normal animals. They showed that 2.2 mg/kg of intravenous flunixin megluine, a short duration NSAID, attenuated mechanical hyperalgesia in cows with

mild mastitis, but only for a short period, and not in moderate cases. These results suggest that NSAIDs were beneficial in reducing the hypersensitivity in bovine mastitis. A larger study with a longer duration NSAIDs and/or repeat dosing may show an extended duration of effect and might produce an effect in more severely affected cases.

1.5.3. Supportive therapy

In addition to NSAIDs and glucocorticoids, a number of other options are available as an adjunct to antibiotics in cases of clinical mastitis. Possible additional treatments include the adminstration of oxytocin and/or frequent milkout of the gland, adminstration of calcium, adminstration of intravenous isotonic or hypertonic saline solutions, oral electrolytes (Ziv 1992; Morin 1999) use of uddermint, herbal treatments, homeopathy and cold water massage (Hovi 1998). Green and others (1997) compared three treatments for toxic clinical mastitis: 45 litres intravenous isotonic electrolyte and flunixin, fluid therapy alone, or flunixin alone. In addition, all cases were treated with parenteral and intramammary oxytetracycline, oxytocin and calcium boroglconate. These authors found that there was no significant difference in the rate of survival between the treatment groups.

1.6. Aims and objectives

Clinical mastitis remains one of the most costly diseases in dairy farming and is associated with adverse animal welfare with the induction of pain and prolonged hyperalgesia. Several different inflammatory mediators have been recognised with APP promising to have good predictive value in early recognition of clinical mastitis. Antimicrobials have been used in the therapy of clinical mastitis for decades but it is only recently that interest has moved to adjunctive therapies such as NSAIDs. Further information on the benefits, both from a welfare and economic point of view, of concurrent non-antimicrobial therapies would be valuable for dairy cattle and farmers in the UK.

Consequently it was considered timely to carry out a study of naturally occurring cases of acute mastitis using commercial dairy farms in order to study :

(1) subjective and objective clinical measurements of pain assessment in cows presenting mild or moderate acute mastitis,

(2) the clinical response *in vivo* of the NSAIDs, meloxicam, in cows with acute clinical mastitis on commercial farms,

(3) to establish, using mechanical stimulation, whether cows with acute mastitis display mechanical hyperalgesia, and if so for how long,

(4) to investigate the production effects of a typical acute incident of mastitis in terms of milk yield and milk quality, and

(5) to study inflammatory mediators and APP in milk and to quantify the effect of NSAIDs.

Chapter 2

Materials and methods

2.1. Study population

The cows in this study were commercial dairy cows milked twice daily, kept at one institute farm and 11 commercial dairy farms. The farms varied in size from 60 to 300 dairy cows, and the majority of cows were Holstein/Friesian. Other breeds included pedigree Holstein, Guernsey, Shorthorn, Ayrshire and crossbreds.

2.1.1. Cow details

On-farm records were referred to for details on the stage of lactation, parity and age of the cows recruited onto the study. The data for daily milk yield in litres per day were recorded from milk meters, where available, or from recording jars by the farmer.

2.2. Case recruitment

Farmers were asked to telephone the author on detection of a cow with mild or moderate clinical mastitis. Clinical mastitis was recognised by the dairyman on each farm by inspection of the milk and by observation and/or palpation of the udder at milking times. The same researcher (MM) visited each cow within two hours of mastitis being detected by the farmer and confirmed the presence of clinical mastitis. Cases were recruited only if they fitted the definition of mild or moderate clinical mastitis and did not include any of the exclusion criteria (Appendix 1). The severity of mastitis was assessed using clinical criteria as previously described (Hogan and others 1989a; Bradley and Green 2001; Eckersall and others 2001). Clinical mastitis was defined as 'mild' when there were changes in the milk appearance but the udder was normal, and 'moderate' when there were changes in the milk appearance and the udder was hot, swollen or painful to touch, but the cow was not off her food, dull or requiring systemic antibiotic therapy

(Figure 2.1). Adult cows that developed acute clinical mastitis were studied for up to six weeks. Cows were excluded from the study if they were suffering from severe acute or peracute mastitis or were suffering from any other inflammatory condition (Appendix 1) in addition to having mild or moderate mastitis. Normal, healthy cows (no history of any inflammatory condition within the last two months) (Appendix 1) were monitored as controls. Normal cows were recruited the same day as case cows and were either being milked directly before or after case cows. Case cows and normal cows were recruited and studied from October 2002 to June 2003.

2.3. Study design

Cows with one quarter affected by clinical mastitis were treated and assessed over a 45 day period. Normal cows (n=45) were recruited to act as controls for day 0 parameters (day of recruitment) and twenty of these cows were followed for a 45 day period to serve as controls for the time period. All cases of clinical mastitis were treated by the researcher according to routine veterinary practice, with intramammary antimicrobial drugs on the day of diagnosis after all measurements and samples had been collected. A broad spectrum antimicrobial preparation was selected, 25mg cefquinome/ml (Cephaguard LC Intramammary, Intervet UK Limited, Milton Keynes), and this was infused every 12 hours for three treatments for every case of mastitis. Cases were allocated randomly to one of three treatment groups using the random data function in Minitab Statistical Software release 13 (Minitab Inc.) (Appendix 2).

Group 1: antibiotics only

Group 2: antibiotics and one dose of 20mg meloxicam/ml (Metacam solution for injection, Boehringer Ingelheim Limited, Bracknell) (at dose rate 0.5mg/kg i.v./s.c.)

Group 3: antibiotics and three doses of meloxicam (at dose rate 0.5mg/kg i.v./s.c. every 3 days)

For cases of mastitis and normal cows, recordings were made on the day of recruitment (day 0), and subsequently on days 1, 2, 6, 20 and 42-45 after initial diagnosis.

Measurements were also taken on days 3 and 7 after initial diagnosis for cases in treatment group 3.

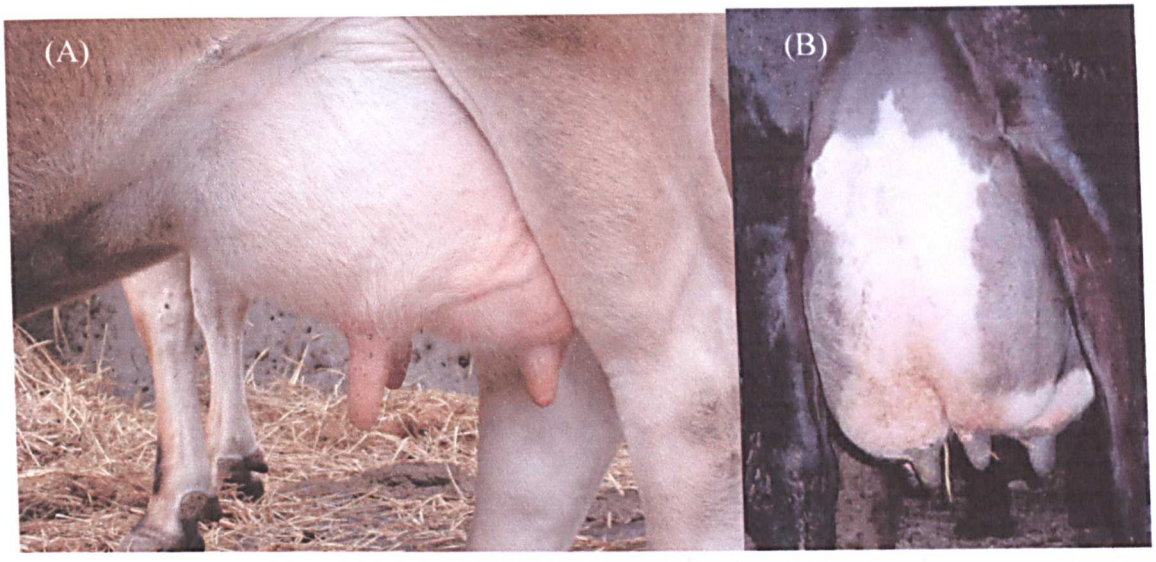


Figure 2.1: Cow with mild (A) mastitis in front left quarter showing no udder changes and cow with moderate (B) mastitis in back left quarter exhibiting signs of udder swelling and redness

2.4. Milk sampling

Milk samples were obtained from the affected mammary gland of each case cow for somatic cell counting and bacteriological examination and additional samples were taken from the unaffected diagonally opposite quarter for somatic cell counting. Differential somatic cell counting was carried out in milk sampled from the mastitic quarter of case cows. Bacteriological examinations were performed on samples collected on days 6, 12, 20 and 42-45. Milk samples from mastitic and diagonally opposite quarters were stored at –20°C and –70°C for analysis of acute phase proteins, prostaglandin E_2 and meloxicam concentrations. From the normal animals (n=20) that were followed for the 45 day study period, a quarter was randomly selected using the random data function in Minitab and milk samples were collected from these quarters and subject to the same tests as the milk samples from the affected quarter of cases of clinical mastitis. The farmers were requested to take the milk samples, using an aseptic technique (Appendix 3), from the affected and diagonally opposite unaffected quarters in the cases of clinical mastitis and from the nominated quarters in the normal cows.

2.5. Bacteriological examination

Milk samples were submitted to University of Glasgow Veterinary Bacteriology laboratory for bacteriological examination. On arrival the samples were mixed thoroughly and 0.05ml sample of the milk was plated, using a 7mm bacteriological wire loop, on 5% sheep blood agar, blood aesculin (Edwards' media) and MacConkey's agar. The plates were incubated at 37°C for 24 hours then examined; the number of colonies, type of haemolysis and probable identification of micro-organisms were noted. Bacteria were identified by recognised standard laboratory techniques, such as Gram, catalase, oxidase and coagulase testing, Rapidec Staph and API strips (Anon 1987). Agar plates were re-incubated for a further 24 hours then re-examined as above. Any agar plates that showed no visible growth after 48 hours were discarded and recorded as no growth.

2.6. Somatic cell counting and milk quality

Milk samples from all the affected and unaffected diagonally opposite quarters of mastitic cases and nominated quarters from control cows (n=20) were submitted for somatic cell counting and milk fat, protein and lactose percentages on all sampling days. The milk was collected into a universal container with a preservative (Potassium dichromate) and sent by first class mail to Scottish Milk (Scottish Milk Laboratories, Paisley, Glasgow) for automated cell counting using the Fossomatic method.

2.7. Differential somatic cell counting

Milk samples from the affected quarters of cases of clinical mastitis and the nominated quarters from the 20 control cows were submitted for differential somatic cell counting on all sampling days. Milk samples were sent fresh by first-class mail to The Clinical Pathology Laboratory, Companion Animal Diagnostic Services at the University of Glasgow Veterinary Faculty. On arrival the samples were mixed thoroughly and a wedge smear was made on a glass microscope slide using one drop of milk. The smear was air dried by fan then fixed in methanol and stained using May Grünwald and Giemsa stains. A concentrated spot of milk cells was prepared by using a Shandon cytospin centrifuge. This centrifuge uses a specialised metallic cassette holder onto which the following is

placed, in order from bottom to top, a labelled glass slide (labelled side up), a filter paper (with a hole cut out of it near the bottom) and finally a funnel shaped sample chamber. 150 µl milk was pipetted into the sample chamber followed by 150 µl saline. The dilution was then centrifuged at 750 rpm for five minutes. The resulting cytospin preparation was air dried by fan. The cytospins were subsequently fixed in methanol and stained using May Grünwald and Giemsa stains.

The cytospin preparation allows a greater number of cells to be readily examined. In some samples, particularly where fat content is high, the cytologist would revert to the wedge smear. The smear and cytospin were microscopically examined, using oil immersion. The cell populations were differentiated to obtain percentages of neutrophils, lymphocytes and macrophages, and report on other findings, e.g. presence of intra-cellular bacteria, fungal hyphae, and degenerate cells.

2.8. Acute phase proteins

Milk samples were stored at –20°C for a mean of 11.7 months (standard deviation ± 0.3 months), median 12.9 months and range 8 – 14.6 months. The samples were transported on ice for analysis to the Centre for Integrated Diagnostic Systems (CIDS) at the University of Glasgow. Haptoglobin (Hp) and mammary-associated serum amyloid A (M-SAA) concentrations were assessed in the milk samples from the 70 complete cases of clinical mastitis, 15 control cows and 20 unaffected diagonally opposite quarters using ELISA kits obtained from Tridelta Development plc (Dublin, Ireland). The control cows and diagonally opposite quarter samples were randomly selected using the random data function in Minitab. Haptoglobin and M-SAA concentrations were measured using an automated ELISA system including on-board analysis of data (Grifols Triturus 4, Biostat Ltd, Stockport, UK). In addition 100 samples were measured using manual ELISA but with the data processed by the analysis programme on the Triturus 4 system. Complete methods for the manual ELISA for Hp and M-SAA are detailed in Appendix 4 and were performed according to the manufacturer's instructions. The samples were initially diluted 1:50 in assay buffer, but if the concentration was greater than the range of the standard curve, they were diluted as necessary. The assay limits of quantification for Hp and M-SAA were 0.26 ± 16 µg/ml and 0.05 ± 0.04 µg/ml (mean ± SD, n=11), respectively. The intra-assay coefficients of variance were 3.7% and 3.5% for milk Hp

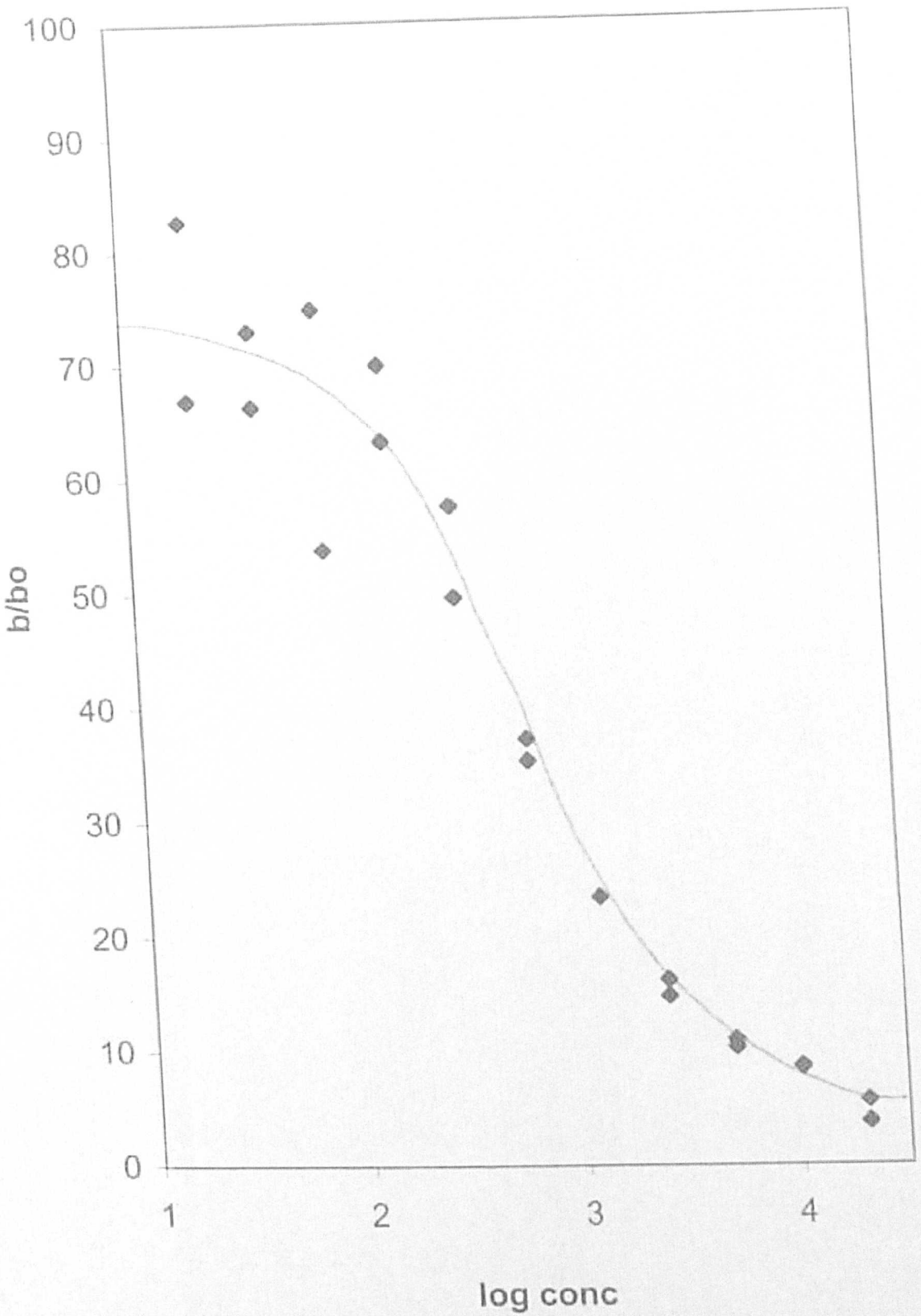
(n=8) at levels of 223 μg/ml and 2.7 μg/ml respectively and were 4.5% and 10% for M-SAA (n=8) at levels of 55 μg/ml and 10.2 μg/ml respectively. Inter-assay coefficients of variance were 22.4% and 21.4% for milk Hp at 5.3 μg/ml (n = 6) and 164 μg/ml (n=19) respectively, and were 15.4% and 14.7% for M-SAA at levels of 24 μg/ml (n=34) and 108 μg/ml (n=33) respectively.

2.9. Prostaglandin E_2

Milk samples were stored at -70 °C for a mean 13.8 months (± 0.3 months), median 15 months and range 10.2 – 16.7 months. Prostaglandin E_2 (PGE_2) concentrations were measured in milk samples from the affected quarter of the 70 complete cases on day 0, 1 and 2 using a radio-immunoassay (RIA). The mean limit of detection across the assays was 0.03 (±0.03). An example of a standard curve (two replicates) is calculated from the normalised percent bound as a function of the $\log_{10}$ PGE_2 concentration and is shown in Figure 2.2. The normalised per cent bound is calculated from (standard/sample counts per minute – buffer counts per minute) / (zero standard counts per minute – background count) × 100. The antibody binding was ~20%, non-specific binding or buffer binding (NSB) was 1.7%. The working range of the standard curve was between 0.08 and 5.00 ng/ml.

2.9.1. Preparation of standards

Five microlitres of the 1mg/ml prostaglandin E_2 (PGE_2) standard (Sigma Chemical Company, Poole, England) was diluted with 5ml phosphate buffer saline (PBS), 0.1% bovine serum albumin (BSA) (Sigma Chemical Company, Poole, England) and 0.1% sodium azide (azide) (Sigma Chemical Company, Poole, England) to produce a PGE_2 concentration of 1.0μg/ml, which was diluted further with 5ml of the PBS, BSA and azide buffer to produce 100ng/ml prostaglandin standard. 3200μl of fresh semi-skimmed milk was added to 800μl of the 100ng/ml standard to give a 20ng/ml prostaglandin solution. Serial dilutions were then performed with milk to produce prostaglandin standards with concentrations of 10, 5, 2.5, 1.25, 0.62, 0.32, 0.16, 0.08, 0.04 and 0.02 ng/ml.



b/b0 normalised per cent bound for each standard/sample log conc $\log_{10}$ PGE_2 concentration

Figure 2.2: Typical antibody binding curve for prostaglandin E_2 standards (in duplicate) prepared with milk as the dilutant

2.9.2. Preparation of milk samples

Milk samples from cases of clinical mastitis were thawed and 2ml placed in a glass test-tube. 2ml of 0.1% tri-fluroacetic acid (TFA) (Sigma Chemical Company, Poole, England) was added to each milk sample and PG standard. The samples and standards were subjected to ultra-sonification using a Vibra Cell ultrasonificator (Sonics & Materials Inc., Connecticut, USA) for approximately five seconds before being centrifuged at 3000g at 4°C for 30 minutes (GS-6R Centrifuge, Beckman, USA).

After centifugation the samples and standards were extracted using Sep-paks (Strata x 33um Polymeric sorbent 200mg/6ml). Sep-paks were initialised using 4ml of 60% Acetonitrile (ACN) (Rathburn Chemicals Limited, Walkerburn, Scotland) in 0.1% TFA then 2ml of the centrifuged milk samples and standards were pipetted into sep-paks. The sep-paks were then washed three times using 5ml 0.1% TFA. Test-tubes were labelled and the elutant generated from adding 4ml of 60% ACN was collected. This elutant was dried under medium using centrifuge and vacuum system (Speed Vac Plus SCZ10A, Savant Instruments Inc., New York, USA). When the samples and standards were dry, 2ml of the PBS, BSA and Azide buffer was added and the samples were ultra-sonificated for approximately five seconds to allow mixing.

2.9.3. Protocol for radio-immunoassay

One hundred µl of each standard and sample was added to labelled tubes. Six hundred, 800 and 100µl of PBS, BSA and Azide buffer to tubes labelled NSB, TC and B0, respectively. Reconstituted freeze-dried antibody was made up to 5ml using PBS, BSA and azide buffer. The antibody was then made up to 50ml (dilution of 1:10) using the PBS, BSA and azide buffer and 5ml of this was diluted further in 50ml (dilution of 1:100). The antibody was kept cool in an ice bucket. Five hundred µl of 1:100 antibody was added to all the tubes except NSB and TC and the tubes were incubated at 4°C for 30 minutes. Eight µl of the radioactive tracer ($^{3H}PGE_2$) was added to 10ml of PBS, BSA and azide buffer to provide sufficient tracer for 100 tests. One hundred µl of the diluted tracer was added to all tubes and the tubes were mixed using an Autovortex Mixer SA2 (Stuart Scientific). The tubes were then incubated overnight at 4°C. Twenty mg dextran was

dissolved in 20ml of PBS, BSA and azide and then 200mg inactivated charcoal was added and the solution stirred for a minimum of 30 minutes on ice. This provided sufficient solution for 100 tests. Two hundred µl of the charcoal/dextran was added to all tubes except TC. The tubes were then mixed using Autovortex Mixer SA2 and incubated at 4°C for 10 minutes. All tubes were then centrifuged at 3000 g at 4°C for 10 minutes. The supernatant was removed from each tubes and placed into a scintillation vial. Five ml of scintillation fluid was added to each tube. The scintillation tubes were then placed in racks and radioactivity was counted using the Liquid Scintillation Analyser 1600TR (Packard, Pangbourne). Results were then entered into Assay zap (Biosoft, Cambridge) computer program.

2.10. Meloxicam

Milk samples were stored at -70 °C for a mean of 8.8 months (± 0.3 months), median 9.9 months and range 5.1 – 11.6 months. Meloxicam concentrations were measured in 16 cases of clinical mastitis: eight cases were randomly selected from treatment Group 2 and eight were randomly selected from treatment Group 3. The meloxicam assay used in this study was a modification of the reverse-phase High Performance Liquid Chromatography (HPLC) method described by Velpandian and others (2000).

2.10.1. Chemical structure

The chemical structure of meloxicam was very similar to piroxicam (Figure 2.3) and the chromatography, extraction and detection properties of piroxicam meant it was an ideal internal standard to control for volume variability.

2.10.2. Reagents

Meloxicam and piroxicam (internal standard) standard compounds were purchased from Sigma Chemical company (Poole, England). All solvents were HPLC grade and were purchased from Rathburn Chemicals Limited (Walkerburn, Scotland). Potassium di-hydrogen orthophosphate was obtained from BDH Laboratory Supplies (Poole, England). Water used in the mobile phase was freshly de-ionised via Purite Select.

2.10.3. Equipment

A Spectra Systems Liquid delivery system pump connected to Auto injector AS3000 and degasser SCM 1000 with a Focus UV Detector Focus were used. For data acquisition and integration Software PC1000 by Spectra Systems was used. In addition, a Beckman GS-6R, GH 3.8 rotor centrifuge, Rotary mixer by B&T, Sample concentrator by Techne DB3, pH meter by Kent 7065, Stirrer hot plate by Corning and an Autovortex mixer SA2 were used.

2.10.4. Chromatographic conditions

Column RP C18 (150 × 4.6 mm; 5μm particle size) was used for analytical separation and C18 ODS, Octadecyl (4.0 × 3.0 mm) was used as a guard column. The mobile phase consisted of an aqueous solution of potassium di-hydrogen orthophosphate (10mM) and methanol: acetonitrile (90:10) at pH 4.5 in the ratio 50:50 delivered at 1ml/min. The instrument was operated at ambient temperature of 20°C. UV detection was achieved at 364nm. The retention times for piroxicam and meloxicam were 5.7 and 7.1 minutes, respectively and a typical chromatogram is shown in Figure 2.4.

2.10.5. Stock solutions

A stock solution was prepared by dissolving 10mg of meloxicam in 100ml of methanol (100μg/ml). Serial dilutions to produce 50.0, 25.0, 10.0, 5.0, 2.5, 0.5, 0.25 μg/ml were used to fortify samples known to be free of the analyte. Dilutions 5.0, 1.0, 0.5, prepared in mobile phase, were used as external standards for the quantification of the unknowns. Piroxicam stock solution was prepared by dissolving 10mg in 100ml of methanol to give 100μg/ml. Serial dilutions to produce 0.5 and 5.0 μg/ml were used as internal standards for the quantification of the unknowns.

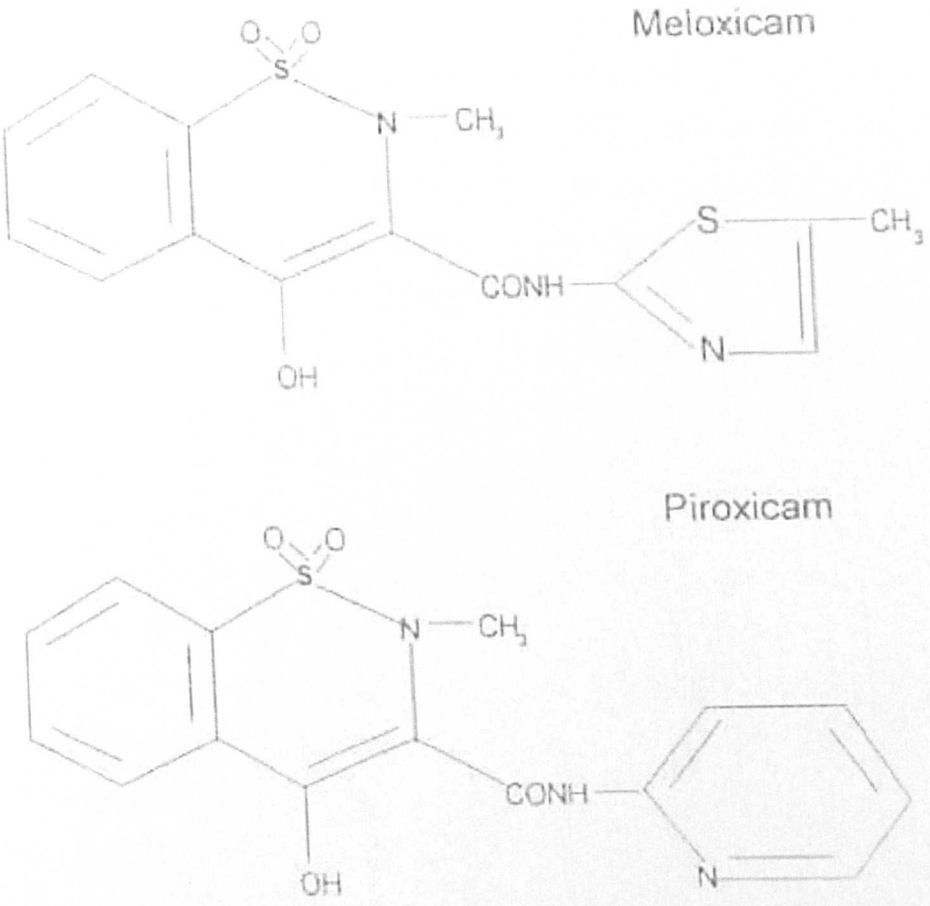


Figure 2.3: Chemical structure of meloxicam and piroxicam (internal standard)

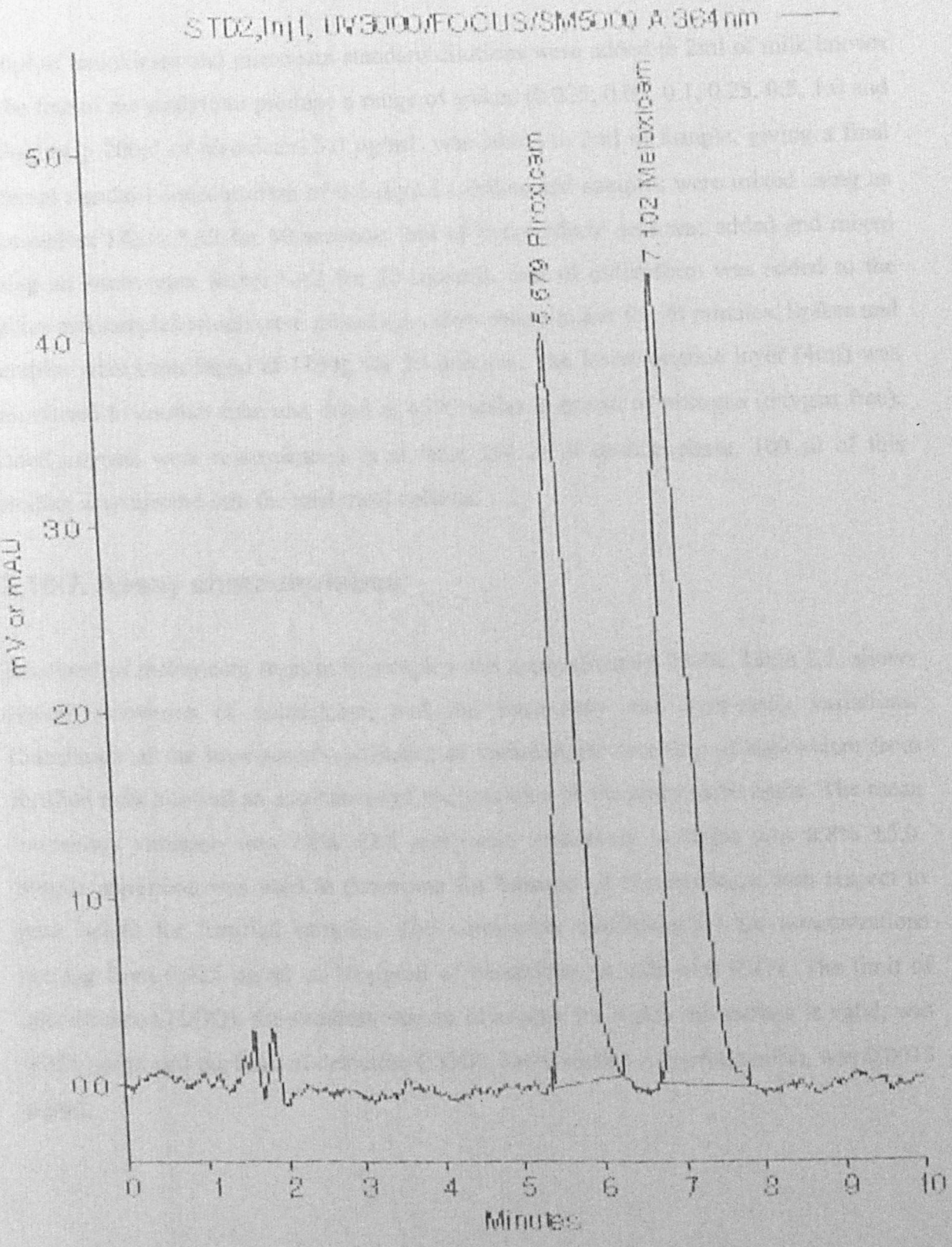


Figure 2.4: Typical chromatogram of milk, spiked with meloxicam (0.5µg/ml) and piroxicam (0.5µg/ml)

2.10.6. Extraction

200µl of meloxicam and piroxicam standard dilutions were added to 2ml of milk known to be free of the analyte to produce a range of spikes (0.025, 0.05, 0.1, 0.25. 0.5, 1.0 and 2.5µg/mL). 200µl of piroxicam 5.0 µg/mL was added to 2ml of sample, giving a final internal standard concentration of 0.5 µg/mL. Spikes and samples were mixed using an Autovortex Mixer SA2 for 30 seconds. 2ml of hydrochloric acid was added and mixed using an Autovortex Mixer SA2 for 30 seconds. 6ml of chloroform was added to the spikes and samples which were mixed on a slow rotary mixer for 20 minutes. Spikes and samples were centrifuged at 1850g for 20 minutes. The lower organic layer (4ml) was transferred to another tube and dried at 45°C under a stream of nitrogen (oxygen free). Dried extracts were re-suspended in at least 250 µl of mobile phase. 100 µl of this product was injected into the analytical column.

2.10.7. Assay characteristics

Recovery of meloxicam from milk samples was approximately 100%. Table 2.1. shows typical recoveries of meloxicam, and the intra-assay and inter-assay variations. Calculation of the inter-assay coefficient of variation for recovery of meloxicam from fortified milk allowed an assessment of the precision of the assay to be made. The mean inter-assay variation was 7.4% ±3.5 and mean intra-assay variation was 8.8% ±5.0. Simple regression was used to determine the linearity of concentration with respect to peak height for fortified samples. The correlation coefficient (r) for concentrations ranging from 0.025 µg/ml to 10 µg/ml of meloxicam in milk was 0.999. The limit of quantification (LOQ), the smallest amount of analyte for which the method is valid, was 0.025 µg/ml and the limit of detection (LOD), based upon 4 × baseline noise, was 0.0013 µg/ml.

Concentration of Meloxicam in milk (μg/ml) (n=4)	Intra-assay recovery (%) Mean ± SD	Intra-assay coefficient variation (%)	Inter-assay recovery (%) Mean ± SD	Inter-assay coefficient variation (%)
0.025	NS		99.0 ± 4.85	4.80
0.05	105.2 ± 13.6	14.3	87.7 ± 16.5	14.5
0.1	NS		106.2 ± 6.50	6.90
0.25	93.0 ± 2.97	2.88	104.1 ± 5.34	5.56
0.5	NS		99.8 ± 9.51	9.49
1.0	102.4 ± 11.7	11.9	105.3 ± 5.20	5.47
2.5	93.79 ± 11.6	10.8	102.2 ± 4.07	4.16
5.0	NS		99.3 ± 4.68	4.65
10.0	NS		106.9 ± 9.91	10.6
Average variation		8.78 ± 4.97		7.35 ± 3.49

NS = no sample

SD = standard deviation

Table 2.1: Mean (± standard deviation) recovery, intra-assay and inter-assay variation in recovery of meloxicam from milk fortified at known concentrations.

2.11. Data handling and statistical analysis

In order to allow information to be stored, collated and analysed, all data were entered into Microsoft Access 2000 (Microsoft Corporation), a relational database management system that allows storage and retrieval of data from related tables. Basic statistical analysis was conducted using Microsoft Excel (Microsoft Corporation) and Minitab Statistical Software version 13 (Minitab INC). One-way ANOVA, Kruskal-Wallis and χ^2-square tests were used as appropriate to compare groups. Where appropriate, data were transformed to improve normality and stabilise variances. Outcome variables for the physiological and laboratory data were analysed in S-Plus 2000 (Professional release 3, MathSoft Inc.) and MlwiN version 1.1 (Rasbach and others 2000) by the linear mixed effects method, which takes the dependency of the data into account and is suitable for the statistical analysis of repeated measurements. A backward stepwise regression model was used; this method used maximum likelihood techniques for estimating the fit of the model, in which variables remained in the model if they significantly improved the fit as assessed by the change in deviance. The farm and identity of the cow were used as random effects. Initial univariable screening used a critical probability of $p \leq 0.25$ to select which variables to offer to the multivariable regressions. Multivariable regressions used a critical probability of $p < 0.05$. Analysis was performed for data up to the first week after recruitment into the study and for the complete 45 day study period. Two-by-two tables were constructed and the sensitivity, specificity, predictive values, and accuracy of physiological and laboratory parameters were calculated (Dohoo and others 2003). Likelihood ratios were calculated using the prevalence of moderate cases of mastitis from the study data.

Chapter 3

On farm assessment of pain in cows with clinical mastitis

3.1. Introduction

Mastitis is a prevalent disease of dairy cows worldwide. A survey conducted in Northern Ireland reported that 17 per cent of dairy cow deaths were attributed to mastitis (Menzies and others 1995). Another study of cows with toxic mastitis in mid-Somerset reported mortality of approximately 50% (Green and others 1997). Cows affected by the peracute form of mastitis are generally considered by veterinary surgeons and farmers to be in severe pain and distress, and veterinary surgeons often use analgesics in the management of the disease, especially the non-steroidal anti-inflammatory drugs (NSAID) in conjunction with antibiotics and other supportive therapies. The NSAID are used for their anti-inflammatory and anti-endoxic properties (Ziv 1992). In stoical species such as ruminants, it is difficult to assess whether less severe mastitis, such as mild or moderate cases, also cause pain, or if NSAID therapy would improve recovery. The Farm Animal Welfare Council's (FAWC) third freedom states 'Freedom from pain, injury or disease', therefore it is important to be able to quantify and control pain in a disease such as mastitis, which has an average incidence of 35-40 cases per 100 cows per year (Booth 1997). A recent study assessed the welfare of dairy cattle in the Midlands and south-west of England by making use of animal-based measurements. A panel of 50 experts reported that over 60% of the study farms needed to take action to reduce the incidence of mastitis (Whay and others 2003), again highlighting mastitis as an important disease from a welfare aspect.

Alterations in the nociceptive pathways frequently accompanies inflammation and has this has led to the development of pain threshold testing, which relies on the induction of physiological pain, to act as a proxy measure for the presence of pain. These methods can be used to assess the level of response to a mechanical stimulus in normal animals compared to animals with inflammatory disease. Studies to measure pain thresholds have

been reported in the horse (Szabuniewicz and Szabuniewicz 1975; Pippi and Lumb 1979), in sheep (Nolan and others 1987; Ley and others 1995) and in cattle (Whay and others 1998). The techniques in cattle and sheep were applied in the assessment of lameness, and Fitzpatrick and others (1998) showed that they could also be applied to study bovine mastitis.

The objective of this study was to assess if physiological and pathological parameters measured on farm could act as objective indicators of pain in cows with clinical mastitis and whether the use of NSAID, meloxicam (Metacam solution for injection, Boehringer Ingelheim Limited, Bracknell) produced a measurable improvement in these parameters. It was previously demonstrated in a smaller study of clinical mastitis that when a threshold measurement device was applied to the ipsi-lateral leg (the same side as mastitis) the threshold measurement would be lower than that for the contra-lateral leg (opposite side to the mastitis) (Fitzpatrick and others 1998), and this was investigated further in this study.

3.2. Materials and Methods

3.2.1. Study population and case recruitment

The cows in this study were commercial dairy cows with naturally occurring clinical mastitis (cases) or clinically healthy cows (normal). Recruitment and sampling were described in Chapter 2.

3.2.2. Study design

Cases were allocated randomly to one of three treatment groups (Chapter 2). Group 1: antibiotics only, Group 2: antibiotics and one dose of meloxicam (0.5mg/kg i.v./s.c.), Group 3: antibiotics and three doses of meloxicam (0.5mg/kg i.v./s.c.) given three days apart.

3.2.3. Clinical examination

All cows were observed walking and examined clinically (cases and normal) on every sampling day, and physiological signs such as heart rate, respiratory rate and rectal temperature were recorded. Body condition score was graded using a scale 1-5, described previously by Edmonson and others (1989). The hock-to-hock distance of the cow was assessed, when the cow was standing squarely, by measuring the distance between the mid-point of the hocks using a 460mm ruler (Figure 3.1), to act as a proxy for the hindleg stance of a cow.

Daily milk yield records to estimate cow milk production were collected, where possible, for the duration of the study. Information on the stage of lactation, milk yield, age and parity of cows were obtained from farm records. An estimate of cow temperament based on ease of handling was made as follows grade 1: easy; grade 2: difficult (difficult to put cow in crush; cow lifting leg when trying to apply strap to leg to enable measurement of nociceptive threshold); grade 3: very difficult (requires kick bar to allow manipulation of udder and attachment of strap to measure nociceptive threshold). The temperament of the cow was recorded as assessed prior to administration of treatment. The presence of teat lesions was recorded and an estimate of udder cleanliness was made using a scale similar to that used by Ward and others (2002): grade 1: clean; grade 2: some dirt on udder; grade 3: very dirty. Udder skin temperature was assessed for all four quarters using an infra-red thermometer (Raytex Minitemp MT); the area used was approximately 1cm caudal to the teat base. The udder temperature difference was calculated as the mean temperature for the three unaffected quarters minus the value from the infected quarter, or in the normal animals the randomly selected quarter. The ambient temperature was also measured using an infra-red thermometer (Raytex Minitemp MT).

Figure 3.1: Measurement of hock-to-hock distance (mm) to assess the hindleg stance of cows with clinical mastitis or normal animals

3.2.4. Mechanical threshold measurement

Mechanical nociceptive threshold responses were measured using a ramped mechanical stimulus, using a modification of the method described by Nolan and others (1987). A mechanical device consisting of a meter connected to compressed gas canister and a strap with a blunt ended pin was used to assess the threshold to mechanical stimulation (Figure 3.2). The strap was attached to the cow below the point of the hock, such that the blunt ended pin was over the bony protuberance of the lateral condyle of the metatarsal bone. The power was turned on, the meter reading was checked and set to zero, if necessary, and then the gas was allowed to flow through tubing to inflate a balloon within the cuff, thus gradually pushing on a plate to apply the blunt ended pin against the cow's leg. This ramped mechanical stimulus was applied to each hind leg in turn, and when the cow reacted by either shifting her weight or lifting her leg, the machine was turned off and the reading was recorded in kilopascals (kPa). A maximum cut off point of 70 kPa was applied, at which point the machine was turned off in those animals that had failed to respond to stimulation below this pressure. Three readings were recorded for each leg and a mean value was calculated. Results were expressed as the difference between the contra-lateral leg (unaffected side) and the ipsi-lateral leg (affected side). The device was calibrated weekly using an electronic hand held calibration meter (Conmark C9500 series pressure meters, Conmark Limited, Hertfordshire, England). For each time point when the threshold measurement was assessed, the start leg was decided at random, prior to the visit, using blocks of four in the random number generator of Minitab. A schematic diagram shows the components of the cuff from the mechanical threshold device (Figure 3.3) and Table 3.1. details the properties of the components within the cuff to allow pressure readings to be converted to force readings, where Pressure = force/unit area, thus 1 Pascal (pressure) = 1 Newton per square metre (force). Using the radius of the plate the area of the plate can be calculated (Area of plate/pin= πr^2), and using this the force at the level of the balloon can be computed. Knowing the force at the balloon, the force transmitted via the pin and the area of the pin from the radius, the pressure applied at the interface of the cow can be calculated.

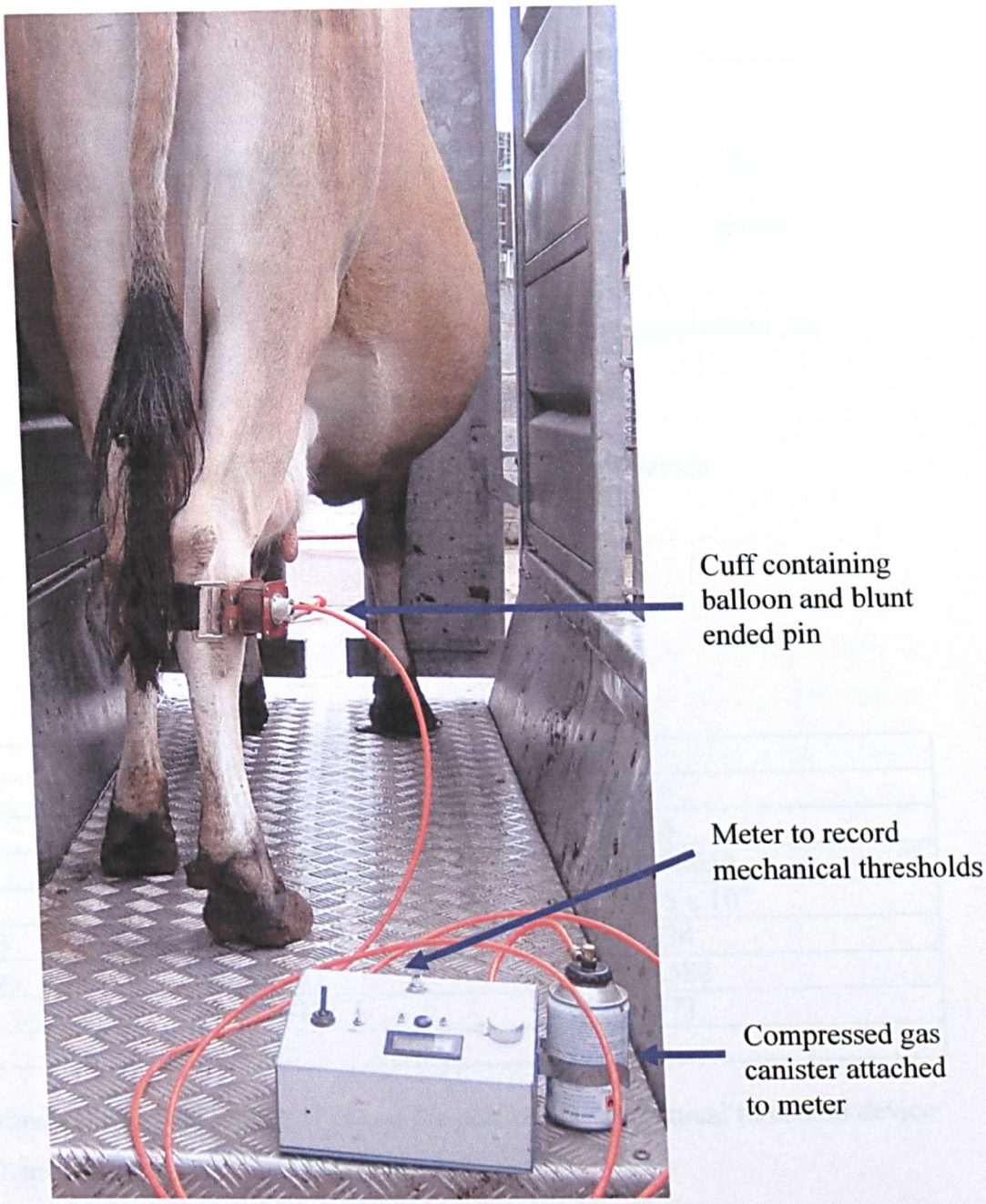


Figure 3.2: Gas-driven ramped mechanical threshold device for the measurement of nociceptive thresholds (kPa) in cows with unilateral clinical mastitis, and in normal animals to calculate the threshold difference between the contra-lateral and ipsi-lateral hindlegs

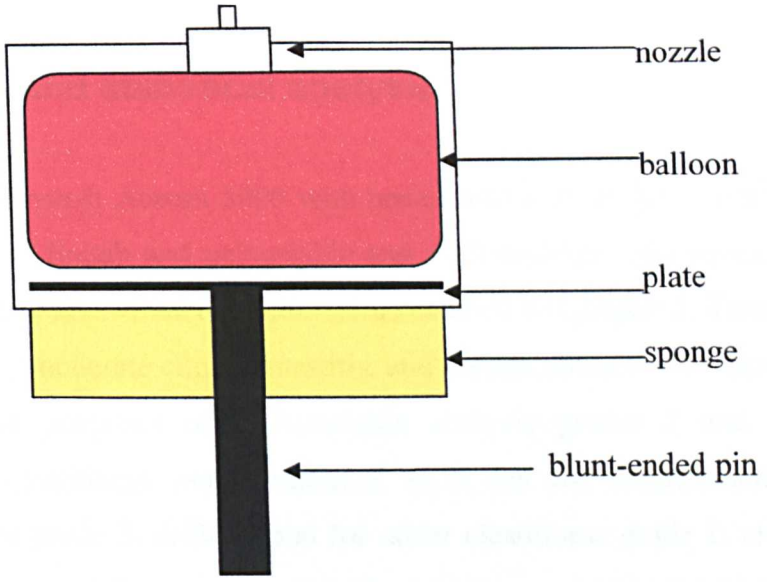


Figure 3.3: Schematic diagram of cuff of the mechanical threshold device

	Plate	Pin
Diameter (m)	0.05000	0.00216
Radius (m)	0.02500	0.00108
Pi π	3.141592654	3.141592654
Area (m^2)	0.001963	3.66435×10^{-6}
Pressure (psi)	10	5359.234
Pressure (kPa)	68.9475	36950.582
Force (kN)	0.135373	0.135373
Force (N)	135.4	135.4

Table 3.1: Dimensions of the plate and pin of the cuff of the mechanical threshold device and the resultant pressure (kPa) and force (N)

3.2.5. Data handling and statistical analysis

Data were entered into Microsoft Access 2000 with basic statistical analysis conducted using Microsoft Excel and Minitab and univariable and multivariable regressions were performed using S-Plus 2000 and MlwiN version 1.1 as detailed in Chapter 2. Treatment groups and cases of mild or moderate clinical mastitis, and normal animals were screened for homogeneity. For the purposes of multivariable analysis grades 2 and 3 for temperament and udder cleanliness were combined, such that for temperament was classed as grade 1: easy or grade 2: difficult and for udder cleanliness grade 1: clean or grade 2: dirty. The goodness of fit was assessed when treatment groups 2 and 3 were combined, such that the treatment groups were antibiotics only (group 1) versus antibiotics and meloxicam (groups 2 and 3) and when data for day 3 and day 6 after diagnosis for treatment group 3 were excluded. Two-by-two tables were constructed to allow the sensitivity, specificity, predictive values and accuracy to be calculated (Dohoo and others 2003) for cases with clinical mastitis versus normal animals and mild versus moderate cases of clinical mastitis. Survival analysis using Kaplan-Meier statistics in Minitab Statistical Software version 13 (Minitab INC) was used to determine whether the treatment altered the time for the threshold levels of cases of clinical mastitis to reach normal levels.

3.3. Results

3.3.1. Cases recruited

One hundred and seventeen cows with clinical mastitis and 45 normal cows were studied. Fifty six of the clinical cases of mastitis were of mild severity and 61 were of moderate severity.

The non-completion rate of cases of clinical mastitis in the study was 40% (47/117) of initially recruited cases. Twenty-one percent (24/117) dropped out on day 6 or before and 33% (39/117) of the cases dropped out of the study before day 20. Reasons for drop-out included the development of mastitis in more than one quarter, recurrent mastitis in the

same quarter, development of lameness, development of endometritis, teat trauma, being culled and adminstration of antibiotics in error within the 45 day study period (Table 3.2). The recurrence case rate was 17% (18 + 2 repeat cases × 100/ 117) and there were 1.3 cases of mastitis per cow (117 + 18 + 10 + 2/ 117). The lameness prevalence in the non-completion group was six percent (3/47). Sixty-eight percent (32/47) of the drop-out cases were moderate mastitis cases and 32% (15/47) were mild mastitis cases, with significantly more moderate cases dropping out compared to mild cases (p=0.005). There was no significant association between the treatment group and the likelihood of a case completing the study or not (p=0.13). Of the 20 animals that were recruited as normal controls for the 45 day period one cow (five percent) dropped out due to a stone being wedged between cleats on day six.

3.3.2. Demographic details

The majority of the recruited cases and normal animals were of the Holstein/Friesian breed (n=134), with small numbers of Pedigree Holstein (n=15), Guernsey (n=5), Shorthorn cross (n=5), Shorthorn (n=1), Ayrshire (n=1) and Ayrshire cross (n=1) cows. The age of the cows ranged from 2-14 years with a median age of 6 years and a mean (±SE) of 5.7 (±0.2) years. The parity of the cows had a similar range of 1 - 10 lactations, with a median of 4 and a mean (±SE) of 3.5 (±0.1) lactations. The median (Inter-quartile range (IQR)) number of days calved for cases of mastitis was 94 (28-163) days and for normal cows 117 (73-163) days. There was no significant difference (p>0.05) in days calved amongst mild or moderate cases of clinical mastitis, or normal cows: the median time after calving when mastitis occurred was 112 (IQR 39-163) days for mild cases and 77 (IQR 9-154) days for moderate cases of clinical mastitis. The median time after calving that normal cows were recruited into the study was 117 (IQR 83–146) days. Descriptive statistics for all the recruited cases categorised into mild and moderate cases of clinical mastitis are detailed in Appendix 5, Table A5.1.

Reason for non-completion	Number	%
Mastitis re-occurred in same quarter	18	38
Mastitis in another quarter	10	21
Mastitis re-occurred in same quarter and another quarter	2	4
Unresponsive mastitis	5	11
Systemically sick	3	6
Lame	3	6
Cut teat	2	4
Endometritis	1	2
Difficult to handle	1	2
Miscellaneous – given antibiotics, culled	2	4

Table 3.2: Reasons for cases dropping out of study before 45 days (n=47)

3.3.3. Homogeneity and goodness of fit

Combining treatment groups 2 and 3 did not affect the goodness of fit of the multivariable model, and therefore for simplicity, was used for this analysis. The omission of data from day 3 and day 7 after diagnosis did not alter the goodness of fit, and for simplicity was included in this analysis.

The initial distribution of cases among treatment groups was assessed. There was no significant difference between treatment groups for age, parity, body condition score, milk yield, heart rate, respiratory rate, rectal temperature, hock-to-hock distance and pain threshold difference when cows were enrolled in the study. There was, however, a significant difference in days calved between treatment groups ($p<0.01$). The median (IQR) days calved were 118 (75-179), 123 (33-186) and 45 (6-98) days for treatment groups 1, 2 and 3, respectively.

3.3.4. Clinical examination

The mean (SE) rectal temperature for non-complete cases and complete cases on the day of recruitment were 38.8°C (±0.1) and 38.4°C (±0.1), ($p=0.017$) while the equivalent values for milk yield (l) 36.5 (±1.6) and 29.5 (±1.6) ($p=0.005$). Fifty-two percent (32/61) of moderate cases of clinical mastitis were in the non-complete group and 48% (29/61) in complete group whereas 27% (15/56) of the mild cases of clinical mastitis were in the non-complete group and 73% (41/56) in the complete group. This difference was significant ($p<0.01$). Descriptive statistics for the complete and non-complete cases are detailed in Appendix 5, Table A5.2.

Table 3.3 shows the mean/median (SE/IQR) heart rate, respiratory rate, rectal temperature, hock-hock distance, udder temperature, and threshold differences on the day of recruitment into the study (day 0), for normal animals and mild and moderate cases of clinical mastitis. There was no significant difference in age, production level and body condition score among normal, mild and moderate cases of clinical mastitis. Descriptive statistics for the complete cases categorised into mild and moderate cases of clinical mastitis and normal animals are detailed in Appendix 5, Table A5.3.

Variables	Normal (n=45)	Mild (n=41)	Moderate (n=29)	p-value
Age (Years)	5.62 (0.37)	5.95 (0.25)	4.96 (0.31)	0.095^{ns}
Parity	3.36 (0.32)	3.83 (0.26)	2.82 (0.28)	0.058^{ns}
Days calved (days)	117 (73-163)	128 (82-198)	70 (6-140)	0.019*
Milk yield (litres)#	32.7 (1.71) (n = 24)	29.3 (2.18) (n = 18)	30 (2.07) (n = 8)	0.399^{ns}
Body condition score	2.0 (2.0-2.5)	2.0 (1.5-2.5)	2.0 (1.5-2.5)	0.125^{ns}
Heart rate (beats/minute)	71 (0.66)	71.2 (1.18)	77.4 (1.25)	0.001**
Respiratory rate (breaths/minute)	22 (0.43)	21 (0.41)	24 (0.67)	<0.001***
Rectal temperature (°C)	38.1 (0.07)	38.2 (0.07)	38.8 (0.16)	<0.001***
Hock-hock distance (mm)	226 (4.13)	270 (7.84)	274(8.1)	<0.001***
Udder skin temperature, affected quarter (°C)	22 (3.2)	26.4 (4.88)	23.9 (4.82)	<0.001***
Udder skin temperature, non-affected quarters (°C)	21.7 (3.2)	26.4 (4.94)	23.9 (4.82)	<0.001***
Udder temperature difference (°C)	-0.23 (0.11)	-0.07 (0.16)	-0.47 (0.26)	0.303^{ns}
Threshold difference (kPa)	-0.58 (0.71)	11.8 (2.73)	17.2 (3.77)	<0.001***

#data only available for limited number of animals
*** = p<0.001, ** = 0.01>p>0.001, * = 0.05>p>0.01, ns = p>0.05

Table 3.3: Mean/median (SE/IQR) for clinical parameters of normal animals (n=45), mild (n=41) and moderate (n=29) cases of clinical mastitis on day of recruitment to study (day 0)

3.3.4.1. Heart rate, respiratory rate and rectal temperature

Moderate cases of clinical mastitis had higher rectal temperatures, heart and respiratory rates compared to mild cases and normal animals ($p<0.001$) (Table 3.3). The heart rate, respiratory rate and rectal temperature of cows over six days were similar for all three treatment groups and the normal animals (Figure 3.4).

Variables that were included in the final multivariable model are listed in Tables 3.3 for heart rate, respiratory rate and rectal temperature as outcome variables, respectively. When variables were explored with multivariable analysis over time, heart rate, respiratory rate and rectal temperature of the mastitis cases all decreased (Tables 3.4-3.9). There was no effect of time in the normal animals (Tables 3.10 & 3.11). Heart rate, respiratory rate and rectal temperature were associated with one another. The heart rate of cases (both day seven and day 45 models) and normal cows (day 7 model) increased as the temperament scale increased (i.e. heart rate increased in animals classed as difficult to handle).

The ability of heart rate, respiratory rate and rectal temperature to differentiate between mild and moderate cases of clinical mastitis and normal animals was determined by estimating the sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy. The 'cut-off' point for cases and normal animals were estimated from the mean of all values of parameters for all cases. Cows with lower heart rate, respiratory rate and rectal temperature than the mean, i.e. <74 beats/minute, <22 breaths/minute and <38.3°C, respectively, were classed as test normal animals and cows ≥74 beats/minute, ≥22 breaths/minute and ≥38.3°C were classed as test cases. Tables 3.12 – 3.14 show that the sensitivity of detecting cases of clinical mastitis using heart rate, respiratory rate and rectal temperature were 60%, 46% and 50%, respectively while the specificities were 78%, 64% and 64%, respectively.

The ability of heart rate, respiratory rate and rectal temperature to differentiate between moderate and mild cases of clinical mastitis was determined by estimating the sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy. The 'cut-off' points for mild and moderate severity was estimated from the mean of the values of the parameters for all cases. Cases with lower values than the mean, i.e. <76

beats/minute, <23 breaths/minute and <38.4°C, were classed as test mild and cases ≥76 beats/minute, ≥23 breaths/minute and ≥38.4°C were classed as test moderate. Tables 3.15 – 3.17 show that the sensitivities of detecting moderate clinical mastitis were 48%, 66% and 59%, while specificities were 73%, 68% and 68%.

3.3.4.2. Udder skin temperature

Normal animals had lower absolute udder skin temperatures compared to mild and moderate cases of clinical mastitis ($p<0.001$) (Table 3.3). There was, however, no significant difference ($p>0.05$) in the udder temperature difference between normal animals and the cases of mastitis (Table 3.3); however, moderate cases did show a larger udder temperature difference compared to mild cases and normal animals (Figure 3.5).

In the multivariable analysis, respiratory rate was the only variable that was associated with udder temperature difference in cases of mild and moderate mastitis over the first seven days (Table 3.18) and respiratory rate and front or back quarter were associated with udder temperature difference in cases of mild and moderate mastitis over the 45 day study period (Table 3.19). The respiratory rate increased as the udder temperature increased and over the 45 day study period, back quarters had larger temperature differences. In normal animals, there was no association with any variables.

3.3.4.3. Hock-to-hock distance

There was a significant difference in the hindleg stance of cows, as measured by hock-to-hock distance, between normal animals and test cases ($p<0.001$) (Table 3.3). There was, however, no difference in the hock-to-hock distance in cases with mastitis in the front quarters compared to cases with mastitis in the hind quarters ($p>0.05$). For all cases of clinical mastitis the hock-to-hock distance decreased over the first six days and then levelled off to near normal (Figure 3.6); however, there was no difference among the three treatment groups over time.

In the multivariable analysis, variables that were positively associated with hock-to-hock distance over the first seven days after recruitment of cases to the study included body condition score, parity and heart rate, while time, udder cleanliness, and threshold

difference were negatively associated with hock-to-hock distance (Table 3.20). Over the 45 day study period, udder temperature difference, body condition score and parity were positively associated with hock-to-hock distance while threshold difference, udder cleanliness and time after recruitment to the study were negatively associated with hock-to-hock distance (Table 3.21). There was no relationship of time or any other variables with hock-to-hock distances of the normal animals over the first seven days.

The ability of hock-to-hock distance to differentiate between mild and moderate cases of clinical mastitis and normal animals was determined by estimating the sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy. The 'cut-off' point for cases and normal animals was estimated from the mean hock-hock distance for all animals. Cows with lower hock-to-hock distance than the mean, i.e. <254 mm, were classed as test normal animals and cows ≥254 mm were classed as test cases. The sensitivity and specificity of detecting cases of clinical mastitis were 64% and 84%, respectively (Table 3.22).

3.3.4.4. Temperament, udder cleanliness and teat lesions

The temperament of the cows was used as a guide to assess if cows with mastitis were more difficult or easy to handle as a result of the clinical mastitis. The majority of cows were classed as having temperament category 1. Figure 3.7 shows the percentage of clinical cases of mastitis, for those cases that were followed for the full 45 days (complete) and those that were not followed for the full 45 days (non-complete) and normal animals in the three categories of temperament (easy, difficult and very difficult). There was no difference between the temperament of normal cows compared to cases of mild and moderate mastitis. The majority of cows were categorised with a cleanliness score of 1 (Figure 3.8). Teat lesions were present in 31% (22/70) of complete cases, 32% (15/47) of non-complete cases and 40% (8/20) of normal animals. The most common teat lesions were hyperkeratosis, papilloma, teat end impaction and teat injury (Figure 3.9).

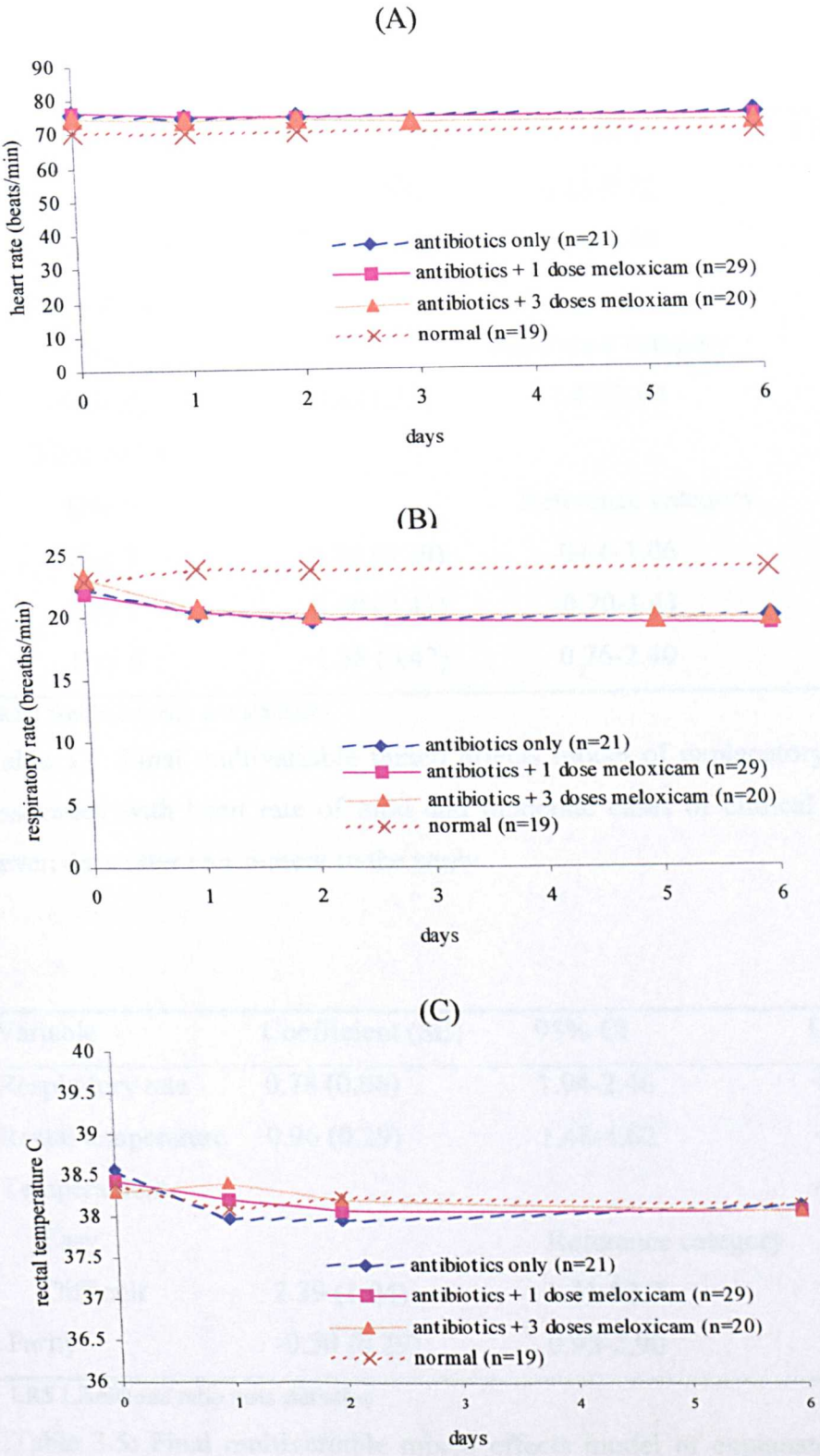


Figure 3.4: Mean (SE) (A) Heart rate (beats/min), (B) respiratory rate (breaths/min), (C) rectal temperatures (°C) for cases of clinical mastitis treated with antibiotics only (n=21), antibiotics and one dose of meloxicam (n=29) and antibiotics and three doses of meloxicam (n=20) and normal animals (n=19) over 6 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Respiratory rate	0.57 (0.07)	0.43-0.72	<0.001
Rectal temperature	0.99 (0.31)	0.38-1.60	<0.01
Temperament			<0.001
Easy		Reference category	
Difficult	3.68 (1.13)	1.47-5.89	
Time point			<0.001
Day 0		Reference category	
Day 1	-0.30 (0.39)	-04.6-1.06	
Day 2	-0.60 (0.41)	-0.20-1.41	
Day 6	-1.58 (0.42)	0.76-2.40	

LRS Likelihood ratio test statistics

Table 3.4: Final multivariable mixed effects model of explanatory variables that were associated with heart rate of mild and moderate cases of clinical mastitis for the first seven days after recruitment to the study

Variable	Coefficient (SE)	95% CI	LRS P value
Respiratory rate	0.78 (0.06)	1.94-2.46	<0.001
Rectal temperature	0.96 (0.29)	1.48-4.62	<0.001
Temperament			<0.001
Easy		Reference category	
Difficult	2.39 (1.04)	1.41-83.7	
Parity	-0.50 (0.29)	0.93-2.90	<0.05

LRS Likelihood ratio tests statistics

Table 3.5: Final multivariable mixed effects model of explanatory variables that were associated with heart rate of mild and moderate cases of clinical mastitis for the 45 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Heart rate	0.25 (0.02)	0.20-0.29	<0.001
Udder temperature difference	0.21 (0.10)	0.01-0.41	<0.001
Time point			<0.001
Day 0		Reference category	
Day 1	-1.41 (0.27)	0.89-1.93	
Day 2	-1.94 (0.27)	1.41-2.47	
Day 6	-2.12 (0.28)	1.57-2.67	

LRS Likelihood ratio test statistics

Table 3.6: Final multivariable mixed effects model of explanatory variables that were associated with respiratory rate of mild and moderate cases of clinical mastitis for the first seven days after recruitment to the study

Variable	Coefficient (SE)	95% CI	LRS P value
Rectal temperature	1.02 (0.18)	1.94-3.99	<0.001
Udder temperature difference	0.11 (0.09)	0.94-1.32	<0.001
Severity			<0.01
Mild		Reference category	
Moderate	1.13 (0.36)	1.54-6.20	
Time point			<0.001
Day 0		Reference category	
Day 1	-1.53 (0.27)	2.74-7.81	
Day 2	-2.12 (0.27)	4.88-14.23	
Day 6	-2.63 (0.27)	8.13-23.78	
Day 20	-2.57 (0.30)	7.25-23.50	
Day 45	-2.74 (0.31)	8.49-28.49	

LRS Likelihood ratio tests statistics

Table 3.7: Final multivariable mixed effects model of explanatory variables that were associated with respiratory rate of mild and moderate cases of clinical mastitis for the 45 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Heart rate	0.03 (0.01)	0.01-0.04	<0.001
Respiratory rate	0.04 (0.01)	0.02-0.06	<0.001
Hock-hock	0.02 (0.01)	0.00-0.03	<0.05
Ambient temperature	0.02 (0.01)	0.00-0.04	<0.001
Time point			<0.05
Day 0		Reference category	
Day 1	-0.16 (0.07)	0.03-0.29	
Day 2	-0.18 (0.07)	0.04-0.32	
Day 6	-0.08 (0.08)	-0.07-0.22	

LRS Likelihood ratio test statistics

Table 3.8: Final multivariable mixed effects model of explanatory variables that were associated with rectal temperature of mild and moderate cases of clinical mastitis for the first seven days after recruitment to the study

Variable	Coefficient (SE)	95% CI	LRS P value
Heart rate	0.02 (0.01)	1.01-1.03	<0.001
Respiratory rate	0.04 (0.01)	1.02-1.06	<0.001
Time point			<0.01
Day 0		Reference category	
Day 1	-0.22 (0.06)	1.11-1.39	
Day 2	-0.25 (0.06)	1.14-1.45	
Day 6	-0.17 (0.06)	1.04-1.34	
Day 20	-0.17 (0.07)	1.04-1.36	
Day 45	-0.16 (0.07)	1.02-1.35	

LRS Likelihood ratio tests statistics

Table 3.9: Final multivariable mixed effects model of explanatory variables that were associated with rectal temperature of mild and moderate cases of clinical mastitis for the 45 days after recruitment to the study

Outcome variable	Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Heart rate	Respiratory rate	0.69 (0.14)	0.41-0.97	<0.001
	Body condition score	5.39 (1.86)	1.76-9.03	<0.005
	Threshold difference	-0.08 (0.04)	0.00-0.16	<0.05
	Temperament			<0.001
	Easy	Reference category		
	Difficult	14.6 (3.75)	7.22-21.9	
Respiratory rate	Heart rate	0.20 (0.04)	0.12-0.28	<0.001
	Udder cleanliness			<0.01
	Clean	Reference category		
	Dirty	-2.10 (0.65)	0.83-3.25	
Rectal temperature	Body condition score	0.17 (0.08)	0.02-0.32	<0.05

LRS Likelihood ratio test statistics

Table 3.10: Final multivariable mixed effects model of explanatory variables that were associated with heart rate, respiratory rate and rectal temperature of normal animals for the first seven days after recruitment to the study

Outcome variable	Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Heart rate	Respiratory rate	2.72 (1.31)	1.17-196.9	<0.05
Respiratory rate	Heart rate	0.25 (0.05)	1.18-1.41	<0.001
Rectal temperature	Heart rate	0.01 (0.01)	1.00-1.02	<0.05

LRS Likelihood ratio tests statistics

Table 3.11: Final multivariable mixed effects model of explanatory variables that were associated with heart rate, respiratory rate and rectal temperature of normal animals for the 45 days after recruitment to the study

		True		Total
		Cases	Normal	
Test	Cases	42	10	52
	Normal	28	35	63
Total		70	45	115

Sensitivity	42/70 = 60%	95% CI 48.5 – 71.5%
Specificity	35/45 = 78%	95% CI 66.0 – 90.0%
Negative predictive value	35/63 = 56%	95% CI 43.8 – 68.2%
Positive predictive value	42/52 = 81%	95% CI 70.4 – 91.6%
Accuracy	77/115 = 67%	95% CI 58.4 – 75.6%

Table 3.12: Two-by-two table to test the ability of heart rate to differentiate between cases of clinical mastitis and normal animals detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Cases	Normal	
Test	Cases	32	16	48
	Normal	38	29	67
Total		70	45	115

Sensitivity	32/70 = 46%	95% CI 34.3 – 57.7%
Specificity	29/45 = 64%	95% CI 50.0 – 78.0%
Negative predictive value	29/67 = 43%	95% CI 31.2 – 43.0%
Positive predictive value	32/48 = 67%	95% CI 53.7 – 80.3%
Accuracy	61/115 = 53%	95% CI 43.9 – 62.1%

Table 3.13: Two-by-two table to test the ability of respiratory rate to differentiate between cases of clinical mastitis and normal animals detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Cases	Normal	
Test	Cases	35	16	51
	Normal	35	29	64
Total		70	45	115

Sensitivity	35/70 = 50%	95% CI 38.3 – 61.7%
Specificity	29/45 = 64%	95% CI 50.0 – 78.0%
Negative predictive value	29/64 = 45%	95% CI 32.8 – 57.2%
Positive predictive value	35/51 = 69%	95% CI 56.3 – 81.7%
Accuracy	64/115 = 56%	95% CI 46.9 – 65.1%

Table 3.14: Two-by-two table to test the ability of rectal temperature to differentiate between cases of clinical mastitis and normal animals detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Moderate	Mild	
Test	Moderate	14	11	25
	Mild	15	30	45
Total		29	41	70

Sensitivity	14/29 = 48%	95% CI 29.8 – 66.2%
Specificity	30/41 = 73%	95% CI 59.4 – 86.6%
Negative predictive value	30/45 = 67%	95% CI 53.3 – 80.7%
Positive predictive value	14/25 = 56%	95% CI 36.5 – 75.5%
Accuracy	44/70 = 63%	95% CI 51.7 – 74.3%

Table 3.15: Two-by-two table to test the ability of heart rate to differentiate between moderate and mild severity clinical mastitis detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Moderate	Mild	
Test	Moderate	19	13	32
	Mild	10	28	38
Total		29	41	70

Sensitivity	19/29 = 66%	95% CI 48.8 – 83.2%
Specificity	28/41 = 68%	95% CI 53.7 – 82.3%
Negative predictive value	28/38 = 74%	95% CI 60.1 – 87.9%
Positive predictive value	19/32 = 59%	95% CI 42.0 – 76.0%
Accuracy	47/70 = 67%	95% CI 56.0 – 78.0%

Table 3.16: Two-by-two table to test the ability of respiratory rate to differentiate between moderate and mild severity clinical mastitis detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Moderate	Mild	
Test	Moderate	17	13	30
	Mild	12	28	40
Total		29	41	70

Sensitivity	17/29 = 59%	95% CI 41.1 – 76.9%
Specificity	28/41 = 68%	95% CI 53.7 – 82.3%
Negative predictive value	28/40 = 70%	95% CI 55.8 – 84.2%
Positive predictive value	17/30 = 57%	95% CI 39.3 – 74.7%
Accuracy	45/70 = 64%	95% CI 52.8 – 75.2%

Table 3.17: Two-by-two table to test the ability of rectal temperature to differentiate between moderate and mild severity clinical mastitis detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

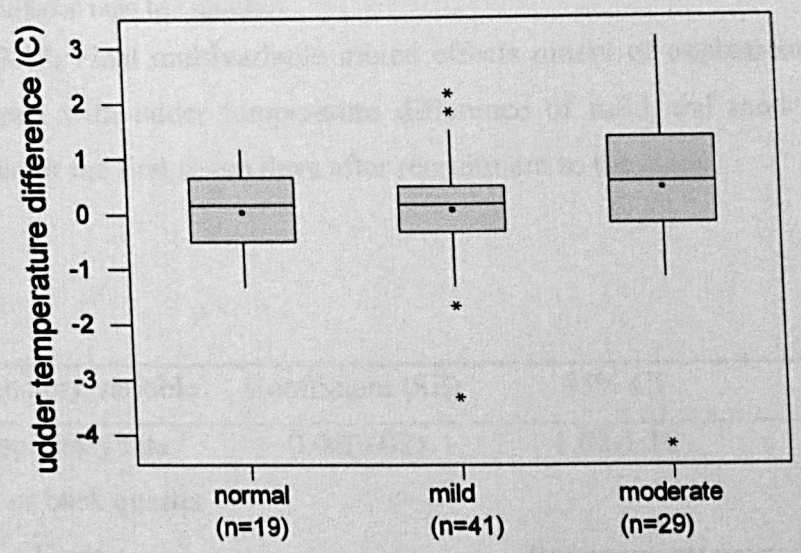


Figure 3.5: Udder temperature difference for mild (n=41) and moderate (n=29) cases of clinical mastitis and normal cows (n=19) on day of recruitment to the study (day 0). For each group, the median value is represented by the horizontal line within the box and the mean value denoted by the dot. The bottom and top of the box represent the first and third quartiles respectively. The vertical lines extending from the box represent the lowest and highest observations within 1.5x the inter-quartile range below and above the first and third quartiles respectively. Outliers are denoted by *.

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Respiratory rate	0.06 (0.02)	0.02-0.09	<0.01

LRS Likelihood ratio test statistics

Table 3.18: Final multivariable mixed effects model of explanatory variables that were associated with udder temperature difference of mild and moderate cases of clinical mastitis for the first seven days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Respiratory rate	0.06(0.02)	1.02-1.10	<0.01
Front or back quarter			<0.05
Front		Reference category	
Back	0.28 (0.12)	1.03-1.68	

LRS Likelihood ratio tests statistics

Table 3.19: Final multivariable mixed effects model of explanatory variables that were associated with udder temperature difference of mild and moderate cases of clinical mastitis for the 45 days after recruitment to the study

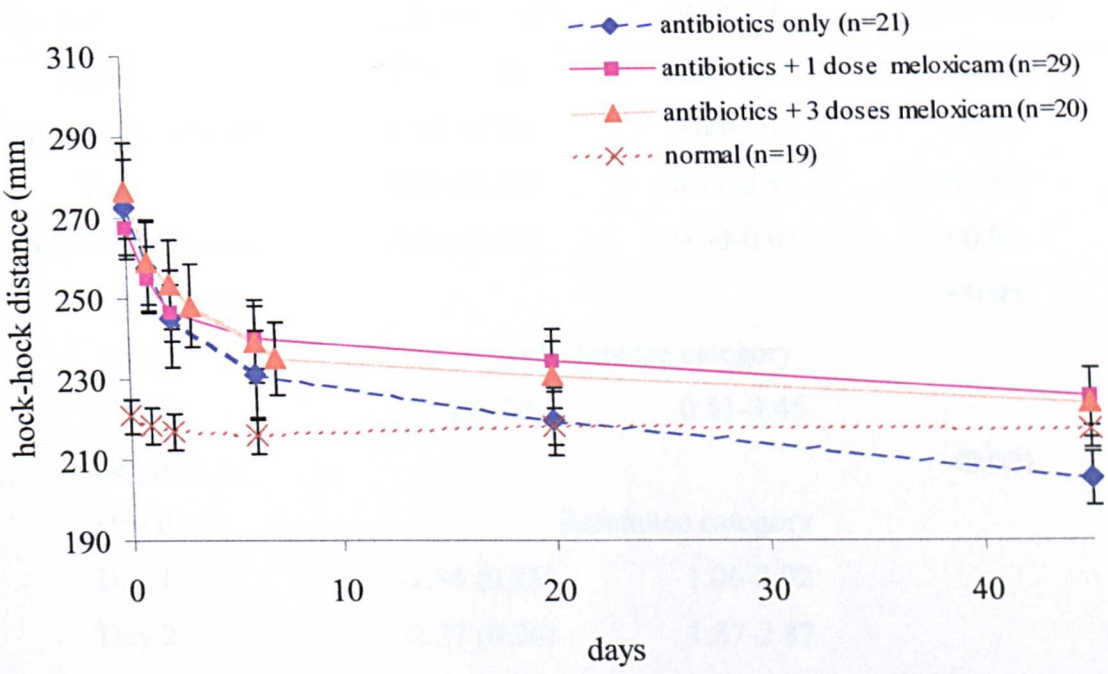


Figure 3.6: Mean hock-to-hock distance ± SE for cases of clinical mastitis treated with antibiotics only (n=21), antibiotics and one dose of meloxicam (n=29) and antibiotics and three doses of meloxicam (n=20) and normal animals (n=19) over 45 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Heart rate	0.08 (0.50)	0.02-0.15	<0.05
Body condition score	1.65 (0.50)	0.66-2.63	<0.001
Parity	0.56 (0.20)	0.17-0.95	<0.005
Threshold difference	-0.02 (0.01)	0.00-0.03	<0.05
Udder cleanliness			<0.05
Clean	Reference category		
Dirty	-1.98 (0.75)	0.51-3.45	
Time point			<0.001
Day 0	Reference category		
Day 1	-1.54 (0.25)	1.06-2.02	
Day 2	-2.37 (0.26)	1.87-2.87	
Day 6	-3.33 (0.27)	2.80-3.86	

LRS Likelihood ratio test statistics

Table 3.20: Final multivariable mixed effects model of explanatory variables that were associated with hock-to-hock distance of mild and moderate cases of clinical mastitis for the first seven days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Udder temperature difference	0.08 (0.08)	0.92-1.27	<0.05
Body condition score	1.64 (0.46)	2.10-12.5	<0.001
Parity	0.48 (0.18)	1.14-2.31	<0.01
Threshold difference	-0.14 (0.06)	1.02-1.31	<0.05
Udder cleanliness			<0.05
Clean		Reference category	
Dirty	-1.94 (0.70)	1.79-26.9	
Time point			<0.001
Day 0		Reference category	
Day 1	-1.63 (0.24)	3.17-8.18	
Day 2	-2.63 (0.25)	8.61-22.6	
Day 6	-3.60 (0.25)	22.3-59.9	
Day 20	4.44 (0.28)	49.1-146.0	
Day 45	5.32 (0.29)	115.7-361.9	

LRS Likelihood ratio tests statistics

Table 3.21: Final multivariable mixed effects model of explanatory variables that were associated with hock-to-hock distance of mild and moderate cases of clinical mastitis for the 45 days after recruitment to the study

		True		Total
		Cases	Normal	
Test	Cases	45	7	52
	Normal	25	38	63
Total		70	45	115

Sensitivity	45/70 = 64%	95% CI 52.8 – 75.2%
Specificity	38/45 = 84%	95% CI 73.3 – 94.7%
Negative predictive value	38/63 = 60%	95% CI 47.9 – 72.1%
Positive predictive value	45/52 = 87%	95% CI 77.9 – 96.1%
Accuracy	83/115 = 72%	95% CI 63.8 – 80.2%

Table 3.22: Two-by-two table to test the ability of hock-to-hock distance to differentiate between cases of clinical mastitis and normal animals detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

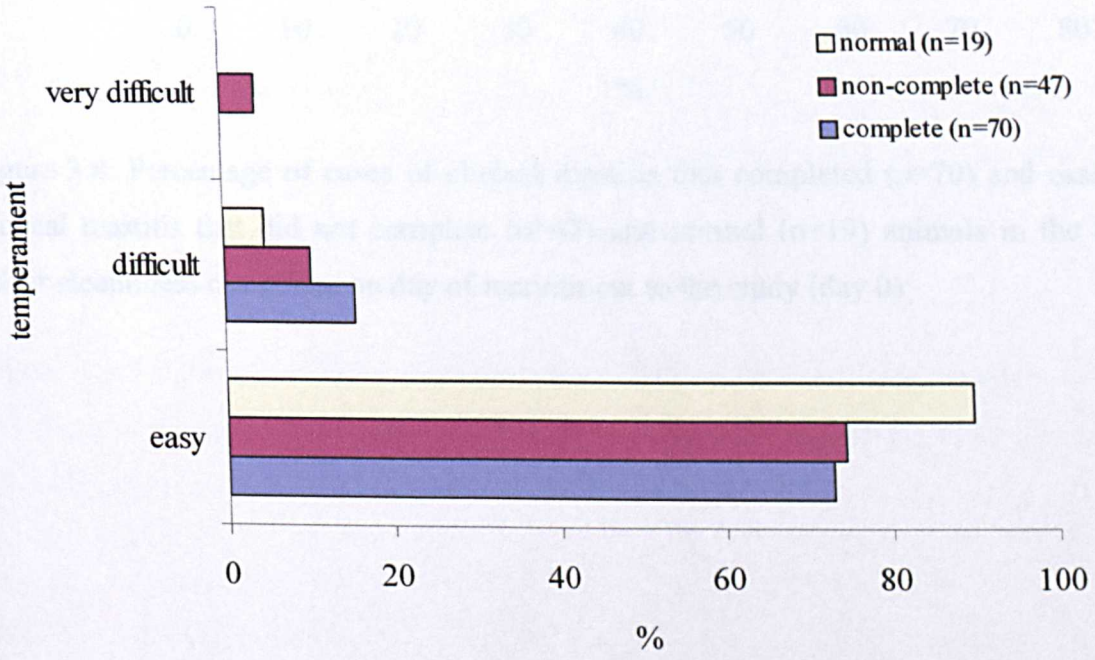


Figure 3.7: Percentage of cases of clinical mastitis that completed (n=70) and cases of clinical mastitis that did not complete (n=47) and normal animals (n=19) in the three temperament categories on day of recruitment to the study (day 0)

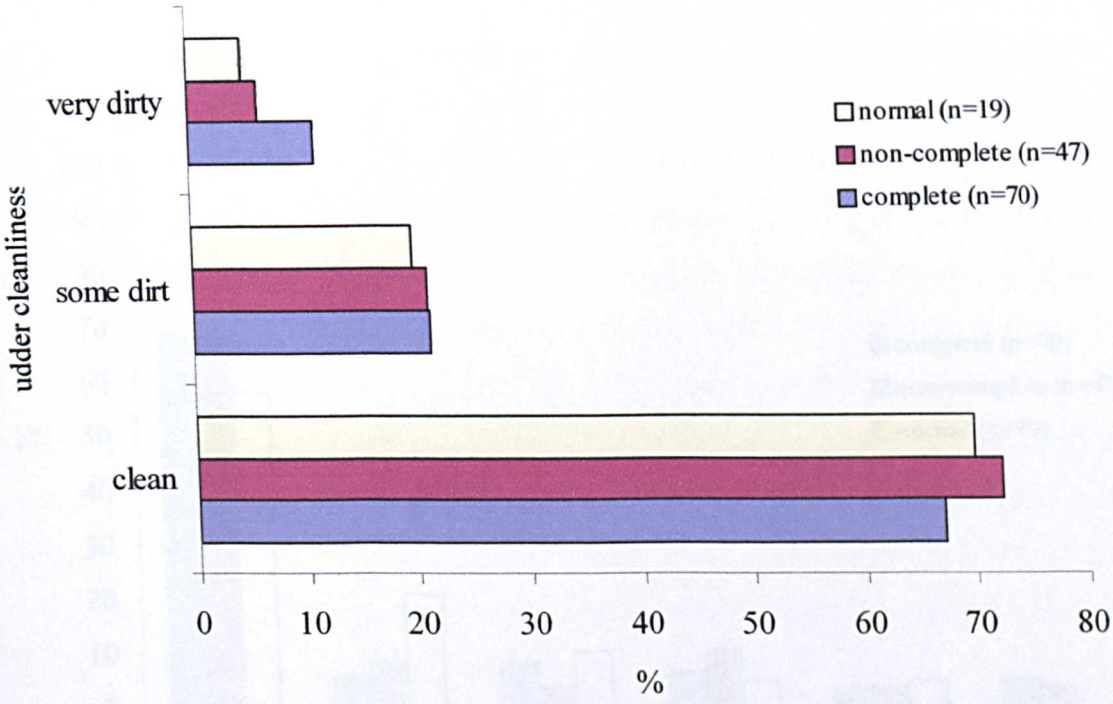


Figure 3.8: Percentage of cases of clinical mastitis that completed (n=70) and cases of clinical mastitis that did not complete (n=47) and normal (n=19) animals in the three udder cleanliness categories on day of recruitment to the study (day 0)

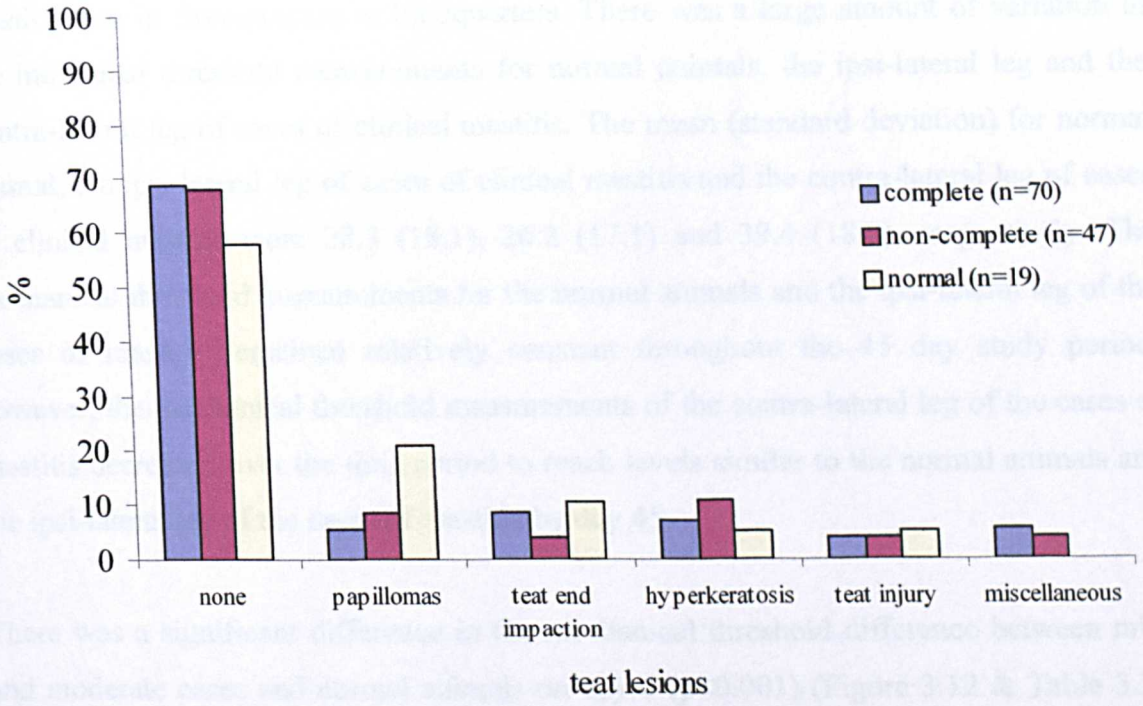


Figure 3.9: Percentage of teat lesions for cases of clinical mastitis that completed (n=70) and cases of clinical mastitis that did not complete (n=47) the 45 day study period and normal animals (n=19) on day recruitment to the study (day 0)

3.3.5. Mechanical threshold measurement

The mechanical threshold measurements for the ipsi-lateral leg were lower ($p<0.001$) than the mechanical threshold measurements for the contra-lateral leg for all cases of mastitis (Figure 3.10). The mechanical threshold measurements on both hindlegs of normal animals were similar to the measurements on the ipsi-lateral leg ($p>0.05$) of the cases of mastitis, but were lower than the measurements for the contra-lateral leg of cases of mastitis ($p<0.05$) (Figure 3.11). There was no significant difference ($p>0.05$) between the measurements for the ipsi-lateral or the contra-lateral leg depending on whether the mastitis was in frontquarters or hindquarters. There was a large amount of variation in the individual threshold measurements for normal animals, the ipsi-lateral leg and the contra-lateral leg of cases of clinical mastitis. The mean (standard deviation) for normal animal, the ipsi-lateral leg of cases of clinical mastitis and the contra-lateral leg of cases of clinical mastitis were 28.3 (18.1), 26.2 (17.1) and 39.4 (18.0), respectively. The mechanical threshold measurements for the normal animals and the ipsi-lateral leg of the cases of mastitis remained relatively constant throughout the 45 day study period, however, the mechanical threshold measurements of the contra-lateral leg of the cases of mastitis decreased over the time period to reach levels similar to the normal animals and the ipsi-lateral leg of the cases of mastitis by day 45.

There was a significant difference in the mechanical threshold difference between mild and moderate cases and normal animals on day 0 ($p<0.001$) (Figure 3.12 & Table 3.3), but there was no significant difference between mild and moderate cases. The mechanical threshold difference on the day of recruitment was significantly lower in cases when mastitis affected the front quarters compared to back quarters ($p<0.05$) (Figure 3.13).

The mechanical threshold difference for mild and moderate cases of clinical mastitis over the 45 day study period was greatest on the day of diagnosis (Figure 3.14). The threshold difference for the normal animals fluctuated around 0kPa (i.e. no difference between the two hind legs) for the 45 day study period. The mechanical threshold difference for the cases given antibiotics alone decreased gradually over the 45 day period with levels similar to that of normal animals reached by day 45. The mechanical threshold difference

of cases given antibiotics and meloxicam decreased rapidly over the first few days and maintained lower levels than the cases that received antibiotics only for the 45 day study period. Mechanical threshold differences of mild and moderate cases of clinical mastitis were similar, but the moderate cases appeared to have a more rapid decrease in threshold difference relative to the mild cases (Figures 3.15). In moderate cases the cows that received antibiotics and meloxicam had mechanical threshold differences that were similar to the normal animals six days after recruitment, whereas the cases given antibiotics only were similar to normal animals 20 days after recruitment. In mild cases the cows given meloxicam and antibiotics had normal level mechanical threshold differences by 45 days after recruitment into the study, whereas the cases given antibiotics only, had an increased mechanical threshold difference even by 45 days after recruitment into study.

Multivariable analysis of data from the first seven days after recruitment to the study showed that mechanical threshold difference increased as the rectal temperature increased, and the mechanical threshold difference decreased with meloxicam treatment and over time (Table 3.23). Multivariable analysis for the 45 day study period showed that the mechanical threshold difference decreased with meloxicam treatment and over time (Table 3.24) There was no effect of time, or any other variables, on mechanical threshold responses in normal animals.

The duration of the alteration in response to mechanical stimulation was investigated. In cases of mild and moderate mastitis the median (IQR) duration of alteration in response to mechanical stimulation for the animals treated with antibiotics only was 45 (2 - 45) days and for animals treated with antibiotics and meloxicam was 6 (1 - 45) days. The cases were subdivided into mild and moderate cases and the median (IQR) duration of alteration in response to mechanical stimulation for animals treated with antibiotics only were 45 (1.5 - 45) and 45 (20 - 45) days, for mild and moderate cases respectively, and for animals treated with antibiotics and meloxicam were 6 (1 - 45) and 2 (1 – 32.5) days, for mild and moderate cases respectively. Survival analysis demonstrated a significant difference between groups in the time to achieve normal threshold levels, such that cases treated with meloxicam reached normal threshold levels earlier than cases treated with antibiotics only ($p=0.04$) (Figure 3.16).

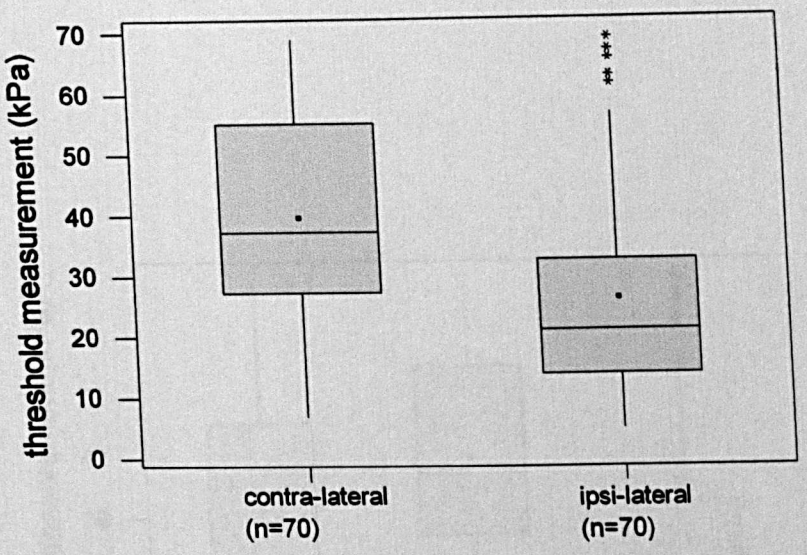


Figure 3.10: Mechanical threshold measurements (kPa) for the leg ipsi-lateral (n=70) and contra-lateral (n=70) to the quarter with clinical mastitis on day of recruitment to the study (day 0)

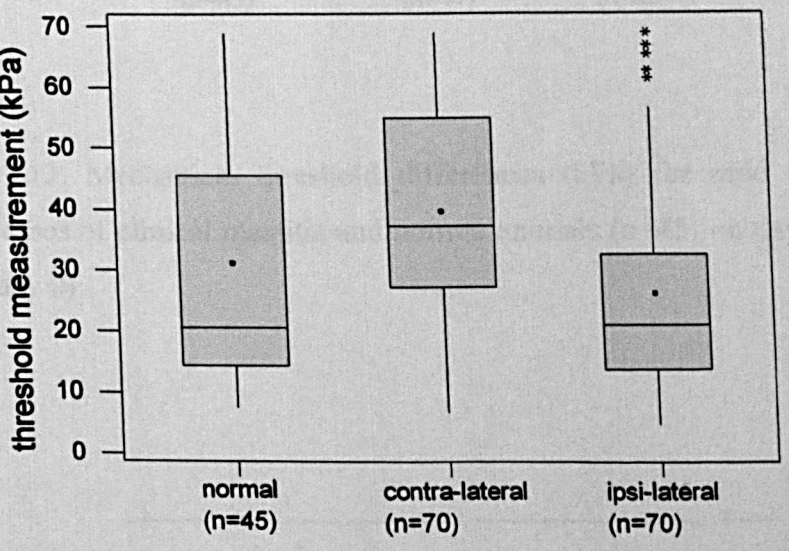


Figure 3.11: Mechanical threshold measurements (kPa) for ipsi-lateral leg (n=70) and contra-lateral leg (n=70) in cases of mild and moderate mastitis and for normal animals (n=45) on day of recruitment to the study (day 0)

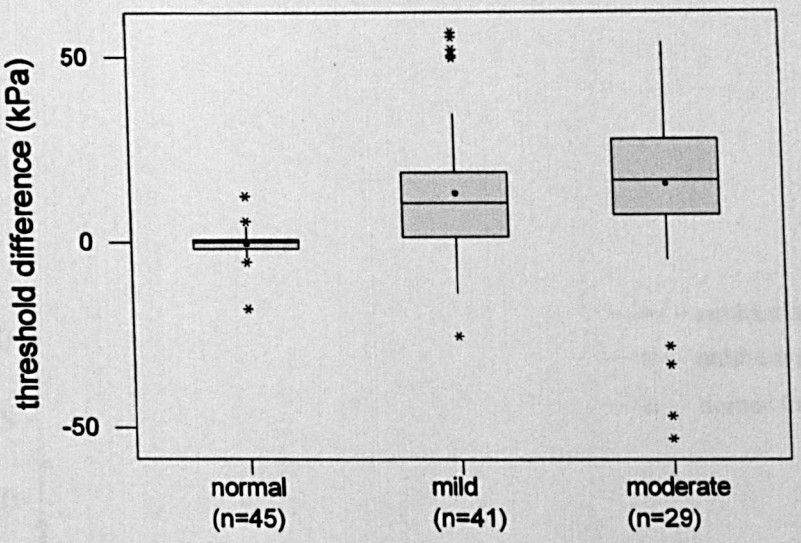


Figure 3.12: Mechanical threshold differences (kPa) for mild (n=41) and moderate (n=29) cases of clinical mastitis and normal animals (n=45) on day of recruitment to the study (day 0)

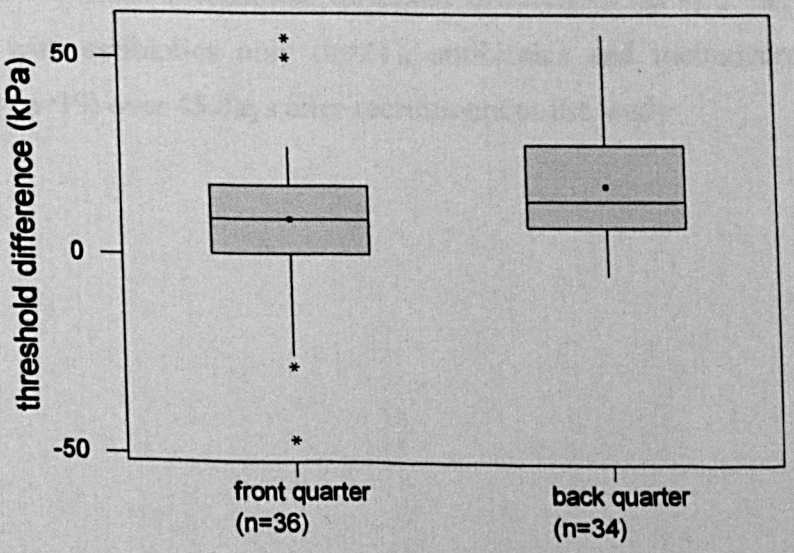


Figure 3.13: Mechanical threshold differences (kPa) for cases of clinical mastitis in the front quarter (n=36) and back quarter (n=34) on day of recruitment to the study (day 0)

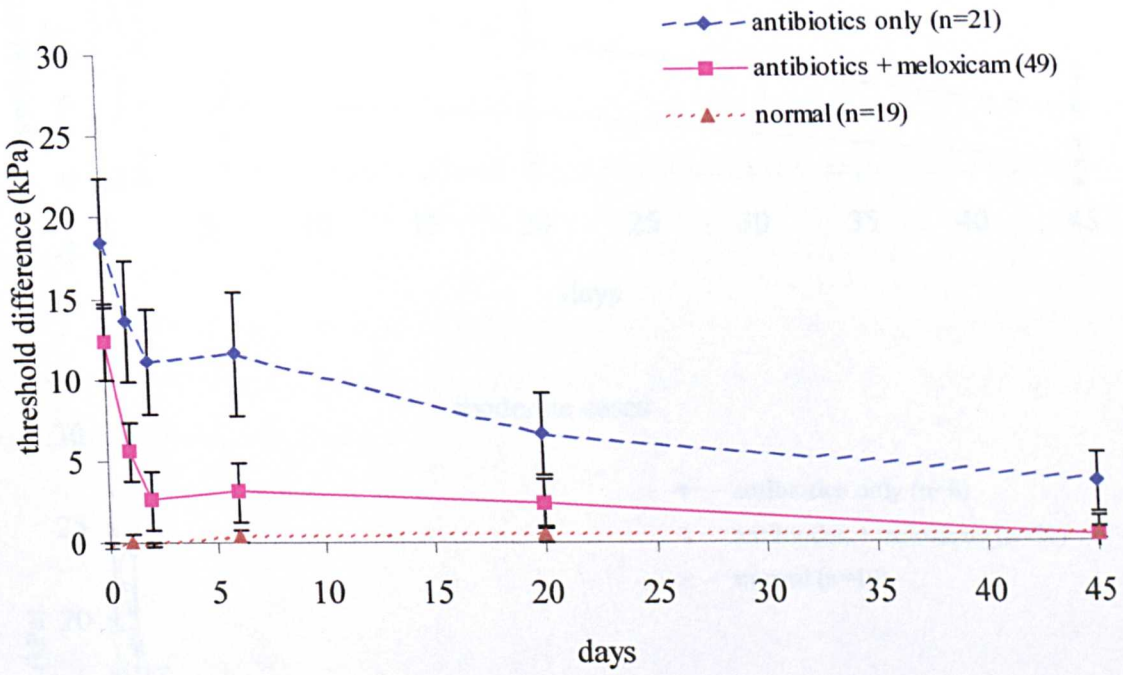


Figure 3.14: Mean mechanical threshold differences (kPa) ± SE for cases of mastitis treated with antibiotics only (n=21), antibiotics and meloxicam (n=49) and normal animals (n=19) over 45 days after recruitment to the study

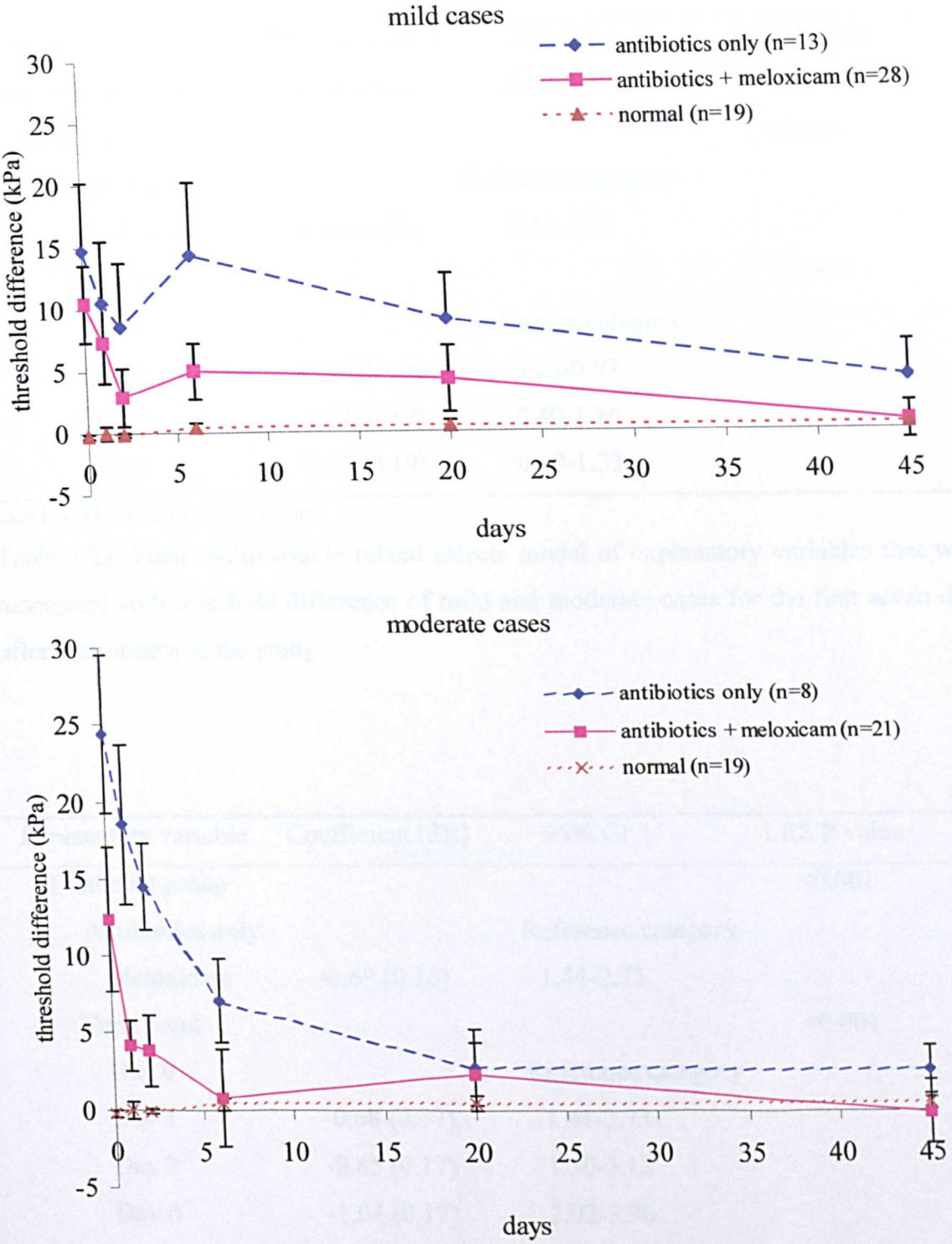


Figure 3.15: Mean threshold difference (kPa) ± SE for cases (mild, moderate) of mastitis treated with antibiotics only (n=13, 8), antibiotics and meloxicam (n=28, 21) and normal animals (n=19, 19) over 45 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Rectal temperature	0.14 (0.12)	-0.10-0.37	<0.001
Treatment group			<0.001
antibiotics only		Reference category	
meloxicam	-0.84 (0.20)	0.45-1.22	
Time point			<0.001
Day 0		Reference category	
Day 1	-0.61 (0.18)	0.25-0.97	
Day 2	-0.77 (0.19)	0.40-1.14	
Day 6	-0.97 (0.19)	0.60-1.33	

LRS Likelihood ratio tests statistics

Table 3.23: Final multivariable mixed effects model of explanatory variables that were associated with threshold difference of mild and moderate cases for the first seven days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Treatment group			<0.001
Antibiotics only		Reference category	
Meloxicam	-0.69 (0.16)	1.44-2.73	
Time point			<0.001
Day 0		Reference category	
Day 1	-0.68 (0.17)	1.41-2.73	
Day 2	-0.85 (0.17)	1.50-3.12	
Day 6	-1.04 (0.17)	2.02-3.96	
Day 20	-1.07 (0.19)	2.01-4.19	
Day 45	-1.30 (0.19)	2.50-5.33	

LRS Likelihood ratio tests statistics

Table 3.24: Final multivariable mixed effects model of explanatory variables that were associated with threshold difference of mild and moderate cases of clinical mastitis for the 45 days after recruitment to the study

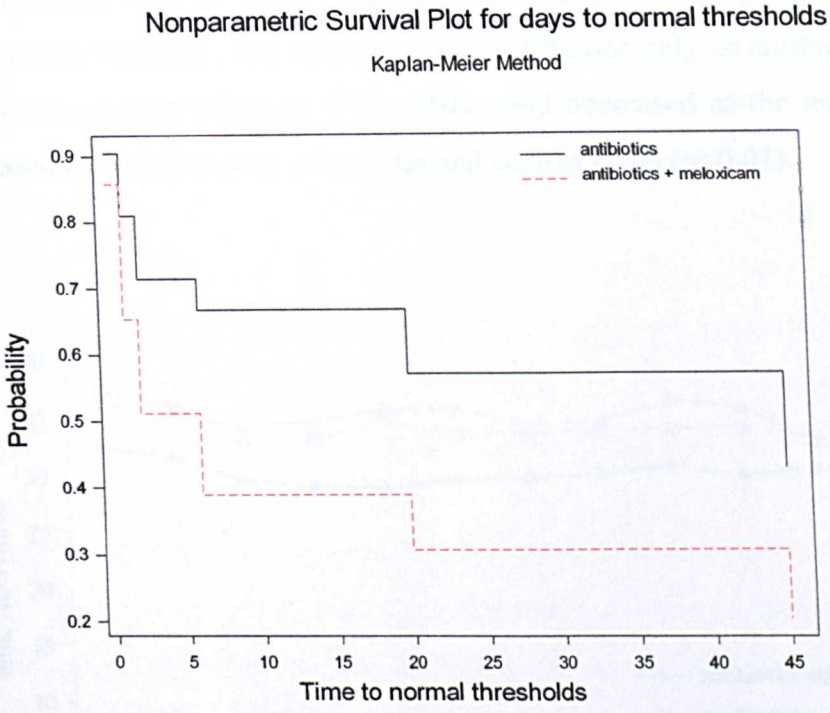


Figure 3.16: Kaplan-Meier survival function for duration of alteration in response to mechanical stimulation in mild and moderate cases of clinical mastitis treated with antibiotics only (n=21) or antibiotics and meloxicam (n=49)

3.3.6. Milk yield

Farmers were requested to record the milk yield for every cases of clinical mastitis for the 45 day study period, however, compliance was poor. Complete milk yield data was available from only 10 cases from five different farms. Of these 10 cases, seven were classified as mild and three as moderate severity. General linear model analysis showed that the milk yield of cases receiving antibiotics and three doses of meloxicam was greater than the milk yield of cows given antibiotics only or antibiotics and one dose of meloxicam ($p<0.05$) (Figure 3.17). Milk yield decreased as the number of days calved increased in both the cases of mastitis and normal cows ($p<0.01$).

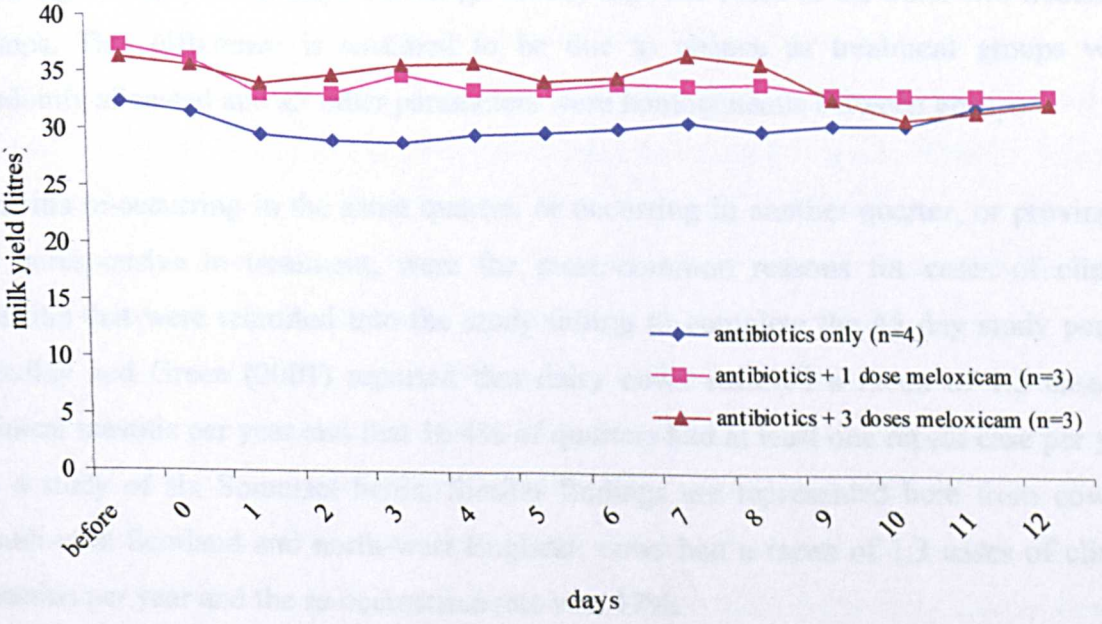


Figure 3.17: Milk yield before the cow had clinical mastitis and for 12 days after clinical mastitis for cases treated with antibiotics only (n=4), antibiotics and one dose of meloxicam (n=3) and antibiotics and three doses of meloxicam (n=3)

3.4. Discussion

Cases of clinical mastitis occurred throughout lactation, but the majority occurred within the first four months, i.e. early lactation, which has been recognised previously as the most common time of mastitis occurrence (Bramley and Dodd 1984; Wilesmith and others 1986; Hogan and others 1989b; Lescourret and others 1995; Miltenberg and others 1996). The age and production demographics were similar for normal animals and cases of clinical mastitis, indicating that recruited normal animals were representative of the population at risk of developing mastitis, and thus could be considered as appropriate controls for the cases of clinical mastitis. On checking the homogeneity of the assignment to treatment groups, cases receiving antibiotics and three doses of meloxicam were significantly fewer days calved ($p=0.002$) than the cases in the other two treatment groups. This difference is assumed to be due to chance as treatment groups were randomly allocated and all other parameters were homogeneous between groups.

Mastitis re-occurring in the same quarter, or occurring in another quarter, or proving to be unresponsive to treatment, were the most common reasons for cases of clinical mastitis that were recruited into the study failing to complete the 45 day study period. Bradley and Green (2001) reported that dairy cows suffered a mean of 1.5 cases of clinical mastitis per year and that 16.4% of quarters had at least one repeat case per year, in a study of six Somerset herds. Similar findings are represented here from cows in south-west Scotland and north-west England: cows had a mean of 1.3 cases of clinical mastitis per year and the re-occurrence rate was 17%.

The development of lameness was the next most common reason for recruited cases failing to complete the 45 day study period. The lameness rate in 434 British dairy herds assessed by Whitaker and others (2004) was 20.7%, demonstrating that lameness is common in dairy cattle and explaining the occurrence of lameness as the second most common reason for cases in the current study failing to complete the 45 day study period. The finding that more moderate cases of clinical mastitis failed to complete the 45 day study period than mild cases of clinical mastitis is biologically plausible as more severely affected cases may be more difficult to cure, more likely to develop mastitis in another

quarter, and more likely to develop concurrent infection. This may reflect the fact that cows that develop clinical mastitis are often immunosupressed, either as a consequence of the mastitis (Taub and others 1989) or prior to the development of mastitis (Oliver and Sordilo 1988). The rectal temperature of non-complete cases was significantly higher than complete cases. This finding is also biologically plausible as it would be expected that more severely affected (moderate) animals would have higher rectal temperatures then less severely affected (mild) and that animals with higher rectal temperatures may be more likely to become systemically ill, as pyrexia is a clinical sign of systemic disease (Radostits and others 2000).

The normal ranges for the physiological parameters of adult cattle are: heart rate 65-80 beats/minute; respiratory rate 15-35 breaths/minute and rectal/body temperature 38.0-39.0°C (Rosenberger 1979; Jackson and Cockcroft 2002). The physiological parameters of clinical cases and normal animals were generally within the normal ranges for cattle on the day of recruitment to the study, with moderate cases being the top end of normal for rectal temperature, heart and respiratory rates. These findings suggest that the subjective grading of mastitis severity by a single observer was good, with the moderate cases having physiological measurements in the top end of normal range and the mild cases and normal animals towards the bottom of the normal range. Physiological parameters may be used to distinguish between mild and moderate cases of clinical mastitis, as suggested by data from this study, and from this treatment could potentially be targeted specifically to severity of mastitis. The use of an accurate rectal thermometer to measure the rectal temperature would be the most convenient and user-friendly parameter for farmers to use to judge severity and could even be adapted for automated systems using body temperature sensors. The physiological parameters for the cases of clinical mastitis in all three treatment groups decreased slightly over the first two days after recruitment and then remained relatively constant for the remaining period of the study. The multivariable analysis showed an association between the physiological parameters; heart rate, respiratory rate and rectal temperature. This inter-relationship would be expected and has biological inference. The relationship is such that it is not clear which is the explanatory and which is the response variable, for example some variance of heart rate can be explained by the association with respiratory rate and some of the variance of respiratory rate can be explained by the association with heart rate. There was no effect of treatment on heart rate, respiratory rate and rectal temperature.

Using the data from this study heart rate, respiratory rate and rectal temperature were assessed as predictors of clinical mastitis and as predictors of mild or moderate severity. As predictors of clinical mastitis they had sensitivities and specificities around 50-60%. Their ability to differentiate between mild and moderate severities had sensitivities and specificities around 60-70%.

Ward and others (2002) stated that cow cleanliness was vital if mastitis is to be controlled in a straw yard, and while factors such as the quality and quantity of straw used, the ventilation rate within the building and stocking density were important, the major factor was faecal consistency. These authors reported a significant relationship between faecal consistency and the cleanliness of the udder, flanks and legs of the early lactation cows ($p<0.05$), suggesting that diets that result in drier faeces may be beneficial in the control of environmental mastitis in straw yard systems (Ward and others 2002). Cook (University of Wisconsin www.vetmed.wisc.edu/dms/fapm/forms.htm) and Hughes (2001) recommended regular hygiene scoring to increase awareness of cow cleanliness. These authors used a similar subjective, ordinal scale to the one used in this study. Data from this current study showed no difference in the udder cleanliness between normal cows and cases of clinical mastitis, however, normal animals were taken from the same farm as cases and therefore a comparison between different farm incidences of clinical mastitis according to cow cleanliness was inappropriate in this instance.

There was no difference in the presence of teat lesions between normal animals and cases of clinical mastitis, a result that differs from previous studies. Neave and others (1969) reported that teat lesions were an important source of Streptococci and Staphylococci spp., and that consequently teat lesions along with the presence of infected milk were important sources for development of clinical mastitis. It may be that the alteration in the pathogen profile from udder based pathogens to environmental organisms over the years (Milne and others 2002) has led to teat lesion colonisation being a less important risk in the development of clinical mastitis, or indeed the type of teat lesions may influence the risk and the type of teat lesions may have altered over the years.

Normal animals had smaller hock-to-hock distances compared to cows with clinical mastitis suggesting that there was an alteration in the hindleg stance of the cow as a result

of disease. Hock-to-hock distance showed promise as a predictor of clinical mastitis, with a sensitivity of 64% and specificity of 84%. There was no difference between mild and moderate cases of mastitis suggesting that increasing severity of mastitis did not result in the cow further altering her hindleg stance. This is surprising as moderate cases were, by definition, associated with udder changes and it would be expected that udder swelling might alter the stance of a cow. The hock-to-hock distance for the normal cows was relatively constant over the study period suggesting that unaffected cows have a uniform stance. Treatment did not affect the hock-to-hock distances over time suggesting that the administration of the NSAID; meloxicam (Metacam, Boehringer-Ingelheim Limited, Bracknell) did not have any beneficial influence on this variable. This again is surprising, as if the alteration in the stance of a cow with mastitis is not associated with the presence of udder swelling (as demonstrated by the lack of difference in hock-to-hock distances between mild and moderate cases), it might have been expected to be due to localised pain. It may be that cows with a wider hindleg stance are more predisposed to mastitis and this could be related to larger udders and greater milk yields. It has been previously recognised that cows with higher genetic milk yield have an increased risk of developing clinical mastitis (Bunch and others 1979; Shook 1989). Unfortunately there was insufficient data on milk yield in this study to assess this further.

Hock-to-hock distance over the first seven days after recruitment of cases to the study was positively associated with body condition score, parity and heart rate, and negatively associated with time, udder cleanliness, and threshold difference. An increased hock-to-hock distance with increased body condition score and parity and a decrease in hock-to-hock distance with time is biologically plausible. The decrease in hock-to-hock distance observed in cows with dirty udders might be explained by the fact that cows that stand with their hindlegs closer together are more inclined to have dirty udders as a result of the hindlegs contacting the udder more closely.

Previous studies relating to the measurement of mechanical stimulation thresholds were based on the absolute values from the lame leg of affected animals and compared to the absolute values of one leg of the normal animal (Nolan and others 1987; Ley and others 1995; Whay and others 1998). Those authors reported that the mechanical thresholds were significantly lower in the lame animals compared to normal animals. In this current study threshold measurements were assessed on both hindlegs and it was found that the

measurements from the ipsi-lateral leg of cases with unilateral mastitis was not significantly different from those of the normal animals, however, the measurements from the contra-lateral leg of the cases of mastitis were significantly higher than those of normal animals and the ips-lateral leg ($p<0.05$). This finding that normal animals reacted at similar thresholds to those of the ipsi-lateral leg was surprising and is contrary to the finding reported by Fitzpatrick and others (unpublished data). Instead the findings from the current study suggest that the threshold to stimulation is actually higher in the contra-lateral leg (hypoalgesia) rather than lower in the ipsi-lateral leg (hyperalgesia) when compared to normal animals. Hypoalgesia has been reported to occur in dairy cows subjected to acute stressors (Herskin and others 2004), but in the current study there is no reason why the case cows were subject to stress and the normal cows were not. Possible biases in the measurement of normal animals can be ruled out regarding time as normal animals were recruited and measured concurrently to the cases of mastitis and the threshold machine was calibrated on a regular basis and was found to be accurate on all occasions. Also biases in the measurement of the contra-lateral leg can be ruled out as the study design was such that the milk samples were collected by the farmer not the researcher therefore the researcher was less likely to recall which quarter was the affected one, especially as the study period was intense with many cows being measured on the same day. Instead this phenomenon might be explained by the low power of the study due to the large variation in the threshold measurements at the individual animal level or might be a chance finding due to statistical analysis or more probably an alternative hypothesis needs to be adopted. It might be that the threshold measurements of the ipsi-lateral leg appear similar to the measurements recorded in normal animals due to the ability to override the pain reflex for longer as a result of the reluctancy of the cow to make any movement. The high contra-lateral leg threshold measurements might be due to the reluctance of the cow to bear weight on the ipsi-lateral side to the mastitis. The complex nature of the innervation of the mammary gland is likely to lead to considerable cross over of nerve impulses at the spinal level making the pain associated with mastits more difficult to interpret compared to unilateral lameness and may explain why the contra-lateral threshold measurements alter with time but the ipsi-lateral measurements remain relativelty constant.

To allow the alterations in threshold response to be monitored further, the difference between the contra-lateral and ipsi-lateral leg of animals affected with unilateral clinical

mastitis and the difference between the two threshold measurements in normal animals were used, and this negates the individual variations in threshold responses. When considering the threshold difference, i.e. the reading from the ipsi-lateral leg subtracted from the contra-lateral leg, normal animals had lower threshold differences compared to mild and moderate cases of mastitis. The threshold difference was higher for moderate cases compared to mild cases, but was not significant ($p>0.05$) on the day of diagnosis of clinical mastitis. Studies in lameness in cattle reported increased sensitisation to mechanical stimulus, i.e. reduced thresholds to mechanical stimulation, with increasing severity of lameness (Whay and others 1997). With a larger dataset it might be that the alteration in nociceptive pathways are more pronounced with increasing severity of mastitis. There was no effect of time on the response of normal cattle to the mechanical threshold device, consistent with findings from Whay and others (1996), who suggested that cows do not adapt to repeated application of mechanical stimulation to the hindlegs.

The alteration in response to mechanical stimulation in this study lasted between six and 45 days for moderate and mild cases of clinical mastitis, respectively, which was a similar duration to that reported in a smaller study by Fitzpatrick and others (1998). These authors reported the alteration in response to mechanical stimulation lasted for up to four days and 20 days for mild and moderate cases of clinical mastitis, respectively. It is interesting to note, however, that in the current study the alteration in response to mechanical stimulation of moderate cases of clinical mastitis lasted for approximately six days, which was a shorter duration than in mild cases where the alteration in response to mechanical stimulation lasted for approximately 45 days. This may be due to the different causal pathogens in the different severities of mastitis and may reflect the pathogenesis of the specific organisms and their response to therapy.

In sheep with severe 'foot rot', alteration in response to mechanical stimulation was present for three months after treatment and apparent resolution of the foot lesion (Nolan and others 1987; Ley and others 1995). Unilaterally lame cattle were examined 28 days after treatment of the lameness and the pain threshold was reported to still be significantly reduced in cows with solar ulcers and white line disease but not in cows with acute tissue infection compared to normal cows assessed on day of recruitment to the study (Whay and others 1998). This indicated that alterations in response to mechanical stimulation were present for at least 28 days in lame cows, but the

persistence of the alteration in response to mechanical stimulation differed between lesion type. In this current study, alterations in response to mechanical stimulation were present for a median of 45 days in cows with mild and moderate clinical mastitis that were treated with antibiotics only. In cases of mild and moderate clinical mastitis treated with antibiotics and meloxicam, alterations in the response to mechanical stimulation were present for a median of 6 days. Treatment with meloxicam in conjunction with antibiotics, therefore, reduced the duration of alteration in response to mechanical stimulation in cows with clinical mastitis by 39 days. The finding that there was no apparent hyperalgesia in the ipsi-lateral leg of the cases of clinical mastitis in this study, but there was a treatment effect with NSAID might be due to the quirk of statistical analysis or due to the central action of NSAID, which would also be consistent with the lack of treatment effect on the hock-to-hock distance.

In the current study the threshold difference also decreased with time and increased with increased rectal temperature, which could indirectly represent increased severity as moderate cases had higher mean rectal temperatures than mild cases. Mean mechanical thresholds for sheep with mild and severe footrot were increased following administration of a local anaesthetic block and local infiltration above the affected foot and below the mechanical testing device using 0.25% bupivacaine HCl (Ley and others 1989). Lascelles and others (1998) used a combination of a VAS scoring system and mechanical pain threshold testing to assess the NSAID, carprofen, in bitches undergoing elective ovariohysterectomy. They reported that both pre- and post-operative carprofen administration significantly reduced the degree of pain exhibited, however, pre-operative carprofen had greater analgesic properties in the early post-operative period compared to post-operative carprofen.

The studies by Welsh and Nolan (1994 &1995) used a maximum cut-off threshold of 16N this is considerably lower than the equivalent maximum threshold of 135N used in this study, however, details of the pin and plate diameters are not specified and thus calculations of pressure at the pin or plate levels can't be made and a direct comparison with this study is not possible. In the studies assessing the mechanical thresholds in lame cows, Whay and others (1997 & 1998) also used a lower maximum force threshold of 20N. These authors used a device with a pin diameter of 2mm, this is marginally smaller than the pin (diameter 2.16mm) used in this current study. They report their results in

units of force and from this and the pin diameter the maximum pressure at the pin level can be calculated as 6,370kPa. This is considerably smaller than the pressure of 36,950kPa, that was recorded in this study. Whay and others (1997 & 1998) give no details of the plate area within the cuff, therefore, the pressure at this level can't be compared. The discrepancy between the pressure and force measurements in this study and published work by Whay and others (1997 & 1998) may be explained by errors in the calculations of the force at the cuff level, however, the absence of details of the dimensions within the cuff makes this difficult to prove. Possible biological reasons for the difference could include variations in the skin thickness at the hock, more stringent exclusion criterion, different genetics, different nutritional status especially with respect to calcium levels, as calcium in involved in nerve function. It does seem unlikely that a biological reason could explain the 10-fold difference in threshold levels between cows in the studies by Whay and others (1997 & 1998) and this current study.

Thermal stimulation and mechanical stimulation were used by Nolan and others (1987) in sheep, who reported that both methods were reliable and reproducible methods for the assessment of the efficacy of analgesic drugs in sheep. In this current study the threshold differences, calculated from the threshold responses to mechanical stimulation of both hindlegs, of normal animals fluctuated around zero for the 45 day study period suggesting that there was no difference in the threshold response between the two hindlegs for the duration of the study. These results confirm that there did not appear to be adaptation due to repeated application of the mechanical device over time in this study. This suggests that application of a mechanical stimulation and calculation of threshold responses is also reliable and useful for the assessment of analgesics in cattle.

The greatest economic consequence of clinical mastitis is loss in milk production, which is a combination of reduction in milk yield, and compulsory discardment of milk following treatment with antibiotics (Kossaibati and Esslemont 1995). Hortet and Seegers (1998) reviewed the literature to assess the milk loss associated with mastitis. These authors estimated that, for an average Holstein cow, the milk losses were 4-6% of lactation yield, with 40% of cases having negligible loss, 30% losing 150-250 kg/lactation, and 30% losing 950-1050 kg/lactation. Lucey and Rowlands (1984) reported that a clinical case of mastitis resulted in a 6.4% drop in yield. It would be beneficial from both economic and welfare aspects if the milk loss associated with

mastitis could be reduced. Although there were limited data on the milk yield and cases that were given antibiotics alone had a lower milk yield relative to the other treatment groups before mastitis, there was a suggested yield benefit from the administration of meloxicam. Multivariable analysis, however, detected an association with days calved and milk yield with no other variables being significant. It should be noted that cases in treatment group 3 were significantly fewer days calved than cases in the other groups, and this alone may explain the suggested yield benefit seen with meloxicam treatment. A previous study (Shpigel and others 1994) demonstrated a positive benefit on the milk yield with the use of NSAID in addition to antibiotics in the treatment of naturally occurring clinical mastitis. The cows recruited into their study were Israeli-Holstein cows from seven commercial farms, which were accommodated in a zero grazing system and milked three times a day. They reported a significant improvement in recovery, judged by return to at least 75% of pre-mastitis daily milk yield, in cows with acute clinical mastitis in both a non-blinded controlled study and a blinded placebo-controlled study where a combination of ketoprofen and antimicrobial was compared to antimicrobial alone. Antimicrobial, 20g sodium sulphadiazine and 4g trimethoprim (Diaziprim Firte, Vitamed) was administered daily by the intramuscular route until milk production started to increase, to a maximum of five treatments. Ketoprofen (Ketofen-RM528 10% solution, Rhone Merieux) was administered intramuscularly once daily for the duration of the antimicrobial treatment. The cases recruited into the study by Shpigel and others (1994) included animals that were systemically sick and the antimicrobial therapy was administered by the intramuscular route and for a longer duration than in the current study. The inclusion of systemically sick animals may have allowed the difference between antibiotics only and antibiotics plus NSAID to be more pronounced, due to NSAIDs anti-endotoxic, anti-inflammatory and anti-pyrexic effects increasing the treatment response of these systemically sick cows. A larger data set, with stage of lactation taken into account is required before a true assessment of the effect on milk yield production by the use of meloxicam can be made.

In summary, threshold difference, udder temperature and hock-to-hock distance are useful parameters to detect disease at the day of recruitment and physiological parameters, such as heart rate, respiratory rate and rectal temperature can differentiate between mild and moderate mastitis on the day of recruitment. These findings may have implications in improving the detection of mastitis in automated systems with sensors

detecting udder temperature, body temperature and hock-to-hock distance being possible innovations. The ability to differentiate between the severities of mastitis can allow the specific targeting of therapy according to severity. The use of meloxicam in conjunction with intramammary antibiotics had a significantly beneficial effect on pain threshold response. Meloxicam, thus, reduced the increased responsiveness to pain measured in cows with mastitis and its' use could have significant welfare improvements due to alleviation of pain and returning the cow to normality more quickly.

Chapter 4

Laboratory assessment of pain and inflammation in cows with clinical mastitis

4.1. Introduction

Many different pathogenic bacteria can infect the mammary gland and there are considerable differences in epidemiological and predisposing factors with different types of infection (Dodd and Neave 1970). The most prevalent pathogens isolated from clinical cases in the UK include *S uberis*, *E coli* and *Staph aureus* (Anon 1999). Mastitis has adverse effects on the economics of milk production by reducing the quantity and quality of milk and manufactured milk products (DeGraves and Fetrow 1993). Intramammary infections are the most important factor influencing the number of somatic cells in milk (Reneau 1986). There is, however, no widely accepted definition of what constitutes a low bulk milk somatic cell count (BMSCC), individual cow somatic cell count (ICSCC) or individual quarter somatic cell count (IQSCC). Published studies have used somatic cell counts (SCC) of less than 250 $x10^3$ cells/ml (Barkema and others 1999; de Haas and others 2002), <200 $x10^3$ cells/ml (Huxley and others 2002), <150 $x10^3$ cells/ml (Schukken and others 1989) <100 $x10^3$ cells/ml (Peeler and others 2000) and <50 $x10^3$ cells/ml (Beaudeau and others 2000) to define both low cell count herds and individual cows. A healthy quarter has been suggested as one with a SCC <100 $x10^3$ cells/ml and which is free of mastitis pathogens on routine bacteriological culture of the milk (Dohoo and Meek 1982; Harmon 1994; Sargeant and others 2001). Increased SCC are associated with compositional changes, such as decreases in lactose, ∝-lactoalbumin and fat, and changes in the types of proteins (Harmon 1994). Mastitic milk has greatly reduced concentrations of casein but increased levels of albumin and globulin (Munro and others 1984).

Milk somatic cells are primarily leukocytes that include macrophages, lymphocytes and polymorphonuclear neutrophils (PMNs). Following bacterial invasion, inflammatory mediators produced by cells in the infected gland elicit neutrophil recruitment. This leads to neutrophil numbers rising from relatively low normal levels observed in the healthy udder to constitute greater than 90% of the total mammary leukocyte population during mastitis (Sordillo and others 1997). The role of PMNs is to phagocytose and kill bacteria (Nickerson 1987; Daley and others 1991) and high concentrations of cells are required to eliminate the bacteria due to a decreased capacity for phagocytosis in milk compared to blood (Wisniowski and others 1965; Kent and Newbould 1969; Paape and others 1979). The dominance of PMNs is short lived as they are not able to reproduce, within 24 hours mononuclear cell numbers increase, while during resolution or progression to chronicity macrophages become the most dominant cell type (Higgins and Lees 1984). The acute phase response to inflammation includes the release of acute phase cytokines which, in turn, lead to the appearance of, or a rapid increase in, the concentration of a number of plasma proteins collectively known as the acute phase proteins (APP) (Baumann and Gauldie 1994; Moshage 1997). In cattle, haptoglobin and serum amyloid A are the most sensitive APP indicating acute inflammation (Horadagoda and others 1999). The concentrations of APP can be used to provide an indication of the severity of disease (Godson and others 1996; Horadagoda and others 1999; Eckersall and others 2001; Sheldon and others 2001; Nielsen and others 2004). The concentrations of APP in serum and milk correlated with the severity of clinical signs of mastitis (Eckersall and others 2001) and with an increase in CMT score (Nielsen and others 2004). The concentrations of APP in serum were increased relative to severity of bacterial contamination of uterus post-calving (Sheldon and others 2001).

The objective of this study was to assess if laboratory parameters could act as quantitative markers of severity of clinical mastitis in cows and whether the use of NSAID, meloxicam altered these parameters.

4.2. Materials and methods

4.2.1. Sampling

Milk samples (~90ml) were obtained from the affected mastitic mammary gland of each cow and from the nominated quarter of the normal cows for somatic cell counting, differential cell counting, bacteriology and measurement of APP concentrations using techniques described in Chapter 2. Samples (~65ml) were also taken from the diagonally opposite quarter to the affected quarter in the mastitic cows for somatic cell counting and APP determination.

4.2.2. Statistical analysis

Data were entered into Microsoft Access 2000 and basic statistical analysis was conducted using Microsoft Excel and Minitab Statistical Software version 13 and univariable and multivariable regressions were performed using S-Plus 2000 and MlwiN version 1.1 as detailed in Chapter 2. Data were assessed for the first seven days after recruitment, as this would be the period when the NSAID and antimicrobial activity were at their peak. Data for the 45 day period was also assessed for IQSCC and data for the first 12 days were assessed for the APP, as these were monitored for 12 days rather than 45 days. The APP were only assessed for the first 12 days to allow the effect of treatment to be evaluated for all the complete cases, a random selection of diagonally opposite quarters and normal animals.

A binomial logistic regression was carried out, using the presence of specified microbiological isolation, or all other microbiological results e.g. *S uberis* versus all other culture results, as the outcome, and cow and farm as random effects. Microbiological groups tested included *S uberis*, *E coli*, no growth, *Staph aureus*, *S dysgalactiae*, *A pyogenes* and miscellaneous. Putative covariates examined were: breed, the quarter affected, body condition score, parity, severity, days calved, teat lesions, udder cleanliness, temperament of cow, heart rate, respiratory rate, rectal temperature, udder temperature difference, hock-hock distance, threshold difference, logIQSCC, fat content, protein content, lactose content, cube root Hp and cube root mammary-associated serum amyloid A (M-SAA).

A maximum value for IQSCC was used for the samples that could not be counted due to the presence of too many clots. The value used was 24,000 $\times 10^3$ cells/ml and the log of this value was used for the univariable and descriptive analysis.

Two-by-two tables were constructed to allow the sensitivity, specificity, predictive values and accuracy to be calculated (Dohoo and others 2003) for cases with clinical mastitis versus normal animals, and mild versus moderate cases of clinical mastitis.

4.3. Results

4.3.1. Cases recruited

As described in Chapter 3, 117 cases with clinical mastitis were recruited into the study, however, only 70 cases were followed for the full 45 day study period. Of these 70 cases, 41 were classified as mild and 29 were classified as moderate clinical mastitis. One moderate case was difficult to sample due to poor milk let-down and therefore provided insufficient samples for many of the tests. Twenty normal cows were recruited into the study; however, only 19 were followed for the full 45 day study period.

4.3.2. Bacteriological examination

On bacteriological examination of the recruited cases, 30% of isolates yielded *S uberis*, 24% yielded no growth, 22% *E coli*, 11% *Staph aureus*, six per cent other microbiological species, four per cent *A pyogenes* and three per cent *S dysgalactiae* (Figure 4.1). The miscellaneous category (six per cent) included other *Streptococcus* spp., *Corynebacterium* spp., coagulase-negative *Staphylococcus* spp., *Pseudomonas* spp., *Candida* spp., and *C* bovis. All normal cows had no significant growth on bacteriological examination of the randomly selected quarter.

The microbiological results of the cases that did not complete the study were compared to the cases that completed the study. There was a greater proportion of cases caused by *S uberis*, *A pyogenes* and the miscellaneous category in the cases that did not complete the study compared to those that did, however, this difference was not significantly different ($p>0.05$) (Figure 4.2).

The microbiological profiles of the cases, mild and moderate clinical mastitis, were assessed for differences. There were significantly more isolations of *S uberis* in the moderate cases of mastitis ($p<0.01$), and significantly more no growths in cases of mild mastitis ($p<0.01$). There tended to be more isolations of *E coli* in moderate cases and more isolations of *Staph aureus* in mild cases, although these were not significantly different ($p>0.05$) (Figure 4.3).

Logistic regression (Table 4.1) was used to identify any associations between the microbiological result and physiological (Chapter 3) and other laboratory parameters on day 0. The isolation of *S uberis* from a case of clinical mastitis was positively associated with severity ($p<0.05$). The odds of *S uberis* mastitis being classed as moderate in severity was approximately three times (OR 3.09 95% CI 1.17-7.40) that of mastitis being classed as mild. On univariable analysis, the isolation of *E coli* from a case of clinical mastitis was positively associated with rectal temperature and heart rate. The association with rectal temperature was, however, not significant ($p>0.05$) at the multivariable level when included with heart rate. The odds of *Staph aureus* mastitis being classed as mild in severity was approximately four times (OR 4.20 95% CI 1.09-16.3) that of mastitis being classed as moderate. The culture result of no growth was negatively associated with IQSCC ($p<0.001$).

4.3.3. Somatic cell counting

The IQSCC of normal cows ranged from 6 – 625 $\times 10^3$ cells/ml with a median of 12 $\times 10^3$ cells/ml and mean of 53.3 $\times 10^3$ cells/ml on the day of recruitment to the study (day 0) (Table 4.2). The milk samples from quarters with mild mastitis had a median IQSCC of 5,966 $\times 10^3$ cells/ml and a range of 23 – 23,478 $\times 10^3$ cells/ml (Table 4.2). Nine of the 41 samples were not suitable for counting due to the presence of too many clots in the milk sample. Milk samples from quarters diagonally opposite to the quarters affected by mild mastitis had a median IQSCC of 170 $\times 10^3$ cells/ml with a range of 16 – 10,048 $\times 10^3$ cells/ml; three out of the 41 samples were unsuitable for analysis. The IQSCC of milk samples from quarters with moderate mastitis ranged from 143 – 22,756 $\times 10^3$ cells/ml with a median of 10,707 $\times 10^3$ cells/ml (Table 4.2). Ten of the 28 milk samples were

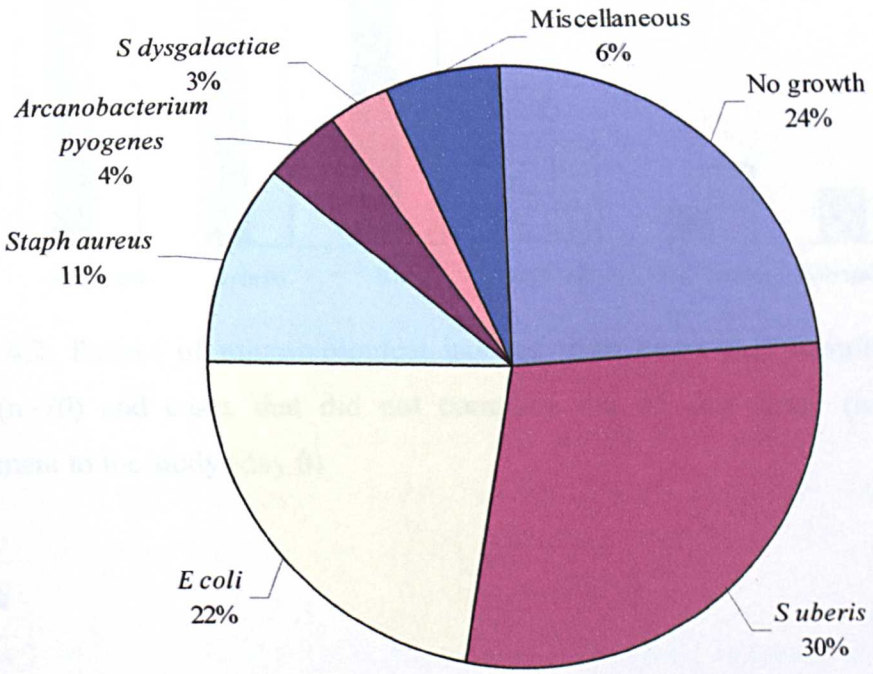


Figure 4.1: Microbiological isolates from 117 cases of clinical mastitis on day of recruitment to the study (day 0)

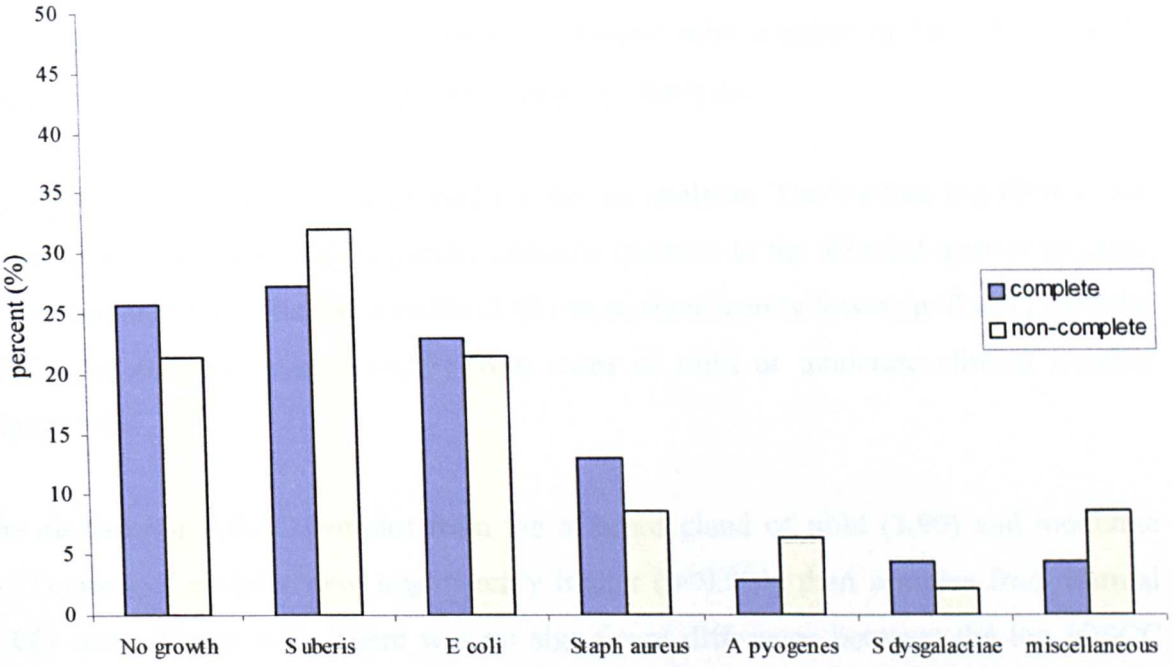
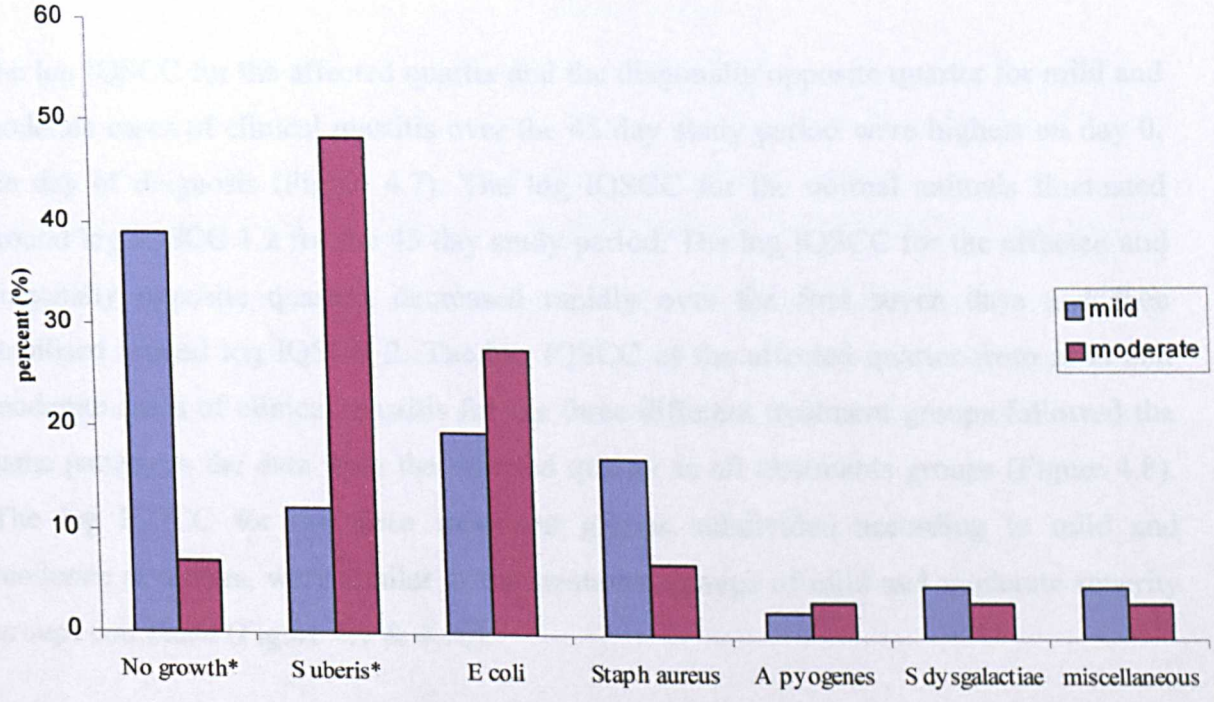


Figure 4.2: Pattern of microbiological isolates from cases that completed the 45 day study (n=70) and cases that did not complete the 45 day study (n=47) on day of recruitment to the study (day 0)



* significant difference between mild and moderate ($p<0.01$)

Figure 4.3: Pattern of microbiological isolates from mild (n=41) and moderate (n=29) cases of clinical mastitis on day of recruitment to the study (day 0)

unsuitable for analysis. Milk samples from quarters diagonally opposite to the moderate mastitis had a median IQSCC of 389 $\times 10^3$ cells/ml with a range of 16 – 4,615 $\times 10^3$ cells/ml; two of the 28 samples were unsuitable for analysis.

The IQSCC data were log transformed for further analysis. The median log IQSCC for normal cows (1.08) and the diagonally opposite quarters to the affected quarter in cases of mild and moderate clinical mastitis (2.42) were significantly lower ($p<0.001$) than the IQSCC for affected quarters (4.03) from cases of mild or moderate clinical mastitis (Figure 4.4).

The median log IQSCC samples from the affected gland of mild (3.90) and moderate (4.21) cases of mastitis were significantly higher ($p<0.001$) than samples from normal (1.08) cows (Figure 4.5). There was no significant difference between the log IQSCC from the affected gland of mild and moderate cases ($p>0.05$), however, 36% (10/28) of samples from moderate cases were not counted, whereas only 22% (9/41) of mild cases were not counted ($p=0.21$) due to the presence of too many clots in the samples. There was no significant difference between the log IQSCC from the diagonally opposite quarter of mild and moderate cases ($p>0.05$) (Figure 4.6).

The log IQSCC for the affected quarter and the diagonally opposite quarter for mild and moderate cases of clinical mastitis over the 45 day study period were highest on day 0, the day of diagnosis (Figure 4.7). The log IQSCC for the normal animals fluctuated around log IQSCC 1.2 for the 45 day study period. The log IQSCC for the affected and diagonally opposite quarters decreased rapidly over the first seven days and then stabilised around log IQSCC 2. The log IQSCC of the affected quarter from mild and moderate cases of clinical mastitis for the three different treatment groups followed the same pattern as the data from the affected quarter in all treatments groups (Figure 4.8). The log IQSCC for the three treatment groups subdivided according to mild and moderate severities, were similar to the treatment groups of mild and moderate severity groups combined (Figure 4.9 & 4.10).

There was no significant difference in the IQSCC of the affected quarter compared to the diagonally opposite quarter for mild and moderate cases at day 45 after recruitment to the study ($p>0.05$). The IQSCC for the affected quarter of mild and moderate cases were

Outcome variable	Explanatory variable	Coefficient (SE)	95% CI	P value	OR (95% CI)
S uberis	Severity			<0.05	
	- mild	Reference category			
	- moderate	1.08 (0.47)	0.25-2.01		3.09 (1.17-7.40)
E coli	Heart rate	0.069 (0.033)	0.00-0.13	<0.05	
	Rectal temperature	0.44 (0.31)	-0.16-1.04	0.15	
Staph aureus	Severity	1.44 (0.69)		<0.05	
	- moderate	Reference category			
	- mild	1.44 (0.69)	0.08-2.79		4.20 (1.09-16.3)
No growth	LogIQSCC	-1.03 (0.36)	0.32-1.73	<0.005	

IQSCC Individual quarter somatic cell count OR Odds ratio

Table 4.1: Final multivariable binary logistic regression model of explanatory variables that were associated with microbiological culture results of *S uberis*, *E coli*, *Staph aureus* or no growth from samples taken on day of recruitment (day 0) of mild and moderate clinical mastitis

Group	Median	Mean (SE)	Range	Samples not tested*
Normal (n=19)	12	53.3 (30.6)	6 – 625	0/19
Mild mastitis (n=41)	5,966	7,050 (1,059)	23 – 23,478	9/41
Diagonally opposite quarter in cows with mild mastitis (n=41)	170	779 (295)	16 – 10,048	3/41
Moderate mastitis (n=28)	10,707	10,707 (1,595)	143 – 22,756	10/28
Diagonally opposite quarter in cows with moderate mastitis (n=41)	389	826 (233)	16 – 4,615	2/28

*Number of samples that were not suitable for counting due to the presence of too many clots in the milk sample /total number of samples

Table 4.2: Median, mean and ranges of individual quarter somatic cell counts (IQSCC) $\times 10^3$ cells/ml of milk in normal animals and cases of mild or moderate mastitis on day of recruitment to the study (day 0)

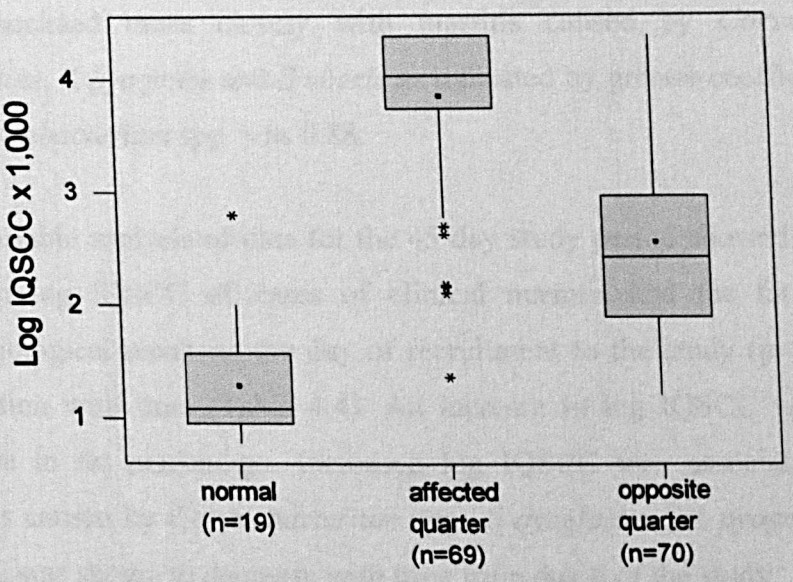


Figure 4.4: Log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml for affected (n=69) and diagonally opposite quarters (n=70) from cases of mild and moderate clinical mastitis and from the selected quarters of normal animals (n=19) on the day of recruitment to the study (day 0).

For each group, the median value is represented by the horizontal line within the box and the mean value denoted by the dot. The bottom and top of the box represent the first and third quartiles respectively. The vertical lines extending from the box represent the lowest and highest observations within 1.5x the inter-quartile range below and above the first and third quartiles respectively. Outliers are denoted by *.

significantly higher than the IQSCC of normal animals at day 45 after recruitment ($p<0.01$ for mild and $p<0.05$ for moderate cases). The IQSCC for diagonally opposite quarters in mild and moderate cases was significantly higher than the IQSCC of normal cows 45 days after recruitment to the study ($p<0.05$).

Multivariable analysis of data from the first seven days after recruitment to the study showed a positive association with log IQSCC of cases of clinical mastitis and the concentration of M-SAA and Hp in milk and the microbiological result on the day of recruitment to the study ($p<0.001$) (Table 4.3). An increase in log IQSCC was associated equally with an increase in the concentration of M-SAA and Hp. Increased log IQSCC was associated more closely with mastitis caused by *Corynebacterium* spp., *S dysglactiae*, *A pyogenes* and *S uberis* as indicated by greater coefficients, e.g. coefficient for *Corynebacterium* spp. was 0.88.

Multivariable analysis of data for the 45 day study period showed a positive association between log IQSCC of cases of clinical mastitis and the fat percentage, and the microbiological result on the day of recruitment to the study ($p<0.001$) and a negative correlation with time (Table 4.4). An increase in log IQSCC was associated with an increase in fat percentage. Increased log IQSCC was associated more closely with mastitis caused by *Corynebacterium* spp., *S dysglactiae*, *A pyogenes* and *S uberis*. Log IQSCC was shown to decrease with time from day 6 of the study.

4.3.3. Milk quality

The mean fat percentage for milk samples from normal cows and cows with mild or moderate mastitis were 2.3, 3.0 and 2.7, respectively (Table 4.5). The fat percentage was not significantly different between normal cows and cases of mild or moderate mastitis ($p>0.05$) (Figure 4.11), and there was also no significant difference between affected quarters and diagonally opposite quarters ($p>0.05$). The fat percentage of milk samples from normal cows and from milk samples from affected quarters of mild and moderate cases of clinical mastitis fluctuated showing no real trend for the duration of the 45 day study period (Figure 4.12), but for all groups, reached maximal levels at day 45 after recruitment to study. There were no significant differences in the fat percentage

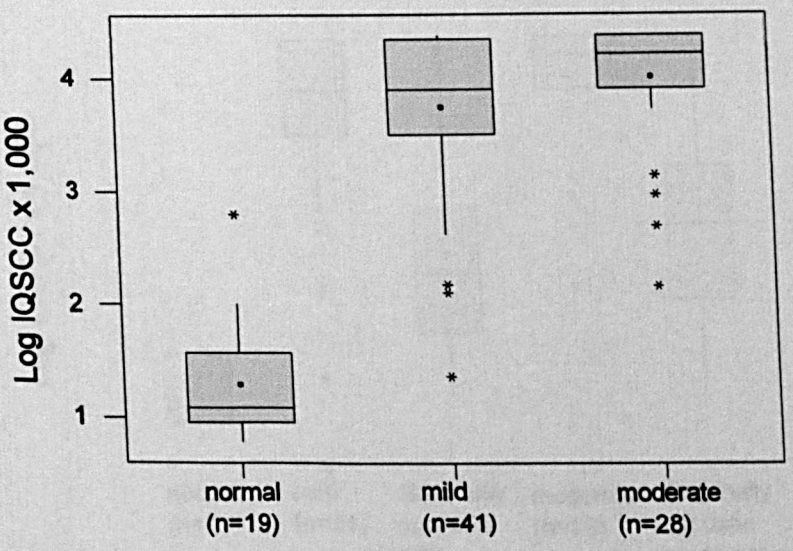


Figure 4.5: Log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml for mild (n=41) and moderate (n=28) cases of clinical mastitis and normal animals (n=19) on the day of recruitment to the study (day 0)

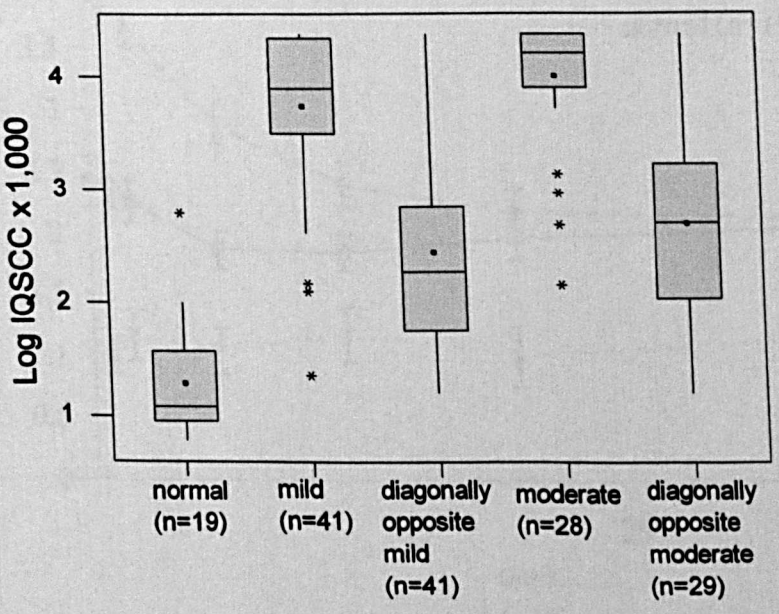


Figure 4.6: Log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml for normal animals (n=19), affected quarters of cows with mild (n=41) or moderate (n=28) clinical mastitis and from quarters diagonally opposite (n=41 for mild and n=29 for moderate) those with clinical mastitis on the day of recruitment to the study (day 0)

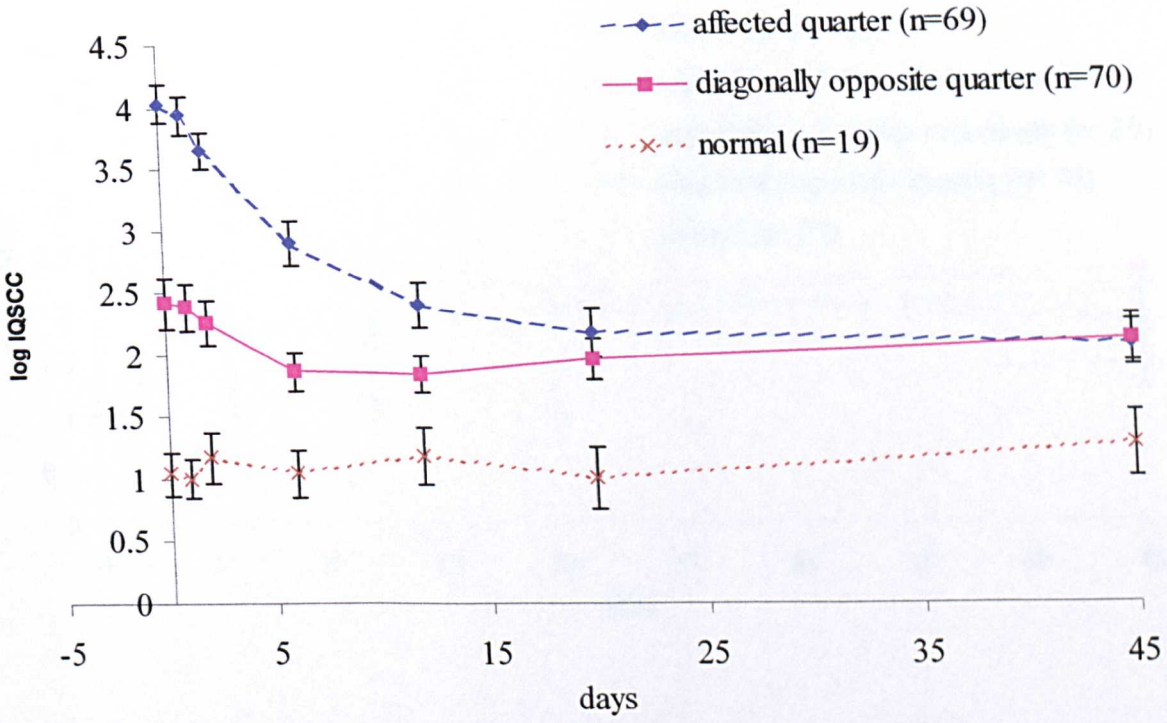


Figure 4.7: Median log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml ± CI for affected (n=69) and diagonally opposite (n=70) quarters from mild and moderate cases and from selected quarters from normal animals (n=19) over the 45 days after recruitment to the study

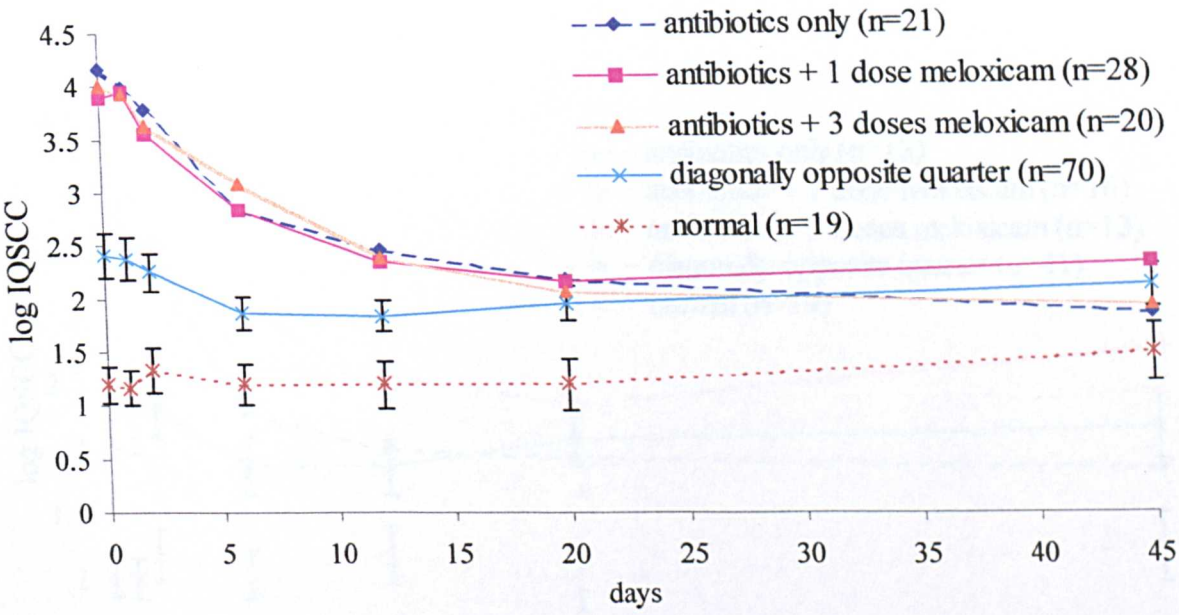


Figure 4.8: Median log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml ± CI for mild and moderate cases of mastitis in antibiotics only (n=21), antibiotics and one dose of meloxicam (n=28) and antibiotics and three doses meloxicam (n=20) treatment groups and diagonally opposite quarters (n=70) and from selected quarters from normal animals (n=19) over 45 days after recruitment to the study

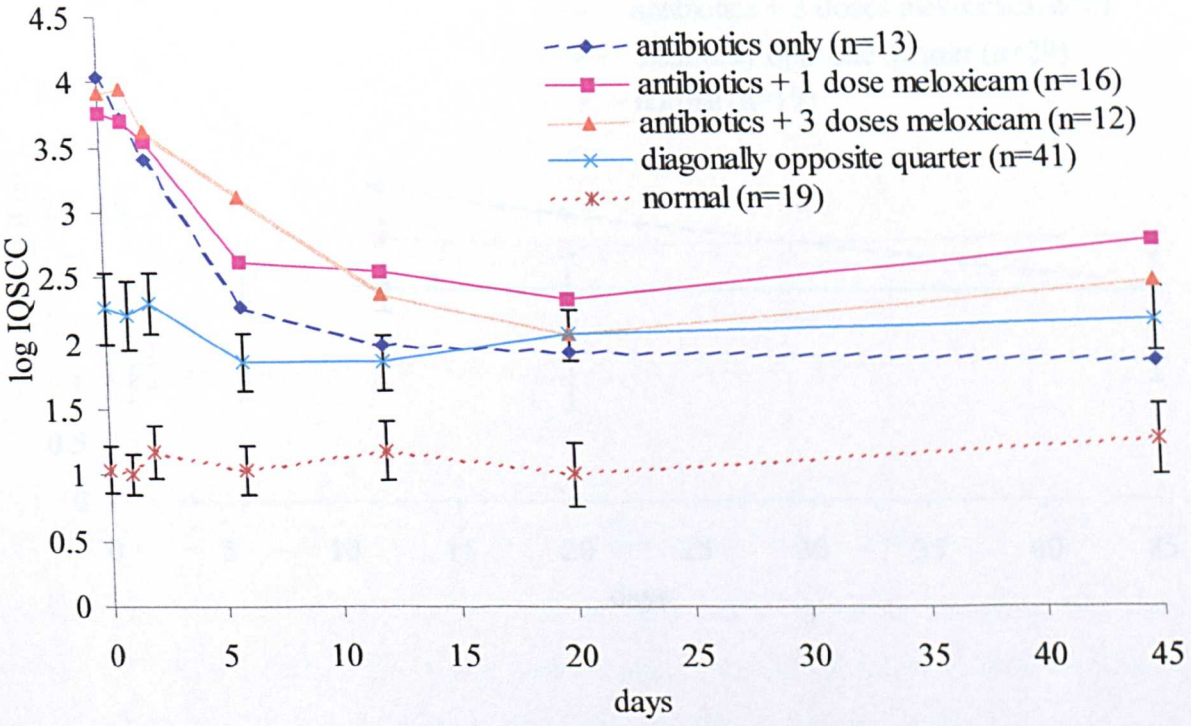


Figure 4.9: Median log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml ± CI for mild cases of mastitis in antibiotics only (n=13), antibiotics and one dose of meloxicam (n=16) and antibiotics and three doses meloxicam (n=12) treatment groups and diagonally opposite quarters (n=41) and from selected quarters from normal animals (n=19) over 45 days after recruitment to the study

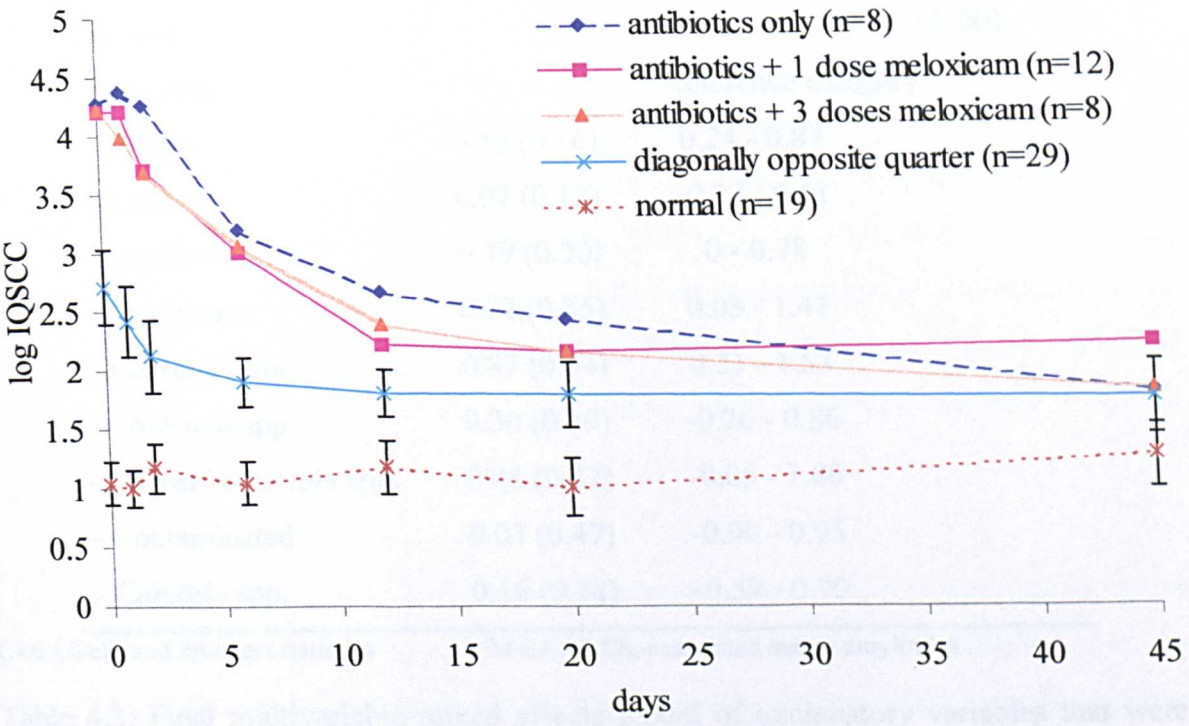


Figure 4.10: Median log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml ± CI for moderate cases of mastitis in antibiotics only (n=8), antibiotics and one dose of meloxicam (n=12) and antibiotics and three doses meloxicam (n=8) treatment groups and diagonally opposite quarters (n=29) and from selected quarters from normal animals (n=19) over 45 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
M-SAA	0.16 (0.04)	0.09 - 0.24	<0.001
Haptoglobin	0.16 (0.04)	0.08 - 0.24	<0.001
Bacteria			<0.001
- no growth		Reference category	
- *S uberis*	0.56 (0.16)	0.24 - 0.87	
- *E coli*	0.07 (0.17)	-0.27 - 0.41	
- *Staph aureus*	0.39 (0.20)	0 - 0.78	
- *A pyogenes*	0.72 (0.35)	0.03 - 1.41	
- *S dysgalactiae*	0.87 (0.34)	0.21 - 1.53	
- CN *Staph* spp.	0.30 (0.29)	-0.26 - 0.86	
- *Corynebacterium* spp.	0.88 (0.47)	-0.05 - 1.80	
- Contaminated	0.03 (0.47)	-0.90 - 0.95	
- *Candida* spp.	0.16 (0.38)	-0.59 - 0.90	

LRS Likelihood ratio test statistics M-SAA Milk-associated serum amyloid A

Table 4.3: Final multivariable mixed effects model of explanatory variables that were associated with log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml of mild and moderate cases for the first seven days after recruitment to the study

at day 45 between the affected quarter and diagonally opposite quarters of mild and moderate cases and normal cows ($p>0.05$).

The protein percentage of milk samples from normal cows ranged from 2.8 – 3.7% with a mean of 3.2% on the day of recruitment to the study (day 0) (Table 4.5). The milk samples from quarters with mild or moderate mastitis had mean protein percentages of 3.9% and 4.5%, respectively (Table 4.5). Milk samples from quarters diagonally opposite to mild or moderate mastitis had mean protein percentages of 3.4% and 3.5%, respectively. The protein percentage for normal cows was significantly lower ($p<0.001$) than the protein percentage for affected quarters from cases of mastitis (Figure 4.13). The protein percentages of the diagonally opposite quarters were also significantly lower than the affected quarters ($p<0.001$), while there was no significant difference between the protein percentage of normal cows and diagonally opposite quarters ($p>0.05$). The protein percentage for the affected quarter of mild and moderate cases of clinical mastitis over the 45 day study period was highest on the day of recruitment (Figure 4.14). The protein percentage for the milk samples from normal animals fluctuated around 3.1-3.2% for the 45 day study period.

Milk samples from the affected quarter of moderate cases showed a larger increase in the protein percentage then mild cases, although this was not significantly different ($p>0.05$). The protein percentage decreased to reach normal levels 20 days after recruitment to the study. There were no significant differences in the protein percentage at day 45 between the affected quarter and diagonally opposite quarters of mild and moderate cases and normal cows ($p>0.05$).

The lactose percentage of milk samples from the normal cows ranged from 3.9 – 5.1% with a mean of 4.7% on the day of recruitment to the study (day 0) (Table 4.5). Lactose percentages for milk samples from quarters with mild or moderate mastitis had means of 3.6 and 2.8%, respectively (Table 4.5). The lactose percentages of milk samples from quarters diagonally opposite to mild or moderate mastitis both had means of 4.4%. The lactose percentage of normal cows was significantly higher ($p<0.001$) than the lactose percentage of affected quarters from cases of mild or moderate mastitis (Figure 4.15). The lactose percentage of the diagonally opposite quarters was also significantly higher than the lactose percent of the affected quarters ($p<0.001$), while

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
Fat	0.07 (0.02)	0.04 - 0.11	<0.001
Timepoint			<0.001
- 0		Reference category	
- 1	0.00 (0.00)	0	
- 2	0.00 (0.00)	0	
- 6	-0.48 (0.13)	0.22 – 0.73	
- 12	-0.85 (0.12)	0.62 – 1.09	
- 20	-0.96 (0.12)	0.72 - 1.19	
- 45	-1.11 (0.13)	0.86 – 1.35	
Bacteria			<0.001
- no growth		Reference category	
- *S uberis*	0.54 (0.15)	0.25 - 0.84	
- *E coli*	0.29 (0.20)	-0.09 - 0.68	
- *Staph aureus*	0.36 (0.16)	0.05 - 0.67	
- *A pyogenes*	0.89 (0.32)	0.26 - 1.51	
- *S dysgalactiae*	0.57 (0.46)	-0.32 - 1.47	
- CN *Staph* spp.	-0.13 (0.20)	-0.27 - 0.53	
- *Corynebacterium* spp.	0.61 (0.38)	-0.13 - 1.34	
- Contaminated	-0.29 (0.64)	-0.96 - 2.53	
- *Candida* spp.	0.47 (0.46)	-0.44 - 1.37	

LRS Likelihood ratio test statistics

Table 4.4: Final multivariable mixed effects model of explanatory variables that were associated with log individual quarter somatic cell count (IQSCC) $\times 10^3$ cells/ml of mild and moderate cases for the 45 day study period

Group	Fat %		Protein %		Lactose %	
	Mean (SE)	Range	Mean (SE)	Range	Mean (SE)	Range
Normal cows (n=19)	2.3 (0.6)	0.5 – 11.4	3.2 (0.1)	2.8 – 3.7	4.7 (0.1)	3.9 – 5.1
Cows with mild mastitis (n=41)	3.0 (0.4)	0.5 – 14.2	3.9 (0.2)	2.6 – 10.4	3.6 (0.2)	1.6 – 4.9
Diagonally opposite quarter in cows with mild mastitis (n=41)	3.4 (0.6)	1.0 – 18.2	3.4 (0.1)	2.1 – 7.6	4.4 (0.1)	2.00 – 5.1
Cows with moderate mastitis (n=28)	2.7 (0.4)	0.4 – 7.4	4.5 (0.2)	3.3 – 7.2	2.8 (0.3)	-0.1 – 4.7
Diagonally opposite quarter in cows with moderate mastitis (n=29)	3.7 (0.5)	0.8 – 9.1	3.5 (0.1)	2.7 – 4.5	4.4 (0.1)	3.2 – 5.0

Table 4.5: Mean (SE) and ranges of percentages of fat, protein and lactose in the milk of normal animals and cases of mild or moderate mastitis on day of recruitment to the study (day 0)

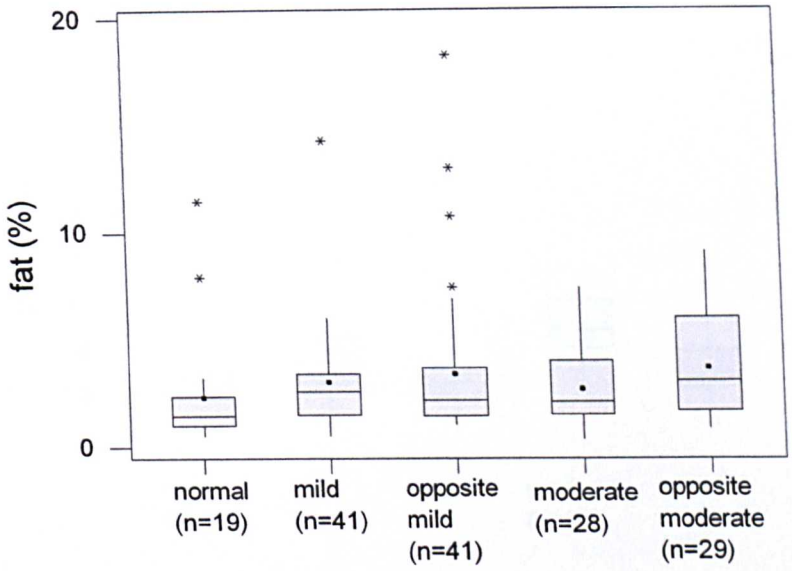


Figure 4.11: Fat percentages (%) in milk from affected quarters of cases with mild (n=41) or moderate (n=28) clinical mastitis, quarters diagonally opposite those with clinical mastitis and from normal animals on the day of recruitment to the study (day 0)

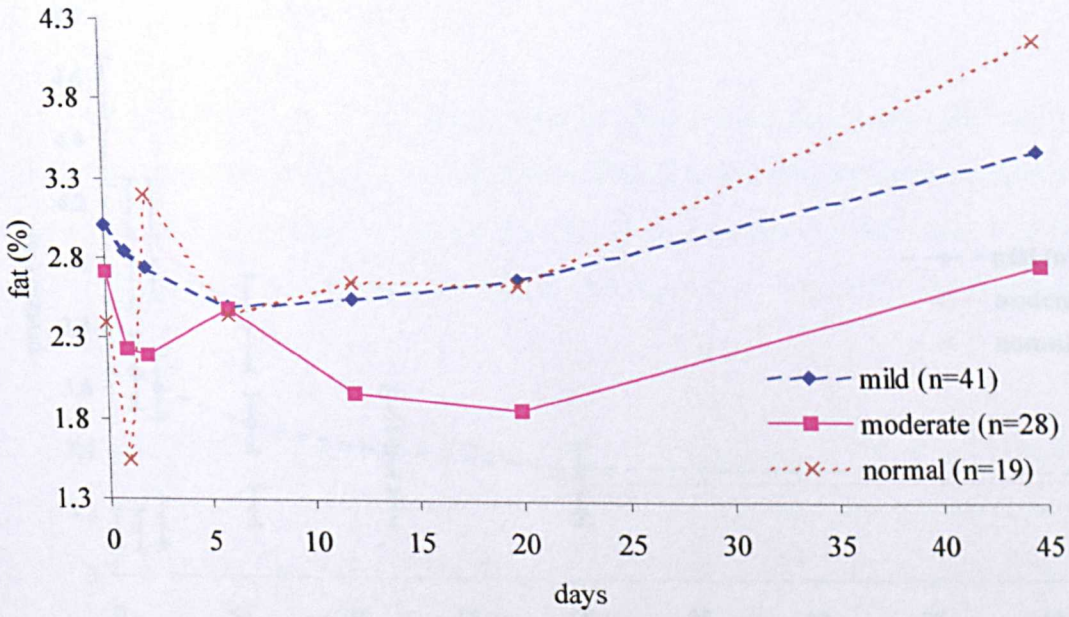


Figure 4.12: Mean fat percent for mild (n=41) and moderate (n=28) cases of clinical mastitis and normal animals (n=19) over the 45 day study period

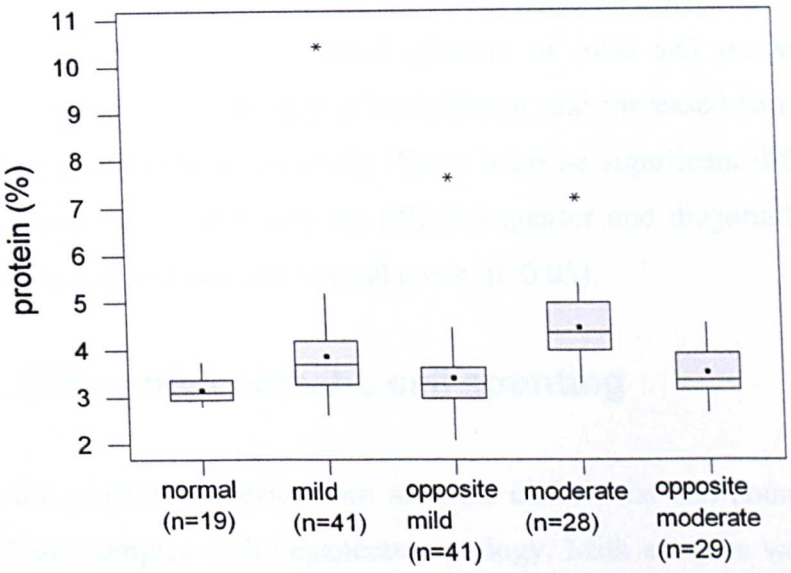


Figure 4.13: Protein percentages (%) in milk from affected quarters of cases with mild (n=41) or moderate (n=28) clinical mastitis, quarters diagonally opposite those with clinical mastitis and from normal cows on the day of recruitment to the study (day 0)

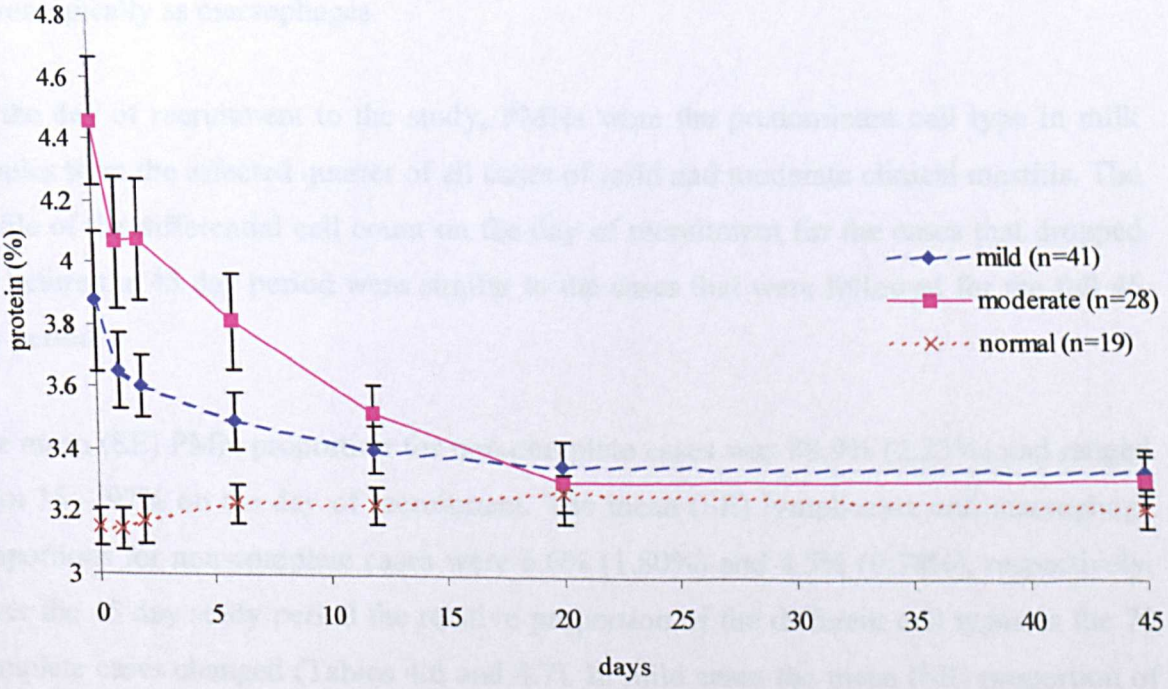


Figure 4.14: Mean protein percent ± SE for mild (n=41) and moderate (n=28) cases of clinical mastitis and normal (n=19) animals over the 45 day study period

there was no significant difference between normal cows and the lactose percentage of diagonally opposite quarters ($p>0.05$). The lactose percentage for the milk samples from normal cows fluctuated around 4.5-4.7% for the 45 day study period (Figure 4.16). The lactose percentage for the affected quarters of mild and moderate cases of clinical mastitis was lowest on the day of recruitment and increased to reach normal levels 12 days after recruitment to the study. There were no significant differences in the lactose percentage at day 45 between the affected quarter and diagonally opposite quarters of mild and moderate cases and normal cows ($p>0.05$).

4.3.3. Differential somatic cell counting

It was not possible to perform an accurate differential cell count on samples with low IQSCC and samples with degenerate cytology. Milk samples were described as having degenerate cytology when it was not possible to determine the cell type. This tended to occur in samples that contained many bacteria or were stored over the weekend prior to examination. All normal animals had low IQSCC at every sampling time, and many of the samples from cases of mild and moderate clinical mastitis on days 20 and 45 after recruitment to the study had low IQSCC. On examination of the milk samples from normal animals there were low numbers of large mononuclear cells, identified microscopically as macrophages.

On the day of recruitment to the study, PMNs were the predominant cell type in milk samples from the affected quarter of all cases of mild and moderate clinical mastitis. The profile of the differential cell count on the day of recruitment for the cases that dropped out before the 45 day period were similar to the cases that were followed for the full 45 day period.

The mean (SE) PMN proportion for non-complete cases was 88.9% (2.23%) and ranged from 15 – 97% on the day of recruitment. The mean (SE) lymphocyte and macrophage proportions for non-complete cases were 6.6% (1.80%) and 4.5% (0.78%), respectively. Over the 45 day study period the relative proportion of the different cell types in the 70 complete cases changed (Tables 4.6 and 4.7). In mild cases the mean (SE) proportion of PMNs decreased from 87.9% (1.7%) to 70.1% (5.5%) and there was an increase in the

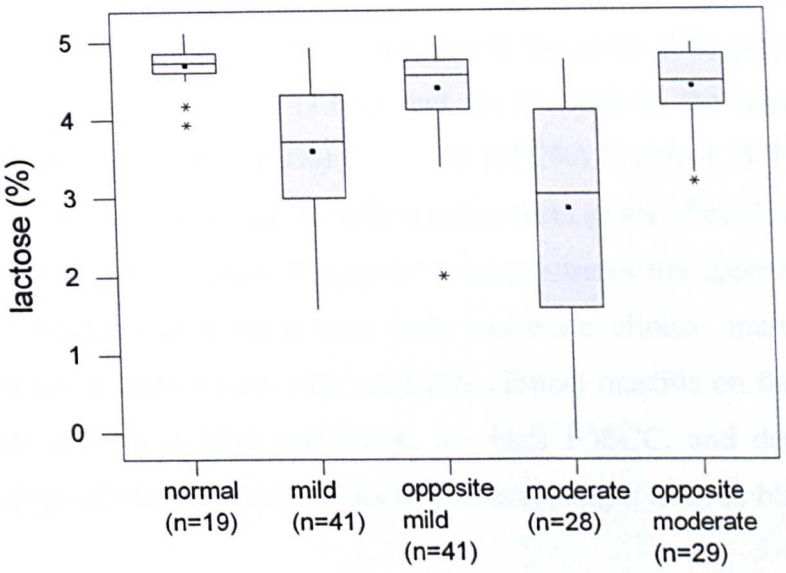


Figure 4.15: Lactose percentages (%) in milk from affected quarters of cases with mild (n=41) or moderate (n=28) clinical mastitis, quarters diagonally opposite those with clinical mastitis and from normal cows on the day of recruitment to the study (day 0)

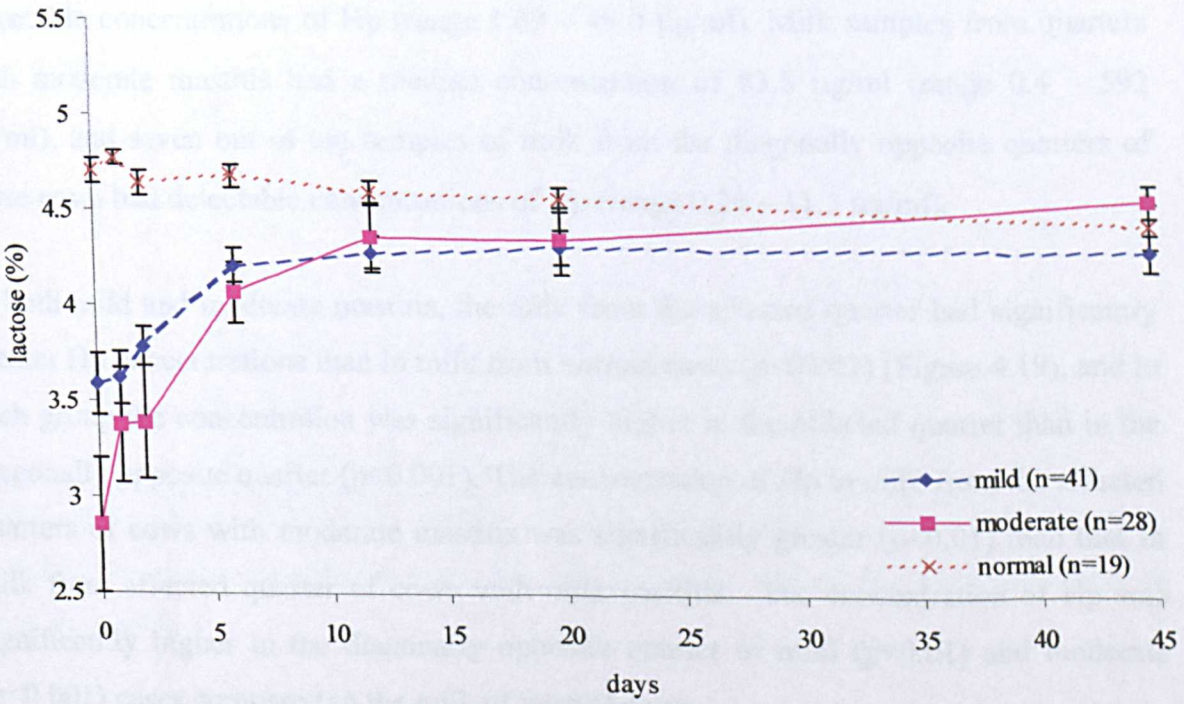


Figure 4.16: Mean lactose percent ± SE for mild (n=41) and moderate (n=28) cases of clinical mastitis and normal (n=19) animals over the 45 day study period

mean (SE) proportion of lymphocytes from 3.4% (0.4%) to 9.6% (1.8%) and an increase in the mean (SE) proportion of macrophages from 8.6% (1.6%) to 19.4% (4.5%) (Table 4.6). In moderate cases the mean (SE) proportion of PMNs decreased from 93.1% (1.5%) to 72.6% (11.2%) and there was an increase in the mean (SE) proportion of lymphocytes from 3.2% (0.4%) to 6.0% (1.6%) and an increase in the mean (SE) proportion of macrophages from 3.9% (1.4%) to 21.3% (11.2%) (Table 4.7) There was considerable variation in cell morphology in milk samples from cases of moderate mastitis on the day of recruitment to the study. Figure 4.17 demonstrates the appearance of neutrophils in cytospin preparation from a case with moderate clinical mastitis. Figure 4.18 is a cytospin smear from a cow with moderate clinical mastitis on the day of recruitment to the study and has a high cellularity, i.e. high IQSCC, and demonstrates the cellular morphology of neutrophils, lymphocytes, macrophages and red blood cells.

4.3.4. Acute phase proteins

Milk samples from normal cows had undetectable concentrations of Hp in milk, i.e. <0.26 μg/ml, the limit of detection (LOD). Milk samples from quarters with mild mastitis had a median concentration of 27.3 μg/ml (range 0.49 – 274 μg/ml) (Table 4.8), and only four of the ten samples of milk from the diagonally opposite quarter of these cows had detectable concentrations of Hp (range 1.69 – 49.0 μg/ml). Milk samples from quarters with moderate mastitis had a median concentration of 83.8 μg/ml (range 0.4 – 592 μg/ml), and seven out of ten samples of milk from the diagonally opposite quarters of these cows had detectable concentrations of Hp (range 0.26 – 11.3 μg/ml).

In both mild and moderate mastitis, the milk from the affected quarter had significantly greater Hp concentrations than in milk from normal cows ($p<0.001$) (Figure 4.19), and in each group the concentration was significantly higher in the affected quarter than in the diagonally opposite quarter ($p<0.001$). The concentration of Hp in milk from the affected quarters of cows with moderate mastitis was significantly greater ($p<0.01$) than that in milk from affected quarter of cows with mild mastitis. The concentration of Hp was significantly higher in the diagonally opposite quarter of mild ($p<0.01$) and moderate ($p<0.001$) cases compared to the milk of normal cows.

Days	PMNs mean (SE)	range	Lymphocytes Mean (SE)	range	Macrophages mean (SE)	range
0	87.9 (1.7)	51 – 98	3.4 (0.4)	0 – 8	8.6 (1.6)	0 – 45
1	88.9 (2.4)	15 – 99	5.9 (1.7)	0 – 60	8.2 (1.2)	0 – 35
2	79.8 (2.8)	14 – 96	6.8 (1.1)	0 – 27	13.4 (2.1)	2 – 62
6	67.6 (4.5)	27 – 96	9.7 (1.7)	0 – 33	22.5 (3.5)	4 – 61
12	65.5 (5.6)	7 – 98	11.5 (2.0)	1 – 36	22.9 (4.5)	1 – 77
20	75.1 (3.9)	37 – 93	5.5 (1.0)	2 – 15	19.3 (3.8)	4 – 61
45	70.1 (5.5)	20 – 98	9.6 (1.8)	0 – 25	19.4 (4.5)	2 – 67

Table 4.6: Mean (SE) and ranges of polymorphonuclear neutrophils (PMNs), lymphocytes and macrophages over the 45 day study period for mild cases of clinical mastitis

Days	PMNs mean (SE)	range	Lymphocytes Mean (SE)	range	Macrophages mean (SE)	range
0	93.1 (1.5)	61 – 99	3.2 (0.4)	0 – 7	3.9 (1.4)	0 – 34
1	89.6 (2.3)	46 – 99	6.3 (2.2)	0 – 54	4.0 (0.6)	0 – 11
2	88.4 (2.2)	14 – 97	4.8 (1.0)	0 – 24	6.6 (1.9)	0 – 54
6	77.4 (3.7)	40 – 93	6.5 (0.9)	0 – 14	15.7 (3.2)	3 – 50
12	69.6 (5.4)	31 – 98	10.4 (2.6)	1 – 33	20.0 (4.5)	1 – 56
20	68.2 (6.4)	22 – 96	14.2 (3.6)	2 – 43	17.4 (4.2)	2 – 54
45	72.6 (11.2)	0 – 94	6.0 (1.6)	3 - 16	21.3 (11.2)	2 – 97

Table 4.7: Mean (SE) and ranges of polymorphonuclear neutrophils (PMNs), lymphocytes and macrophages over the 45 day study period for moderate cases of clinical mastitis

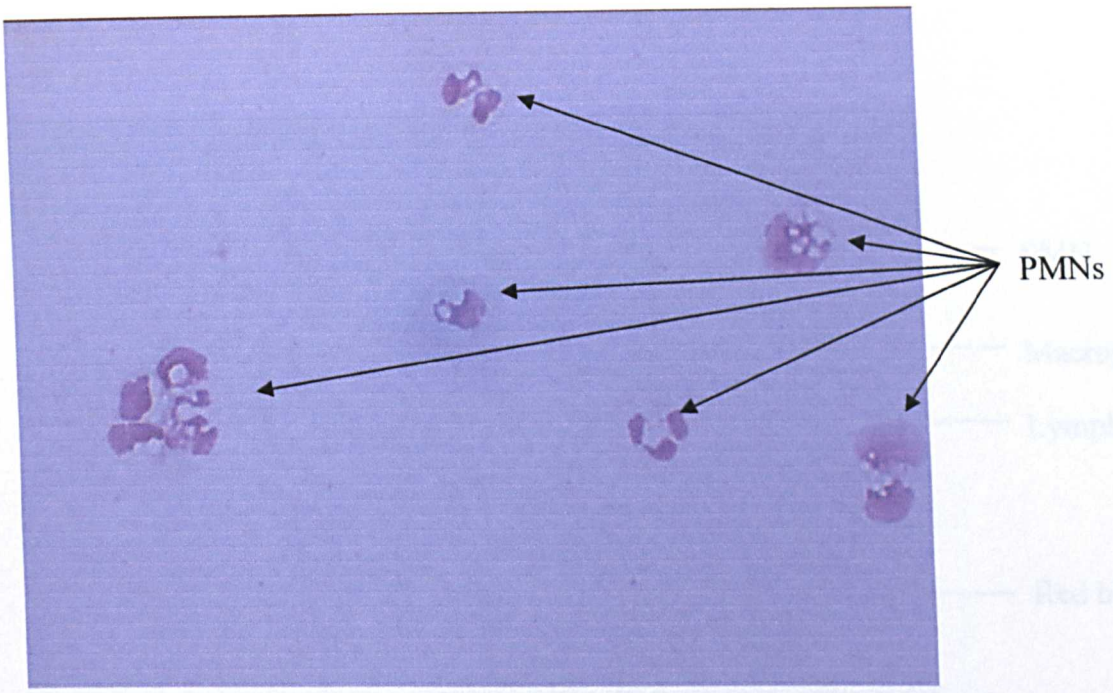


Figure 4.17: Polymorphonuclear neutrophils (PMNs) in milk sample from cow 189 with moderate clinical mastitis on the day of recruitment to study (day 0) at magnification ×400 and stained with May Grünwald and Giemsa

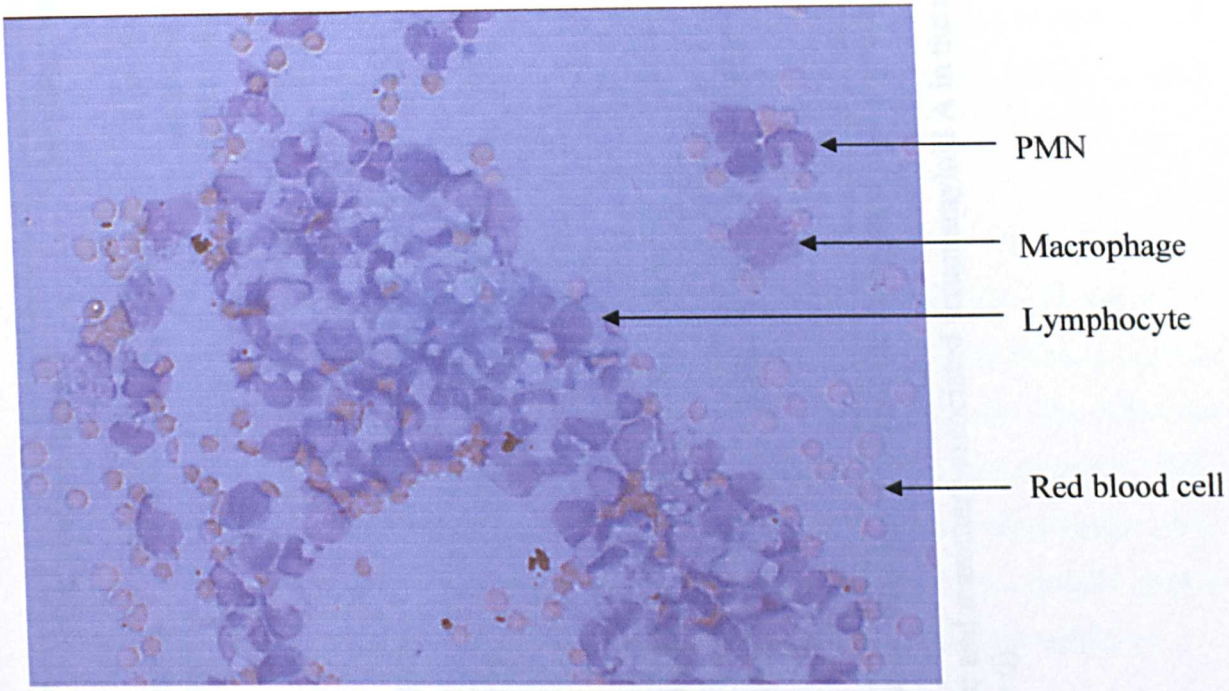


Figure 4.18: Milk sample from cow 171 with moderate clinical mastitis on day of recruitment to study (day 0) showing high cellularity with mainly polymorphonuclear neutrophils (PMNs) but also lymphocytes and macrophages with a background of red blood cells at magnification ×400 and stained with May Grünwald and Giemsa

Group	Haptoglobin (μg/ml)				Mammary-associated serum amyloid A (μg/ml)			
	Samples detectable* (LOD = 0.26)	Median	Mean	Range	Samples detectable* (LOD = 0.05)	Median	Mean	Range
Normal (n=15)	0/15	0	0	all<0.26	4/15	0.09	0.42	0.05-1.11
Mild mastitis (n=41)	40/41	27.3	48.5	0.49-274	41/41	20.0	57.1	1-362
Diagonally opposite quarter in cows with mild mastitis + (n=10)	4/10	5.99	15.6	1.69-49.0	9/10	0.5	16.1	0.06-87.2
Moderate mastitis (n=28)	28/28	83.8	118.1	0.4-592	28/28	8.5	79.9	0.2-1085
Diagonally opposite quarter in cows with moderate mastitis (n=10)	7/11	2.72	3.85	0.26-11.3	8/11	1.17	1.77	0.11-4.29

*Number of samples with a concentration greater than the lower limit of detection (LOD) of the assay used/total number of samples

Table 4.8: Median, mean and ranges of concentrations of haptoglobin and mammary-associated serum amyloid A in the milk of normal animals and cases of mild or moderate mastitis on the day of recruitment to the study (day 0)

Mammary-associated serum amyloid A (M-SAA) was present in detectable concentrations (>0.05 μg/ml) in four of the 15 samples of milk from normal cows and the median concentration for these four normal cows was 0.09 μg/ml (range of 0.05 – 1.11 μg/ml). Milk samples from quarters with mild mastitis had a median M-SAA concentration of 20.0 μg/ml (range 1 – 362 μg/ml) (Table 4.8), and nine out of the ten samples from the diagonally opposite quarter of these cows had detectable concentrations (range 0.06 – 87.2 μg/ml). Milk samples from quarters with moderate mastitis had a median M-SAA concentration of 79.9 μg/ml (range of 0.2 – 1,085 μg/ml) and eight out of ten samples of milk from diagonally opposite quarters in these cows had detectable concentrations (range 0.11 – 4.29 μg/ml).

In both mild and moderate mastitis, the milk from affected quarters had significantly higher concentrations of M-SAA than milk from normal cows ($p<0.001$) (Figure 4.20), and within each group the concentration was significantly greater in the affected quarter than the diagonally opposite quarter ($p<0.05$ for mild and $p<0.01$ for the moderate mastitis group). There was no significant difference between the concentrations of M-SAA in the milk from affected quarters of cows with mild or moderate mastitis ($p>0.05$). The concentration of M-SAA was significantly higher in the diagonally opposite quarter of mild ($p<0.01$) and moderate ($p<0.001$) cases compared to the milk of normal cows.

There was a significant positive correlation ($r=0.66$, $p<0.001$) between the concentration of Hp and M-SAA in milk.

The Hp concentrations in the affected quarter and the diagonally opposite quarter for mild and moderate cases of clinical mastitis during the 12 day period, were greatest on the day of recruitment (Figure 4.21), with milk from the affected quarter of moderate cases having significantly greater Hp concentrations than mild cases. Haptoglobin concentrations in normal animals were below the limit of detection.

In mastitic cases, the Hp concentrations decreased slightly over the 2 days after recruitment and then decreased more markedly by day 6 and again by day 12. The Hp concentrations in the diagonally opposite quarters decreased to reach normal

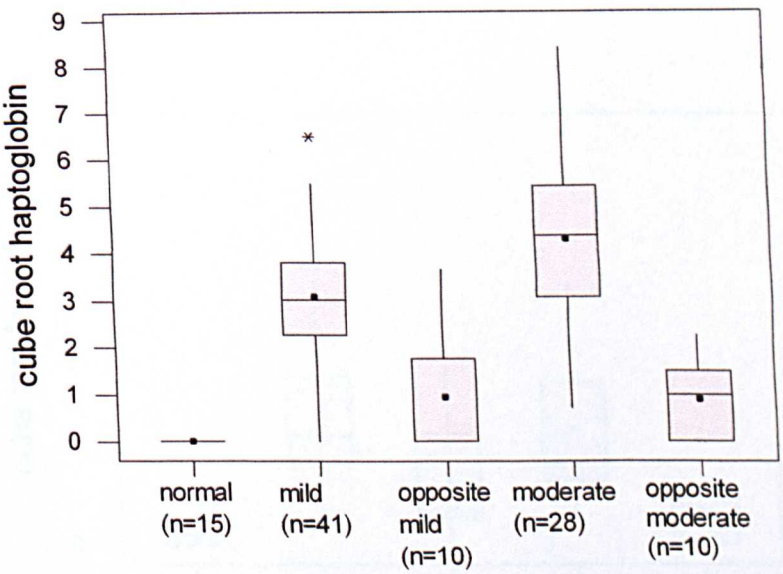


Figure 4.19: Haptoglobin concentrations (Cube root) in milk from normal animals (n=15), affected quarters of cows with mild (n=41) or moderate (n=28) clinical mastitis and from quarters diagonally opposite those with clinical mastitis (n=10 for mild and n=10 for moderate) on day of recruitment to the study (day 0)

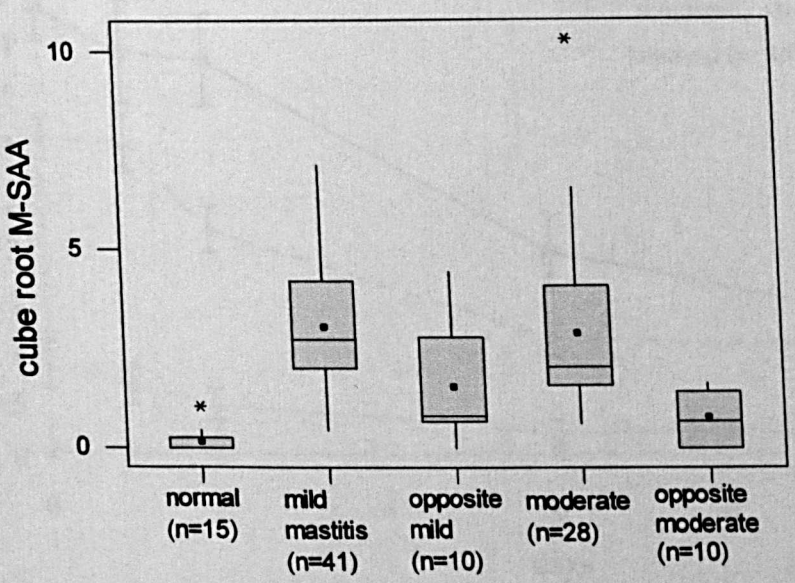


Figure 4.20: Mammary-associated Serum Amyloid A (M-SAA) concentrations (Cube root) in milk from normal animals (n=15), affected quarters of cows with mild (n=41) or moderate (n=28) clinical mastitis and from quarters diagonally opposite those with clinical mastitis (n=10 for mild and n=10 for moderate) on day of recruitment to the study (day 0)

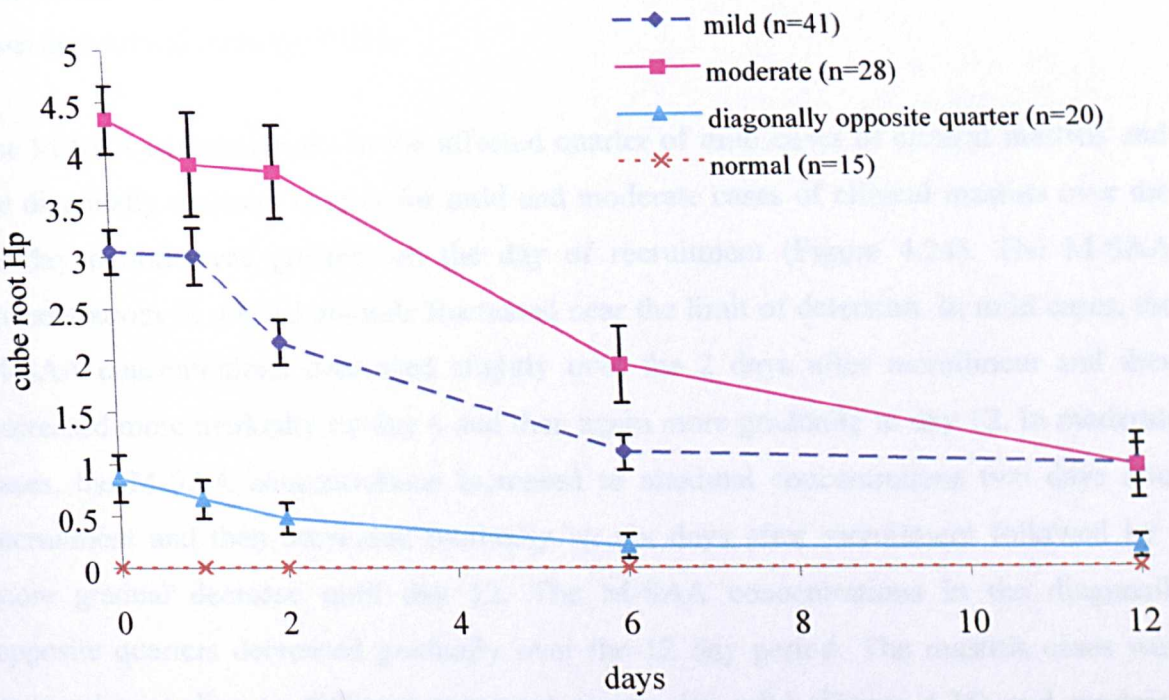


Figure 4.21: Mean haptoglobin (Hp) concentrations (Cube root) ± SE for mild (n=41) and moderate (n=28) cases of clinical mastitis and the diagonally opposite quarters (n=20) and normal animals (n=15)

concentrations 6 days after recruitment. The mastitis cases were assessed according to different treatment groups for mild (Figure 4.22) and moderate (Figure 4.23) cases. There was no obvious effect of meloxicam treatment, as in general the Hp concentrations for the affected quarters of all three different treatment groups followed the same pattern as the data from the affected quarter in all treatments groups (Figure 4.21). There was a significant difference in the Hp concentration at day 12 between the affected quarter of mild and moderate cases and normal cows ($p<0.01$). The Hp concentration in affected quarters was significantly higher than the concentration in the diagonally opposite quarters at day 12 ($p<0.05$). There was, however, no significant difference in the Hp concentration at day 12 between the diagonally opposite quarters of mild and moderate cases and normal cows ($p>0.05$).

The M-SAA concentrations in the affected quarter of mild cases of clinical mastitis and the diagonally opposite quarter for mild and moderate cases of clinical mastitis over the 12 day period were greatest on the day of recruitment (Figure 4.24). The M-SAA concentrations in normal animals fluctuated near the limit of detection. In mild cases, the M-SAA concentrations decreased slightly over the 2 days after recruitment and then decreased more markedly by day 6 and then again more gradually to day 12. In moderate cases, the M-SAA concentrations increased to maximal concentrations two days after recruitment and then decreased markedly by six days after recruitment followed by a more gradual decrease until day 12. The M-SAA concentrations in the diagonally opposite quarters decreased gradually over the 12 day period. The mastitis cases were assessed according to different treatment groups for mild (Figure 4.25) and moderate (Figure 4.26) cases. There was no obvious treatment effect; in general the M-SAA concentrations for the affected quarters of all three different treatment groups followed the same pattern as the data from the affected quarter in all treatments groups (Figure 4.24). The exception was mild cases given antibiotics and three doses of meloxicam; that treatment group showed an increase in M-SAA from day 0 to day 1, after which they followed the same pattern as the other two treatment groups. There was a significant difference in the M-SAA concentration between the affected quarter of mild and moderate cases and normal cows at day 12 ($p<0.001$). The M-SAA concentrations of the affected quarters were significantly higher than the concentration in the diagonally opposite quarters at day 12 ($p<0.01$). There was, however, no significant difference in the M-SAA

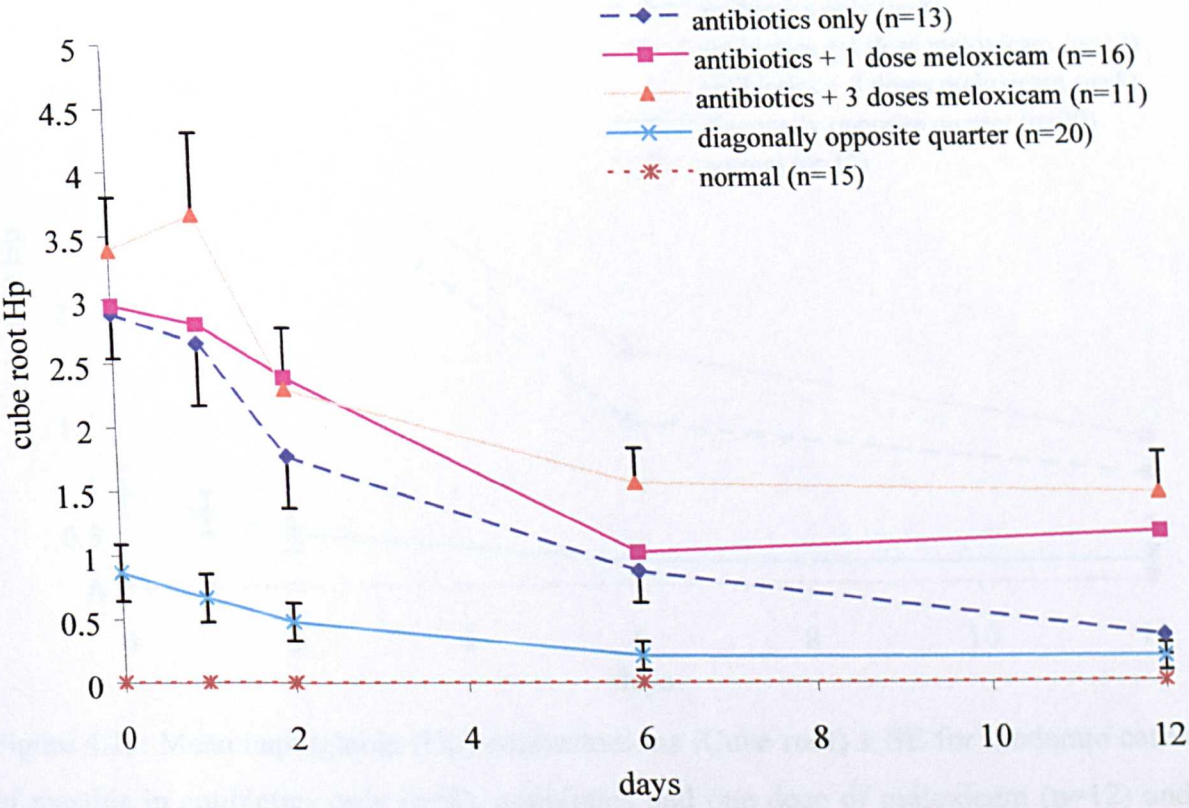


Figure 4.22: Mean haptoglobin (Hp) concentrations (Cube root) ± SE for mild cases of mastitis in antibiotics only (n=13), antibiotics and one dose of meloxicam (n=16) and antibiotics and three doses meloxicam (n=11) treatment groups and the diagonally opposite quarters (n=20) and normal animals (n=15)

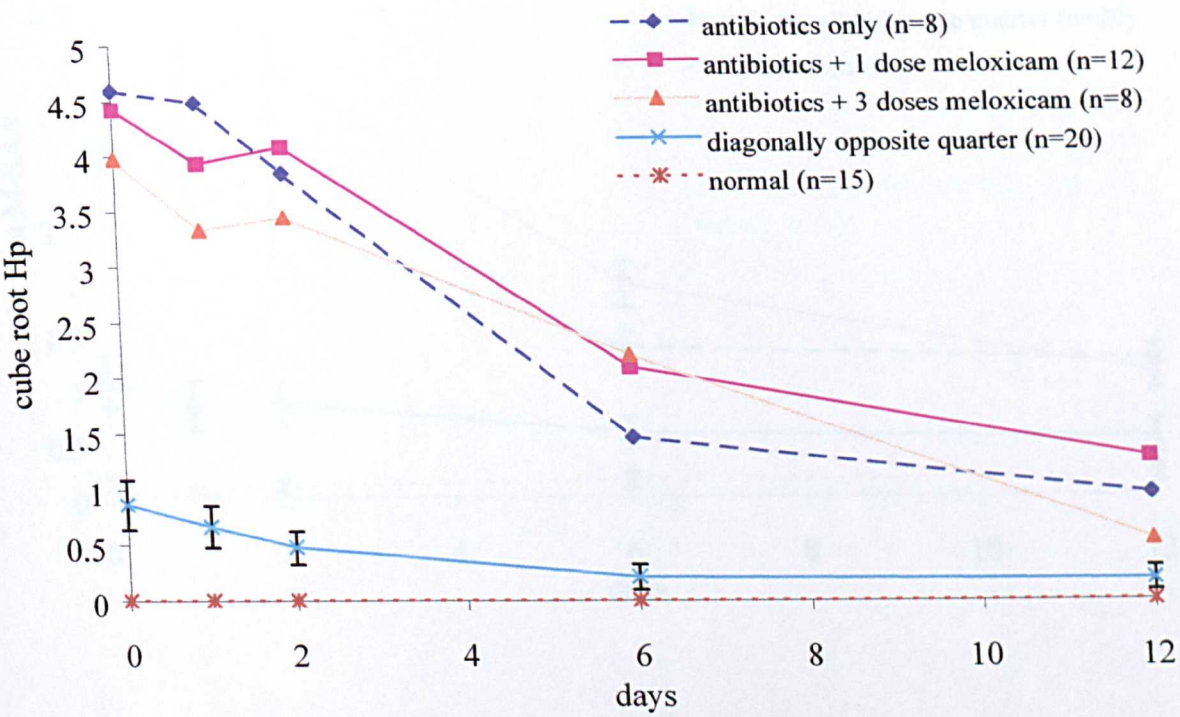


Figure 4.23: Mean haptoglobin (Hp) concentrations (Cube root) ± SE for moderate cases of mastitis in antibiotics only (n=8), antibiotics and one dose of meloxicam (n=12) and antibiotics and three doses meloxicam (n=8) treatment groups and the diagonally opposite quarters (n=20) and normal (n=15) cows

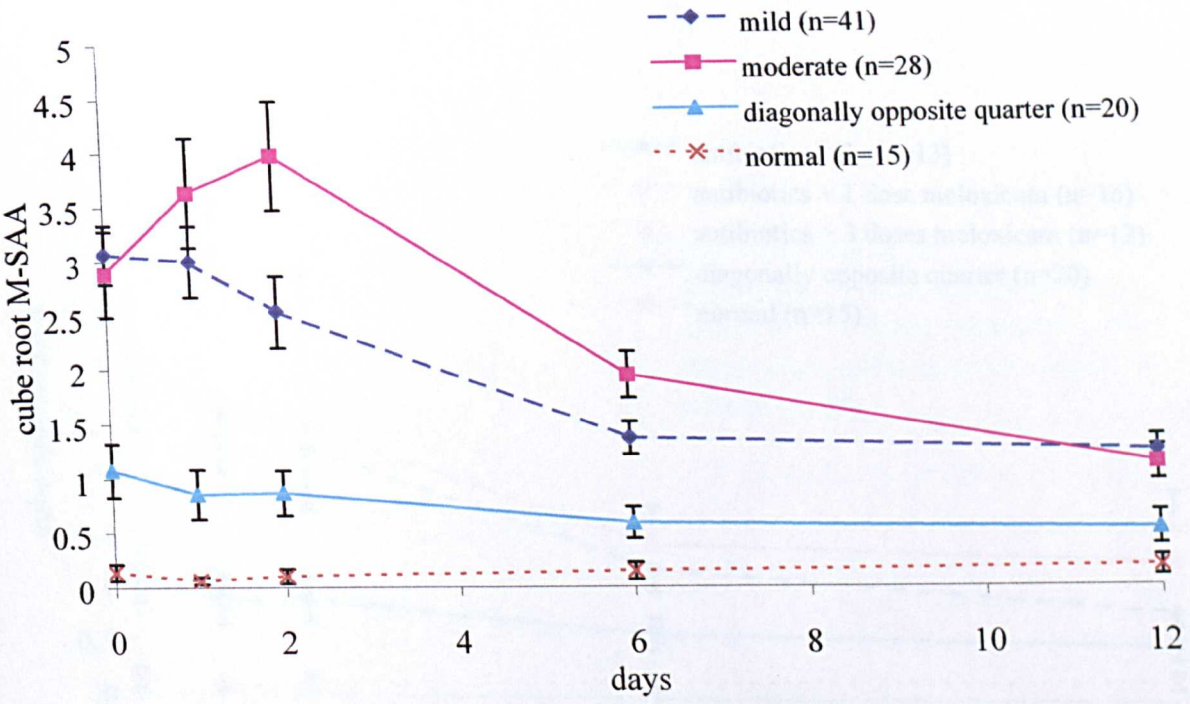


Figure 4.24: Mean mammary-associated Serum Amyloid A (M-SAA) concentrations (Cube root) ± SE for mild (n=41) and moderate (n=28) cases of clinical mastitis and the diagonally opposite quarters (n=20) and normal animals (n=15)

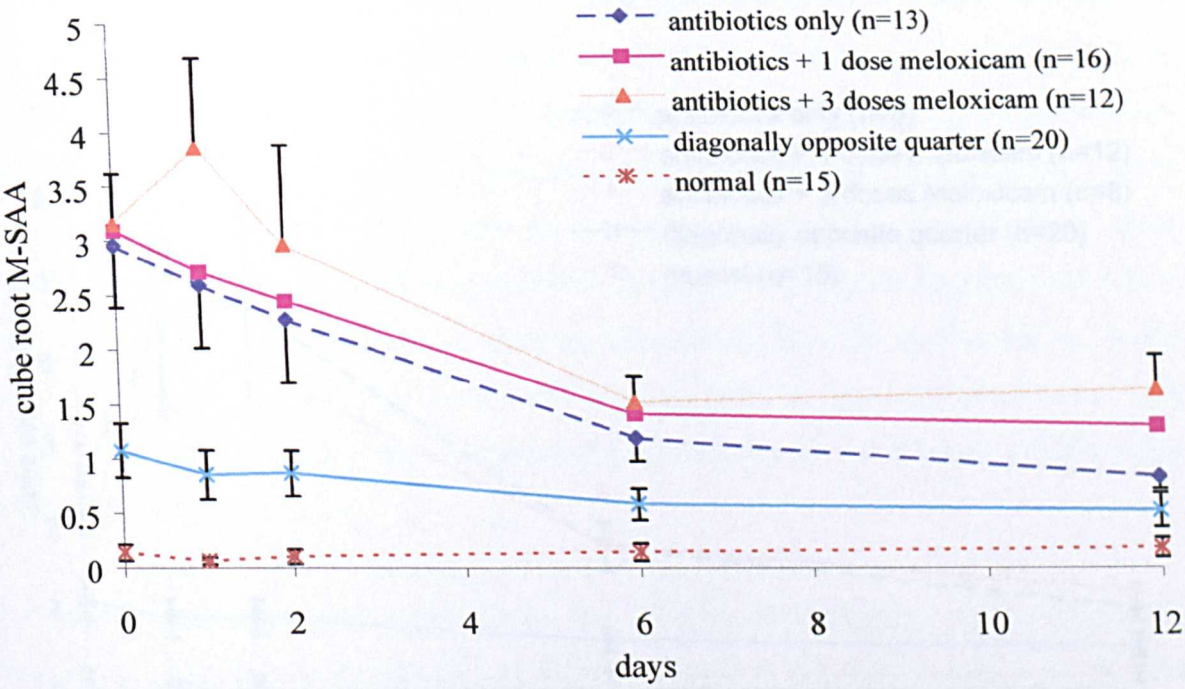


Figure 4.25: Mean mammary-associated Serum Amyloid A (M-SAA) concentrations (Cube root) ± SE for mild cases of mastitis in antibiotics only (n=13), antibiotics and one dose of meloxicam (n=16) and antibiotics and three doses meloxicam (n=11) treatment groups and the diagonally opposite quarters (n=20) and normal (n=15) cows

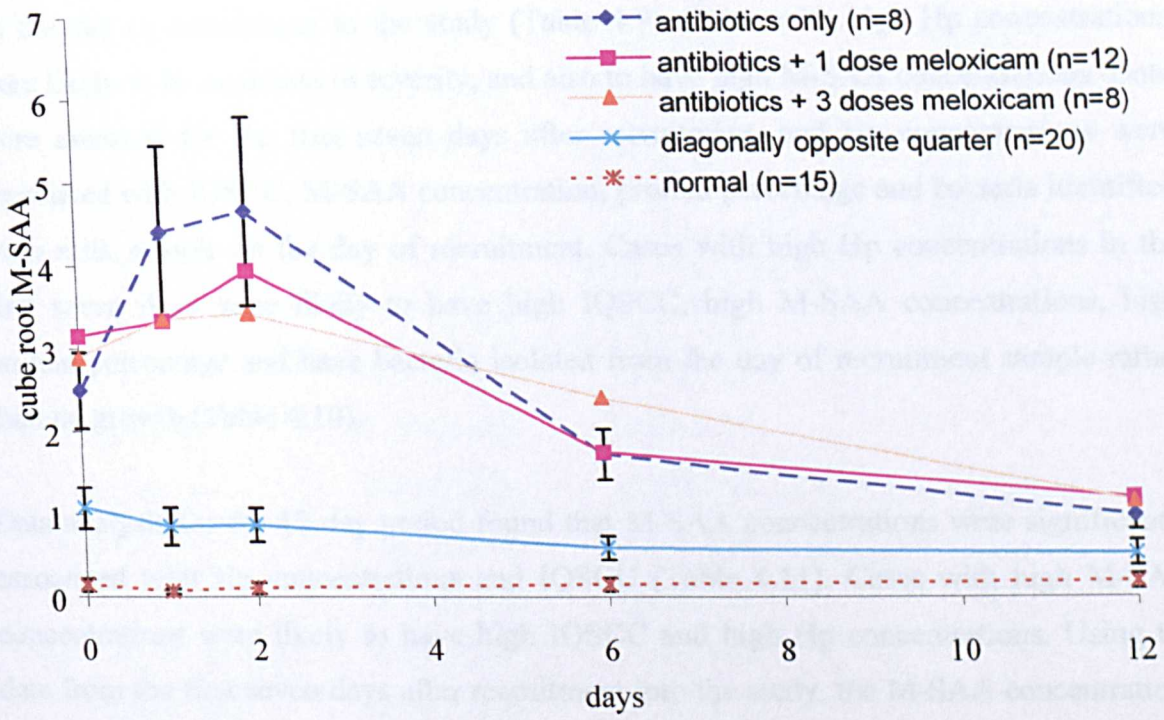


Figure 4.26: Mean mammary-associated Serum Amyloid A (M-SAA) concentrations (Cube root) ± SE for moderate cases of mastitis in antibiotics only (n=8), antibiotics and one dose of meloxicam (n=12) and antibiotics and three doses meloxicam (n=8) treatment groups and the diagonally opposite quarters (n=20) and normal (n=15) cows

concentration at day 12 between the diagonally opposite quarters of mild and moderate cases and normal cows ($p>0.05$).

Multilevel analysis examined the association between APP in milk and physiological (severity grading, days calved, body condition score, parity, quarter affected, treatment group, heart rate, respiratory rate, rectal temperature, udder temperature difference, threshold difference, hock-to-hock distance) and laboratory parameters (bacteriological isolate, IQSCC, fat percentage, protein percentage, lactose percentage) for the 12 day period and separately for the first seven days after diagnosis.

The analysis for the 12 day period showed a significant positive association between Hp concentrations and M-SAA concentrations and the severity of the mastitis, as categorised on the day of recruitment to the study (Table 4.9). Cases with high Hp concentrations were likely to be moderate in severity, and also to have high M-SAA concentrations. Data were assessed for the first seven days after recruitment, and Hp concentrations were associated with IQSCC, M-SAA concentration, protein percentage and bacteria identified from milk sample on the day of recruitment. Cases with high Hp concentrations in the first seven days were likely to have high IQSCC, high M-SAA concentrations, high protein percentage and have bacteria isolated from the day of recruitment sample rather than no growth (Table 4.10).

Data analysis for the 12 day period found that M-SAA concentrations were significantly associated with Hp concentrations and IQSCC (Table 4.11). Cases with high M-SAA concentrations were likely to have high IQSCC and high Hp concentrations. Using the data from the first seven days after recruitment into the study, the M-SAA concentrations were associated with IQSCC, haptoglobin concentrations and protein percentages, such that cases with higher M-SAA concentrations were likely to have high IQSCC, high Hp concentrations and high protein percentages (Table 4.12).

The ability of APP concentrations in milk to differentiate between normal animals and cases of clinical mastitis, and between moderate and mild cases of clinical mastitis, was determined by estimating the sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy of the proteins as diagnostic tests.

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
M-SAA	0.72 (0.04)	0.64 - 0.79	<0.001
Severity			<0.05
Mild		Reference category	
Moderate	0.53 (0.25)	0.05-1.01	

LRS Likelihood ratio test statistics M-SAA Mammary-associated Serum Amyloid A

Table 4.9: Final multivariable mixed effects model of variables that were associated with haptoglobin concentrations in milk from mild and moderate cases of clinical mastitis for the first 12 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
IQSCC	0.96 (0.25)	0.46-1.45	<0.001
M-SAA	0.24 (1.08)	0.02-0.45	<0.005
Protein	0.31 (0.16)	0.01-0.61	<0.05
Bacteria			<0.001
No growth		Reference category	
S uberis	0.77 (0.42)	-0.05-1.59	
E coli	0.75 (0.42)	-0.07-1.57	
Staph aureus	0.79 (0.50)	-0.20-1.77	
A pyogenes	0.75 (0.91)	-1.03-2.53	
S dysgalactiae	0.15 (0.88)	-1.56-1.87	
CN Staph	0.18 (0.72)	-1.23-1.58	
Corynebacterium spp.	0.88 (1.20)	-1.47-3.23	
Contaminated	0.95 (1.16)	-1.32-3.23	
Candida spp.	0.18 (1.00)	-1.77-2.14	

LRS Likelihood ratio test statistics IQSCC Individual Quarter Somatic Cell Count M-SAA Mammary-associated Serum Amyloid A

Table 4.10: Final multivariable mixed effects model of variables that were associated with haptoglobin concentrations in milk from mild and moderate cases of clinical mastitis for the first seven days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
IQSCC	0.58 (0.13)	0.33 - 0.83	<0.001
Haptoglobin	0.51 (0.07)	0.38 – 0.63	<0.001

LRS Likelihood ratio test statistics IQSCC Individual Quarter Somatic Cell Count

Table 4.11: Final multivariable mixed effects model of variables that were associated with mammary-associated serum amyloid A (M-SAA) concentrations of mild and moderate cases for the first 12 days after recruitment to the study

Explanatory variable	Coefficient (SE)	95% CI	LRS P value
IQSCC	0.70 (0.17)	0.36-1.03	<0.001
Haptoglobin	0.41 (0.08)	0.25-0.57	<0.005
Protein	0.32 (0.13)	0.07-0.57	<0.005

LRS Likelihood ratio test statistics IQSCC Individual Quarter Somatic Cell Count

Table 4.12: Final multivariable mixed effects model of variables that were associated with mammary-associated serum amyloid A (M-SAA) concentrations of mild and moderate cases for the first seven days after recruitment to the study

		True		Total
		Cases	Normal	
Test	Cases	68	0	68
	Normal	1	15	16
Total		69	15	84

Sensitivity	68/69 = 98.6%
Specificity	15/15 = 100%
Negative predictive value	15/16 = 94%
Positive predictive value	68/68 = 100%
Accuracy	83/84 = 98.8%

Table 4.13: Two-by-two table to test the ability of Hp concentrations to differentiate between cases of clinical mastitis and normal animals detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

The Hp concentrations 'cut-off' point for cases and normal animals was zero, as all normal animals had Hp concentration < LOD. Table 4.13 showed that the sensitivity of detecting cases of clinical mastitis from normal animals was 98.6%, while the specificity was 100%.

The Hp concentrations 'cut-off' point for moderate and mild cases was estimated from the mean of cube root Hp concentrations for all cases. Cases with lower Hp concentrations than the mean, <3.584, were classed as test mild and cases ≥3.584 were classed as test moderate. Table 4.14 showed that the sensitivity of detecting moderate clinical mastitis from mild cases of clinical mastitis was 68%, while the specificity was also 68%.

The M-SAA concentrations 'cut-off' point for cases and normal animals was taken as the highest value for a normal animal, 1.0354. Animals with M-SAA concentrations lower than 1.0354 were classed as normal and animals with concentrations ≥1.0354 were classed as cases of clinical mastitis. The M-SAA concentrations 'cut-off' point for moderate and mild cases was estimated from the mean of cube root M-SAA concentrations for all cases. Cases with lower M-SAA concentrations than the mean, <2.987, were classed as test mild and cases ≥2.987 were classed as test moderate. Tables 4.15 & 4.16 show that the sensitivity of detecting cases of clinical mastitis from normal animals was 88%, while the specificity was 100%. The sensitivity of detecting moderate clinical mastitis from mild cases of clinical mastitis was 36%, while its specificity was also 63%.

		True		Total
		Moderate	Mild	
Test	Moderate	19	13	32
	Mild	9	28	37
Total		28	41	69

Sensitivity	19/28 = 68%
Specificity	28/41 = 68%
Negative predictive value	28/37 = 76%
Positive predictive value	19/32 = 59%
Accuracy	47/69 = 68%

Table 4.14: Two-by-two table to test the ability of Hp concentrations to differentiate between moderate and mild severity clinical mastitis detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Cases	Normal	
Test	Cases	61	0	61
	Normal	8	15	23
Total		69	15	84

Sensitivity	61/69 = 88%
Specificity	15/15 = 100%
Negative predictive value	15/23 = 65%
Positive predictive value	61/61 = 100%
Accuracy	76/84 = 90%

Table 4.15: Two-by-two table to test the ability of M-SAA concentrations to differentiate between cases of clinical mastitis and normal animals detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

		True		Total
		Moderate	Mild	
Test	Moderate	10	15	25
	Mild	18	26	44
Total		28	41	69

Sensitivity	10/28 = 36%
Specificity	26/41 = 63%
Negative predictive value	26/44 = 59%
Positive predictive value	10/25 = 40%
Accuracy	36/69 = 55%

Table 4.16: Two-by-two table to test the ability of log M-SAA concentrations to differentiate between moderate and mild severity clinical mastitis detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

4.4. Discussion

The pathogen profile of clinical mastitis for cases recruited in this study showed that the most prevalent isolates were *S uberis* (30%), *E coli* (22%), *Staph aureus* (11%), other microbiological isolates (six per cent), *A pyogenes* (four per cent) and *S dysgalactiae* (three per cent). No growth was reported in 24% of the submitted samples. These microbiological results are consistent with the findings of an investigation of the prevalence and aetiology of clinical mastitis in Devon by Milne and others (2002), which highlighted that environmental pathogens, including *S uberis* and coliforms, were more commonly isolated from cases of clinical mastitis than contagious pathogens. In the study by Milne and others (2002), *S uberis* was isolated in 37% of cases, enterobacteriaceae in 23%, coagulase-negative staphylococci in 10%, other isolates in nine per cent, *S dysgalactiae* in four per cent and *Staph aureus* in three per cent and no growth in 14% of samples. The Veterinary Investigation Diagnosis Analysis reports (Anon 1982; Anon 1989; Anon 1993; Anon 1999) also demonstrate an increasing prevalence of mastitis caused by environmental streptococci and coliforms in recent years. This trend seems to be seen nationally within the UK and is likely to reflect the improvement in the control and treatment of the contagious pathogens.

The high proportion of no growth from mastitic samples was similar to that found in other studies of clinical mastitis (Wilson and Kingwill 1975; Wilesmith and others 1986; Milne and others 2002) and could have been due to rapid elimination of the pathogen by the host defences or continued activity of natural inhibitors in the milk between sampling and culturing, i.e. presence of non-viable bacteria, or the presence of exogenous inhibitory substances. Zorah and others (1993) showed that 51% of 84 samples yielded no growth on bacteriological culture were identified as containing antigen for *E coli*. Therefore, the 22% of *E coli* detected in this study could be an underestimate of the true prevalence. In the present study farm records were consulted to determine whether any antimicrobial drugs had been administered to cases of clinical mastitis prior to recruitment to the study, therefore the presence of inhibitory substances in the milk was unlikely.

A greater proportion of cases of mastitis caused by *S uberis* failed to complete the 45 day study period compared to all other isolates, although the difference was not significant. The high drop out rate of cases of clinical mastitis due to *S uberis* is consistent with the

reports suggesting that *S uberis* is a major problem in the control of clinical mastitis (Bramley 1982), due to its' ubiquitous nature (Leigh 1999) and its' ability to persist in the mammary gland (Milne and others 2004).

Moderate cases of clinical mastitis had significantly ($p<0.01$) more cases caused by *S uberis* and there tended to be more cases caused by *E coli* than in mild cases of clinical mastitis. A smaller study of mild and moderate clinical mastitis reported that *E coli* was isolated in 38% of moderate cases but only in 12.5% of mild cases (Eckersall and others 2001), however, those authors reported a very low incidence of *S uberis* mastitis. Published reports confirm that *E coli* mastitis is often more severe than other causes of mastitis. Bradley and Green (2001) reported that cases of *E coli* mastitis were more likely to have systemic signs than other causes of mastitis, and White and others (1986) noted that cows with 'general weakness', swollen udder and high body temperature were more likely to have coliform infection than any other types of mastitis. In a study investigating the cause of clinical mastitis and associated clinical signs in dairy cattle, Milne and others (2003) reported that the presence of an abnormal udder texture had approximately twice the odds of being caused by enterobacteriaceae than by any other group of organisms. At present, there have been no studies suggesting that *S uberis* is associated with increasing severity of disease, however, the results from this current study strongly suggested that *S uberis* is more likely to result in abnormalities of the udder in addition to changes in the milk appearance. The finding that more *Staph aureus* infections were mild is consistent with the observation that *Staph aureus* resides mainly in the udder (McDonald 1977) and that the majority of *Staph aureus* infections are chronic subclinical infections, with occasional clinical cases of mild to moderate severity (Cullor and Tyler 1996).

Clinical signs alone are considered to be insufficient to differentiate between infections caused by Gram-positive and Gram-negative bacteria with certainty (Morin and others 1998), but recent work indicates that some clinical signs are more likely to be associated with certain types of pathogens than others (Milne and others 2003). Animals infected with enterobacteriaceae had the greatest odds of having 'reduced' milk yield, 'swollen' or 'hard' udders, 'watery' milk and/or being systemically 'sick', as judged by the farmer using carefully defined criteria from all cases of clinical mastitis that occurred in Devon dairy herds over a 15 month period (Milne and others 2003). In this study, predictors for Gram-staining characteristics, i.e. Gram-negative or Gram-positive, of the bacteria

causing clinical mastitis were 'cow demeanour' and 'milk yield'. Thus, a cow with clinical mastitis that was 'sick' and had a 'reduced' milk yield was likely to have clinical mastitis caused by a Gram-negative pathogen.

Multilevel analysis of physiological and laboratory data in this study revealed significant associations with pathogen type. The microbiological finding of no growth from milk samples taken on the day of recruitment was associated with low IQSCC, and the isolation of *Staph aureus* was more likely in mild cases of mastitis. These results suggest that cases with microbiological result of no growth or *Staph aureus* in the sample taken on the day of recruitment are associated with less severe damage to the mammary tissue, and indeed this links to the finding that there were significantly more culture results of no growth and tended to be more isolations of *Staph aureus* in mild cases of mastitis compared to moderate cases of clinical mastitis. *Streptococcus uberis* was associated with increasing severity and is consistent with the occurrence of significantly more *S uberis* isolations in moderate cases. *Escherichia coli* mastitis was associated with higher heart rates, which agrees with the results of previous studies indicating that *E coli* intramammary infections were more likely to induce systemic signs (Bradley and Green 2001). A systemic bacteraemia was identified in 32% of dairy cows with clinical mastitis caused by coliforms (Wenz and others 2001).

The median log IQSCC were significantly greater in the quarter from the affected gland of cows with mild or moderate mastitis than the diagonally opposite quarter of these cows and normal cows ($p<0.001$). There was no significant difference between the median log IQSCC of the affected quarter from mild and moderate cases of mastitis ($p>0.05$). This result is likely to be unreliable as more samples from moderate (10/28) cases than mild (9/41) cases could not be tested due to the presence of clots, and thus more moderate cases were given the maximum value for log IQSCC. It would be expected that milk from the affected quarter of moderate cases would have a greater IQSCC compared to milk for the affected quarter of mild cases. The median log IQSCC for affected and diagonally opposite quarters remained significantly higher than that of normal animals for the duration of the study, however, there was no significant difference between the affected quarter and the diagonally opposite quarter in cases of clinical mastitis 45 days after recruitment to the study. There was no prior information on the cell count of the animals before recruitment to the study and it may be that the cows that went on to develop

clinical mastitis had higher cells counts that the normal animals prior to infection. There was no significant difference in parity and stage of lactation of the clinical cases of mastitis and the normal cows, and latter were selected from the farms that provided the clinical cases, suggesting that the normal cows were representative of the population at risk. Somatic cell counts are used as indicators of disease, but there are a number of other factors, such as breed, age, stage of lactation and physiological stresses such as excitement, high temperatures and trauma that can affect levels, making a single result inconclusive (Cullen 1966; Tolle 1975; Reneau 1986). Multiple SCC records for individual cows or quarters, however, are useful to monitor the dynamics of udder health and response to treatment (Reneau 1986). Results from the study by Reneau (1986) showed that following a clinical incident, the IQSCC decreased after treatment but remained high for prolonged period and with less frequent sampling and monitoring, cows recovering from clinical mastitis could have been classed as having subclinical infections. Hillerton (1997) reported that at a cow level (ICSCC) a 'good' cure results in the cell count being below 400,000 cells/ml in approximately 20 milkings although pre-infection cell count levels may not be recovered in some animals within 50 milkings. The SCC data in this study was at the quarter level and explains why the cell count of the affected quarters was higher for longer period than in the study by Hillerton (1997).

There was no significant effect of mastitis on the fat percentage of milk, as judged by the comparison of affected quarters to diagonally opposite quarters and to those of normal cows. The protein content of milk increased with mastitis and the lactose content decreased compared to that in diagonally opposite quarters and in those of normal cows. Literature regarding the changes in milk fat and protein per cent were reviewed by Hortet and Seegers (1998) and were reported to be contradictory. A small decrease in fat production due to mastitis was reported, but was considered to be trivial for short duration infections and only important for persistent infections that were carried over into the next lactation and recurring severe infections (Houben and others 1993). The lack of significant change in the fat content in this study may have been due to the fact that the cases studied were all acute infections.

In cows with mastitis the protein concentration was reported to increase slightly (Houben and others 1993). This was reported more with infections that persisted into the next lactation, and the milk volume was reduced and therefore the overall total protein

production was reduced (Houben and others 1993). Other studies (Myllys and Rautala 1995) showed a decrease in protein content, however, the cases with mastitis were of higher genetic potential and had lower protein and fat contents genetically compared to the animals used as controls, thereby making comparison difficult. The main importance of milk compositional changes with mastitis is for manufacturing companies, however, clinically altered milk has to be removed from human consumption for legislative reasons and the use of antimicrobials results in the milk being withheld to avoid residues in milk.

In the present study, examination of milk samples from normal cows and from cases of clinical mastitis 20 and 45 days after the clinical incident demonstrated that the predominant cells present were macrophages. This is consistent with previous studies reporting that macrophages were the predominant cell type in milk tissues of healthy mammary glands (Outteridge and Lee 1981; Riollet and others 2000). The most common somatic cell type in quarters affected with mild and moderate mastitis on the day of recruitment to the study were PMNs. Daley and others (1991), Riollet and others (2000) and Sordillo and Streicher (2002) also reported that PMNs were the principal cell type present in mammary tissues and secretions during early inflammation. The relative proportion of PMNs then decreased over the 45 days after recruitment to the study, and there were consequent increases in lymphocyte and macrophage proportions. The immune response is activated within 24 hours of initial PMNs influx and results in the increase in lymphocytes numbers. There was no significant difference in the differential cell count of mild cases versus moderate cases of clinical mastitis, and there was no effect of meloxicam treatment.

The median concentrations of Hp and M-SAA measured in the milk of cows with mild and moderate mastitis in this study were 27.3 μg/ml and 83.8 μg/ml and 20 μg/ml and 8.5 μg/ml, respectively. These results are similar to those reported in other studies of naturally occurring mastitis in dairy cattle (Eckersall and others 2001; Nielsen and others 2004). Eckersall and others (2001) reported a median Hp concentration of 90 μg/ml and 110 μg/ml in mild and moderate cases of mastitis, respectively, and median M-SAA concentrations of 2.6 μg/ml and 20.6 μg/ml in mild and moderate cases of mastitis, respectively. In the current study the median Hp concentrations in the diagonally opposite quarter to the quarter affected with mastitis were 6.0 μg/ml and 2.7 μg/ml for mild and moderate cases of mastitis respectively. The median M-SAA concentrations in the

diagonally opposite quarter were 0.5 μg/ml and 1.2 μg/ml for mild and moderate cases of mastitis respectively. Eckersall and others (2001) reported the Hp range for the diagonally opposite quarter to the quarter affected with mastitis to be 0.02 – 0.56 μg/ml for the 2/16 cases of mild clinical mastitis and 0.02 – 0.80 μg/ml for the 2/13 cases of moderate clinical mastitis that had detectable levels. The range of M-SAA concentrations in the 5/16 cases of mild clinical mastitis with detectable levels was 0.2 – 9.4 μg/ml and in the 7/13 cases of moderate clinical mastitis with detectable levels was 0.2 – 27 μg/ml. As was seen in this study, Eckersall and others (2001) also reported that the Hp concentrations in all normal animals were below the limit of detection. The M-SAA concentrations of 4/15 normal animals with detectable levels had a range of 0.005 – 1.11 μg/ml in this study and in the study by Eckersall and others (2001) the M-SAA concentrations were detectable in 8/32 normal animals with a range of 0.2 – 0.54 μg/ml in normal animals. Nielsen and others (2004) reported a mean haptoglobin concentration of 110 μg/ml for the infected quarter and a mean 1.0 μg/ml for the diagonally opposite quarter and mean M-SAA concentration of 47.5 μg/ml in the mastitic quarter and a mean of 40.2 μg/ml in the diagonally opposite quarter. In mastitis, experimentally induced by infusion of *Staphylococcus epidermidis* into the mammary glands of sheep, the mean M-SAA concentration in the milk of the affected gland was 2,496 μg/ml (Winter and others 2003), which was much higher than that measured in naturally occurring mastitis in cattle.

The detection of Hp and M-SAA in the milk of affected quarters of cows with mild and moderate clinical mastitis indicated that an acute phase response had occurred. In the normal cows no haptoglobin was detected and very low M-SAA concentrations were detected in only 4/15 normal cows. Haptoglobin and M-SAA concentrations were detectable in the majority of the diagonally opposite quarters of cows with mastitis, suggesting release of acute phase proteins from a systemic source or local production in these quarters due to local activation of an acute phase response. The study conducted by Nielsen and others (2004) demonstrated that extramammary inflammatory conditions that induced increases in the serum concentration of SAA were not accompanied by increases in M-SAA suggesting that an intramammary inflammatory stimulus is required for concentrations of M-SAA to increase, however, there were only eleven animals with extramammary infection in the study. These authors could not assess whether it was also the case with Hp due to only one animal with extramammary inflammation having

detectable serum Hp concentrations. Eckersall and others (2001), however, reported a significant correlation with Hp concentration in serum and in milk but no association with SAA and M-SAA. We can conclude that increases in M-SAA in the diagonally opposite quarters were likely to be due to inflammatory processes in that quarter but the increase in the milk Hp concentrations could be due to leakage across the blood/mammary gland barrier from the systemic acute phase response. The hypothesis that there were inflammatory responses in the diagonally opposite quarters was also indicated by the increased IQSCC.

In an experimental study (Hirvonen and others 1996) where micro-organisms were infused into both hindquarters of dairy heifers to induce mastitis, Hp concentrations in blood increased the day after infusion and maximum levels were reached in 2-3 days. The levels in heifers with moderate mastitis returned to normal within five days whereas the levels remained increased for two weeks after inoculation in the heifers with severe mastitis. In this study concentrations of APP were assessed only in milk and it was found that Hp concentrations were maximal on the day of recruitment to the study, whereas M-SAA concentrations reached peak concentrations two days after recruitment to the study. A difference in the kinetics of Hp and M-SAA was not reported by Hogarth (2004). Somatic cell counts, Hp and M-SAA concentrations were monitored in milk prior to the development of clinical mastitis and it was reported that before and after clinical mastitis, both M-SAA and Hp showed similar kinetics and were similar to SCC. Peak concentrations of M-SAA and Hp occurred in conjunction with SCC and on the same day as clinical signs in three out of seven cases of clinical mastitis, while in the other four cases the increase in concentration occurred either prior, to or in conjunction with the rise in SCC.

Haptoglobin and M-SAA concentrations in milk were sensitive and specific markers of disease. The aim of this study was to differentiate between cases of clinical mastitis and normal animals. Haptoglobin was shown to have a sensitivity and specificity of 98.6% and 100%, respectively, while M-SAA had a sensitivity and specificity of 88% and 100%, respectively. Haptoglobin concentrations from cases of mild clinical mastitis were significantly different from cases of moderate clinical mastitis. Haptoglobin concentrations can therefore be used to differentiate between mild and moderate severities of clinical mastitis, but with PPV of 59% and NPV of 76% there would be a considerable

degree of misclassification. In the absence of skilled labour this test could aid in the targeting of specific therapy. The concentrations of Hp and M-SAA in the affected quarter of mild and moderate clinical mastitis decreased over the study period but were still significantly greater than the concentrations in diagonally opposite quarters and in normal animals. Thus, milk APP concentrations could therefore be valuable and rapid markers to aid in the clinical diagnosis, prognosis and treatment monitoring of dairy cattle with intramammary infections and could be used in automated milking systems in the future.

There was no effect of meloxicam treatment on IQSCC and differential cell count, however, meloxicam would not be expected to alter cellular immune responses. Meloxicam acts to reduce inflammation, and it was therefore hypothesised that there may be a treatment effect on the acute phase protein concentrations. This was not seen in this study and can be explained by the fact that there are many different mediators and pathways involved in the acute phase response, with meloxicam only acting on the COX enzymes.

In conclusion, the pathogen profile differed with severity of the clinical mastitis. The protein percentage decreased and the lactose per cent increased in the presence of mastitis. There was a dramatic increase in SCC, which was largely due to an influx of PMNs into the mammary gland. Acute phase protein concentrations in the milk increased rapidly and haptoglobin concentration on the day of clinical signs of the mastitis could be used to differentiate between mild and moderate cases of mastitis. Differentiation between different severities of mastitis could be important for therapeutic decisions, such that analgesics could be administered to the more severe cases of clinical mastitis as a routine treatment along with intramammary antimicrobial preparations.

Chapter 5

Prostaglandin E_2 and meloxicam concentrations in milk from cows with clinical mastitis

5.1. Introduction

Prostaglandins (PGs) are ubiquitous in most mammalian tissue and organs where they have numerous diverse biological effects on a variety of physiological and pathological processes (Uda and others 1990). The production of PGs begins with the liberation of arachidonic acid from membrane phospsholipids by phospholipase A_2 in response to inflammatory stimuli, following which arachidonic acid is then converted to PGH_2 by the cyclooxygenase enymes COX-1 and COX-2 (Harris and others 2002). Cell-specific prostaglandin synthases convert PGH_2 into a series of PGs including PGI_2, $PGF_{2\alpha}$, PGD_2 and PGE_2. Prostaglandin E_2, PGI_2 and PGD_2 are powerful vasodilators and contribute to the redness and increased blood flow in areas of acute inflammation. They also sensitise pain receptors, thus lowering the pain tolerance threshold (Reyes and others 2002). Increased concentrations of PGE_2 were reported in experimental models of bovine inflammation (Thomas and others 1995) and in naturally occurring mastitis cases (Atroshi and others 1987). Anderson and others (1985) reported an increase $PGF_{2\alpha}$ and TXB_2 in bovine mastitis and showed that increases in concentrations corresponded to the clinical severity of mastitis.

Anti-inflammatory drugs are widely used in veterinary practice in order to provide symptomatic relief in the treatment of both acute and chronic inflammatory conditions (Cunningham and Lees 1994). The principal value of these drugs is to relieve pain and reduce swelling. The most commonly used drugs are the corticosteroids (glucocorticoids) and the NSAID. Non-steroidal anti-inflammatory drugs inhibit the transformation of arachidonic acid to stable PG (i.e. PGE_2 and prostacyclin) (Abramson and Weissmann

1989). Meloxicam is a NSAID within the group of enolic acids that inhibit COX enzymes and thus inhibit the production of PGs and other inflammatory mediators (Reyes and others 2002). Meloxicam therefore has anti-inflammatory, anti-exudative, analgesic and antipyretic properties (Rang and others 1999).

The objective of this study was to assess if PGE_2 concentrations in milk could act as quantitative markers of severity of clinical mastitis in cows and whether the use of meloxicam reduced the concentrations of PGE_2 in milk following treatment.

5.2. Materials and methods

5.2.2. Sample selection

Prostaglandin E_2 (PGE_2) was measured by radio-immunoassay (Chapter 2) in samples from the mastitic (affected) quarter of all 69 complete cases of clinical mastitis (there was an inadequate volume of milk from one cow) on 0, 1 and 2 days after diagnosis of mastitis. Meloxicam concentrations were measured using High Performance Liquid Chromatography (HPLC) (Chapter 2) in 16 cases that were selected randomly from all complete cases using random number generator in Minitab Statistical Software release 13 (Minitab INC). These 16 cases included eight cases from the treatment group that were given antibiotics and one dose of meloxicam (group 2) and eight cases that were given antibiotics and three doses of meloxicam (group 3) (Chapter 2). In both treatment groups, meloxicam was administered by intravenously route, at the rate of 0.5mg/kg. In Group 2 the meloxicam was administered on the day of diagnosis of the mastitis (day of recruitment) and in group 3 meloxicam was administered on the day of recruitment, three days and six days after recruitment.

5.2.2. Data analysis

Data were entered into Microsoft Access 2000 and basic statistical analysis was conducted using Microsoft Excel and Minitab Statistical Software version 13 and univariable and multivariable regressions were performed using S-Plus 2000 and MlwiN version 1.1 as detailed in Chapter 2. Data for PGE_2 were transformed to improve normality and stabilise variance. To examine the effect of meloxicam treatment on the

PGE_2 concentrations treatment groups 2 and 3 were combined, such that the treatment groups were antibiotics only (group 1) versus antibiotics and meloxicam (groups 2 and 3). Groups 2 and 3 were merged as PGE_2 concentrations were measured on the day of recruitment, day 1 and day 2, which was before the second and third doses of meloxicam were administered for cases in group 3.

Two-by-two tables were constructed to allow the sensitivity, specificity, predictive values and accuracy to be calculated (Dohoo and others 2003) for cases with clinical mastitis versus normal animals and mild versus moderate cases of clinical mastitis.

5.3. Results

5.3.1. Prostaglandin E_2

Radio-immunoassay (Chapter 2) was performed on milk samples from the day of recruitment, one day and two days after recruitment for 69 cows with mild and moderate clinical mastitis. The PGE_2 concentrations in the milk from the affected quarter for the 69 cases varied widely (Table 5.1), but, in general, the concentrations were higher in the moderate cases on the day of recruitment compared to mild cases, and higher on the day of recruitment compared to days one and two after the day of recruitment. The PGE_2 concentration in the affected quarter from mild cases ranged from 0 – 3.08 ng/ml with a median of 0.0 ng/ml and a mean of 0.148 ng/ml on the day of recruitment to the study (day 0). The PGE_2 concentration in the affected quarter from moderate cases ranged from 0 – 10.2 ng/ml with a median of 0.095 ng/ml and a mean of 1.01 ng/ml on the day of recruitment to the study (day 0). The mean log PGE_2 concentration for moderate cases was 0.16 (SE±0.06) and was significantly higher ($p<0.05$) than for mild cases, where the mean log PGE_2 concentration was 0.04 (SE±0.02) on the day of recruitment to the study (Figure 5.1).

The mean log PGE_2 concentrations were highest on the day of recruitment and decreased over the next two days for mild and moderate cases of clinical mastitis (Figure 5.2). The log PGE_2 concentrations for the treatment groups that received antibiotics only (group 1) and antibiotics plus meloxicam (groups 2 and 3) showed similar patterns (Figure 5.3).

		PGE_2 concentration (ng/ml)		
Treatment	Severity	Day 0	Day 1	Day 2
Antibiotics	Mild (n=13)	0 (0 – 0.105)	0 (0 – 0.015)	0 (0 – 0.09)
	Moderate (n=8)	0.23 (0.08 – 3.09)	0.02 (0 – 0.158)	0 (0 – 0.023)
Antibiotics +	Mild (n=28)	0.06 (0 – 0.158)	0 (0 – 0.038)	0 (0 – 0.08)
Meloxicam	Moderate (n=20)	0.05 (0 – 0.275)	0 (0 – 0.038)	0 (0 – 0.105)
All cases (n=69)		0.07 (0 – 10.2)	0 (0 – 4.1)	0 (0 – 6.29)

Table 5.1: Median and inter-quartile ranges of prostaglandin E_2 (ng/ml) concentrations in the milk of cases of mild and moderate clinical mastitis treated with antibiotics only (group 1) or antibiotics plus meloxicam (group 2 and 3) on the day of recruitment to the study (day 0) and on the following two days (day 1 & 2)

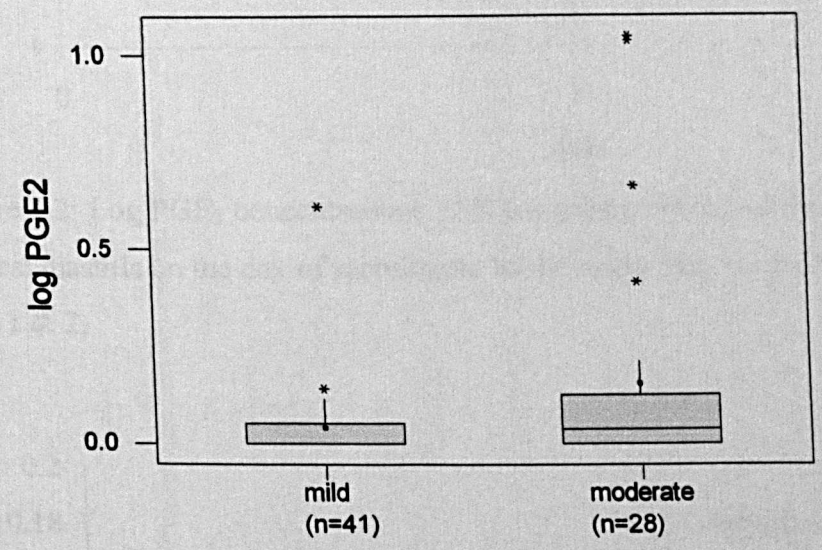


Figure 5.1: Log PGE_2 concentrations for mild (n=41) and moderate (n=28) cases of clinical mastitis on day of recruitment to the study (day 0).

For each group, the median value is represented by the horizontal line within the box and the mean value denoted by the dot. The bottom and top of the box represent the first and third quartiles respectively. The vertical lines extending from the box represent the lowest and highest observations within 1.5x the inter-quartile range below and above the first and third quartiles respectively. Outliers are denoted by *.

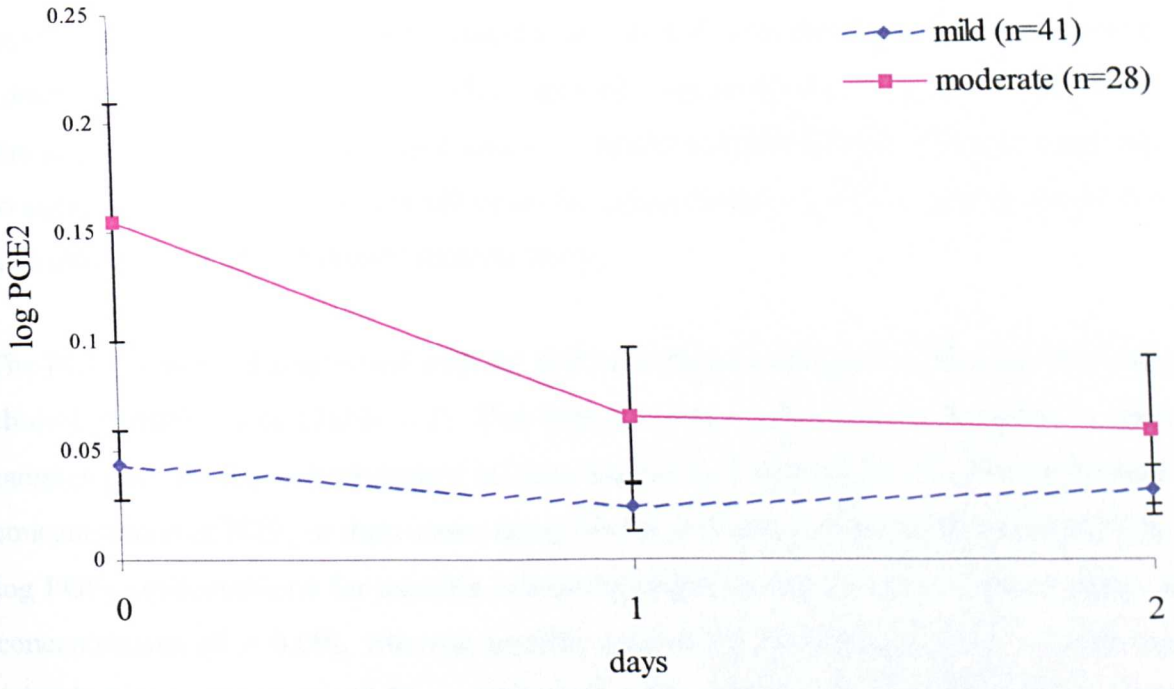


Figure 5.2: Log PGE_2 concentrations ±SE for mild (n=41) and moderate (n=28) cases of clinical mastitis on the day of recruitment to the study (day 0) and the following two days (day 1 & 2)

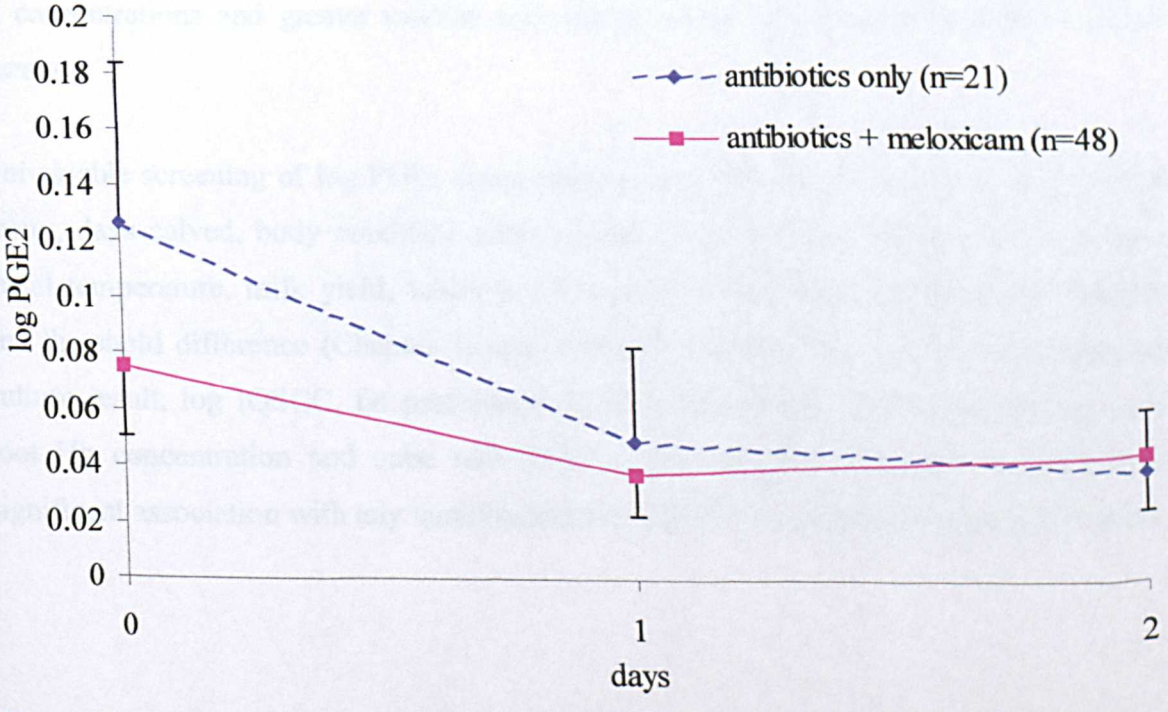


Figure 5.3: Log PGE_2 concentrations ±SE for mild and moderate cases of clinical mastitis given antibiotics only (n=21) and antibiotics plus meloxicam (n=48) on the day of recruitment to the study (day 0) and the following two days (day 1 & 2)

When cases were separated into different severities, the treatment groups again showed the same general trend of highest concentrations of PGE_2 on the day of recruitment with a decrease over the next two days. Mild cases of clinical mastitis (Figure 5.4) had lower concentrations PGE_2 than moderate cases of clinical mastitis (Figure 5.5), but there was no significant ($p>0.05$) treatment effect on the concentrations of PGE_2, either one or two days after the day of recruitment into the study.

The PGE_2 concentrations varied with the different causal pathogen of mild and moderate clinical mastitis cases (Table 5.2). The highest concentrations were detected in milk samples from the day of recruitment in cases caused by *S uberis* and *E coli*, with the peak concentrations of PGE_2 in these cases being 10.2 ng/ml and 9.86 ng/ml, respectively. The log PGE_2 concentrations for mastitis caused by *Staph aureus* showed a narrow range of concentrations (0 – 0.09), whereas mastitis caused by *S dysgalactiae* (0 – 0.42) and miscellaneous organisms (0.04 – 0.66) had wide concentration ranges. There were, however, only three cases caused by *S dysgalactiae* and three cases caused by miscellaneous organisms (Figure 5.6). There was no significant difference in the log PGE_2 concentrations between any of the bacteriological groups, but the environmental organisms, such as *S uberis*, *E coli* and *A pyogenes*, and no growths had a greater range in concentrations and greater median and mean values than mastitis caused by *Staph aureus*.

Univariable screening of log PGE_2 concentrations and physiological parameters, such as parity, days calved, body condition score, treatment group, heart rate, respiratory rate, rectal temperature, milk yield, udder lesions, udder cleanliness, hock-to-hock distance and threshold difference (Chapter 3) and laboratory parameters, such as bacteriological culture result, log IQSCC, fat percentage, protein percentage, lactose percentage, cube root Hp concentration and cube root M-SAA concentration (Chapter 4) showed no significant association with any variable and the log PGE_2 concentrations in this data set.

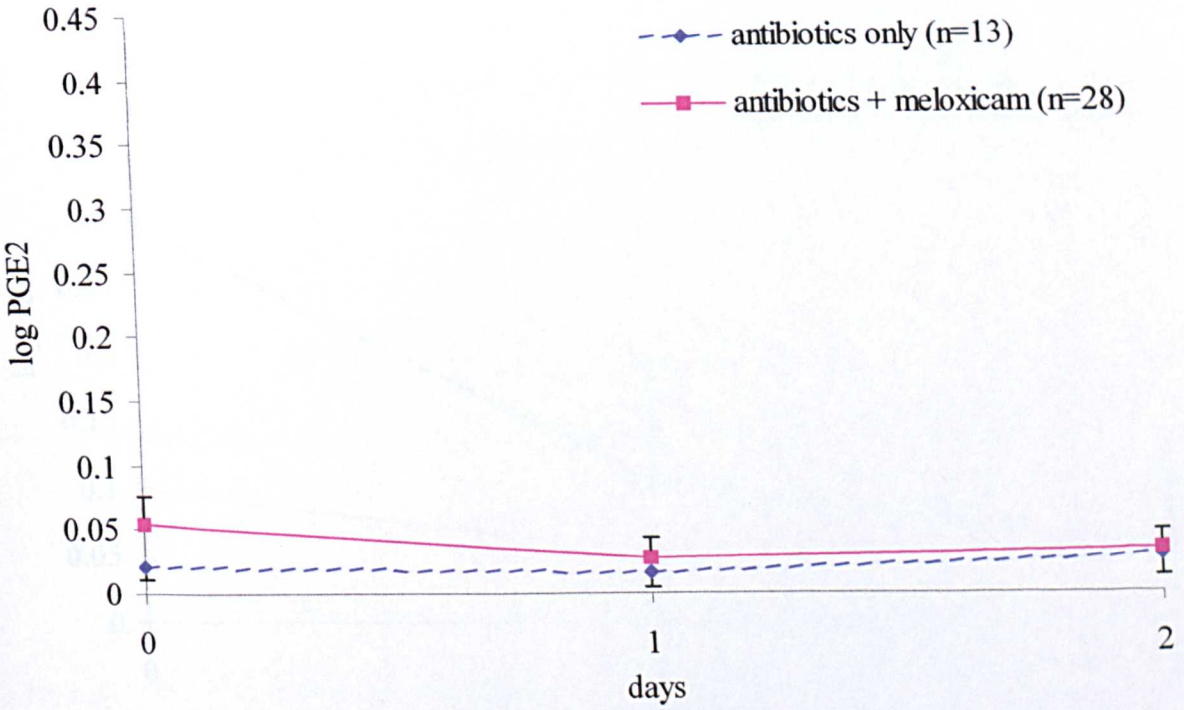


Figure 5.4: Log PGE_2 concentrations ±SE for mild cases of clinical mastitis given antibiotics only (n=13) and antibiotics plus meloxicam (n=28) on the day of recruitment to the study (day 0) and the following two days (day 1 & 2)

	PGE_2 concentration (ng/ml)		
Isolated pathogen	Day 0	Day 1	Day 2
S uberis (n=19)	0.05 (0 – 0.15)	0 (0 – 0.13)	0 (0 – 0.11)
E coli (n=15)	0.10 (0 – 0.28)	0.02 (0 – 0.06)	0 (0 – 0.07)
No growth (n=18)	0.07 (0 – 0.23)	0 (0 – 0.06)	0 (0 – 0.09)
Staph aureus (n=9)	0.0 (0 – 0)	0 (0 – 0)	0 (0 – 0.08)
S dysgalactiae (n=3)	0.07 (0 – 1.6)	0 (0 – 0)	0 (0 – 0.04)
A pyogenes (n=2)	0.16 (NA)	0.08 (NA)	0.08 (NA)
Miscellaneous (n=3)	0.16	0.03 (0 – 4.07)	0 (0 – 1.65)

NA not applicable as there were only two samples

Table 5.2: Median and inter-quartile ranges of PGE_2 (ng/ml) concentrations in the milk of cases of mild and moderate clinical mastitis caused by *S uberis* (n=19), *E coli* (n=15), no growth (n=18), *Staph aureus* (n=9), *S dysgalactiae* (n=3), *A pyogenes* (n=2) and miscellaneous organisms (n=3) on the day of recruitment to the study (day 0) and the following two days (day 1 & 2)

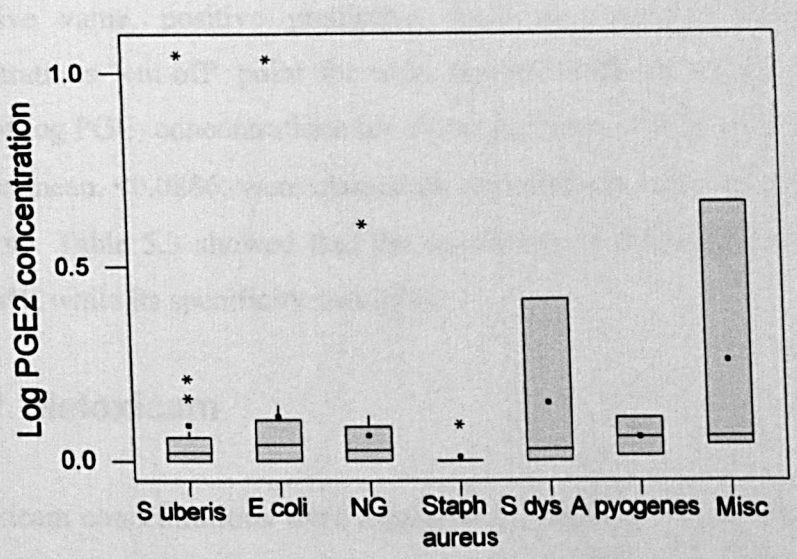


Figure 5.6: Log PGE_2 concentrations for clinical mastitis caused by *S uberis* (n=19), *E coli* (n=15), no growth (NG) (n=18), *Staph aureus* (n=9), *S dysgalactiae* (S dys) (n=3), *A pyogenes* (n=2) and miscellaneous organisms (Misc) (n=3) on day of recruitment to the study (day 0)

		True		Total
		Moderate	Mild	
Test	Moderate	10	6	16
	Mild	18	35	53
Total		28	41	69
Sensitivity		10/28 = 36%		95% CI 18.2 – 53.8%
Specificity		35/41 = 85%		95% CI 74.1 – 95.9%
Negative predictive value		35/53 = 66%		95% CI 53.2 – 78.8%
Positive predictive value		10/16 = 63%		95% CI 39.3 – 86.7%
Accuracy		45/69 = 65%		95% CI 53.7 – 76.3%

Table 5.3: Two-by-two table to test the ability of log PGE_2 concentrations to differentiate between moderate and mild severity clinical mastitis detailing the sensitivity, specificity, negative predictive value, positive predictive value and accuracy

The ability of PGE_2 concentrations to differentiate between moderate and mild cases of clinical mastitis was determined by estimating the sensitivity, specificity, negative predictive value, positive predictive value and overall accuracy. The log PGE_2 concentrations 'cut-off' point for mild and moderate severity was estimated from the mean of log PGE_2 concentrations for all cases. Cases with lower log PGE_2 concentrations than the mean, <0.0886, were classed as test mild and cases ≥0.0886 were classed as test moderate. Table 5.3 showed that the sensitivity of detecting moderate clinical mastitis was 36%, while its specificity was 85%.

5.3.2. Meloxicam

Meloxicam concentrations were measured for eight cows given antibiotics and one dose of meloxicam (group 2) and eight cows given antibiotics and three doses of meloxicam (group 3). The retention times for meloxicam and piroxicam were approximately 7.1 and 5.7 minutes, respectively (Chapter 2) and this knowledge, along with spiking of the samples with prioxicam, allowed the concentration of meloxicam from samples of the cases of clinical mastitis to be estimated. A typical chromatogram for a cow (cow 14) treated with antibiotics and three doses of meloxicam is shown in Figure 5.7.

There were no detectable levels of meloxicam in milk samples collected on the day of recruitment or day 12. There were high levels of meloxciam in the samples taken on day one and day seven and there were low levels of meloxicam detectable in milk samples from day three and day six after recruitment to the study.

5.3.2.1. One dose of meloxicam

In the eight cases of clinical mastitis that were treated with antibiotics and one dose of meloxicam, the meloxicam concentration peaked one day after treatment and subsequently decreased to low levels two days later and six days after treatment concentrations were undetectable (Figure 5.8 and Table 5.4). The mean concentration of meloxicam measured in the milk one day after treatment was 0.11 μg/ml. The inter-animal range was more variable at 0.03 – 0.38 μg/ml. The mean concentration of meloxicam two days after drug administration was 0.04 μg/ml, with the inter-animal

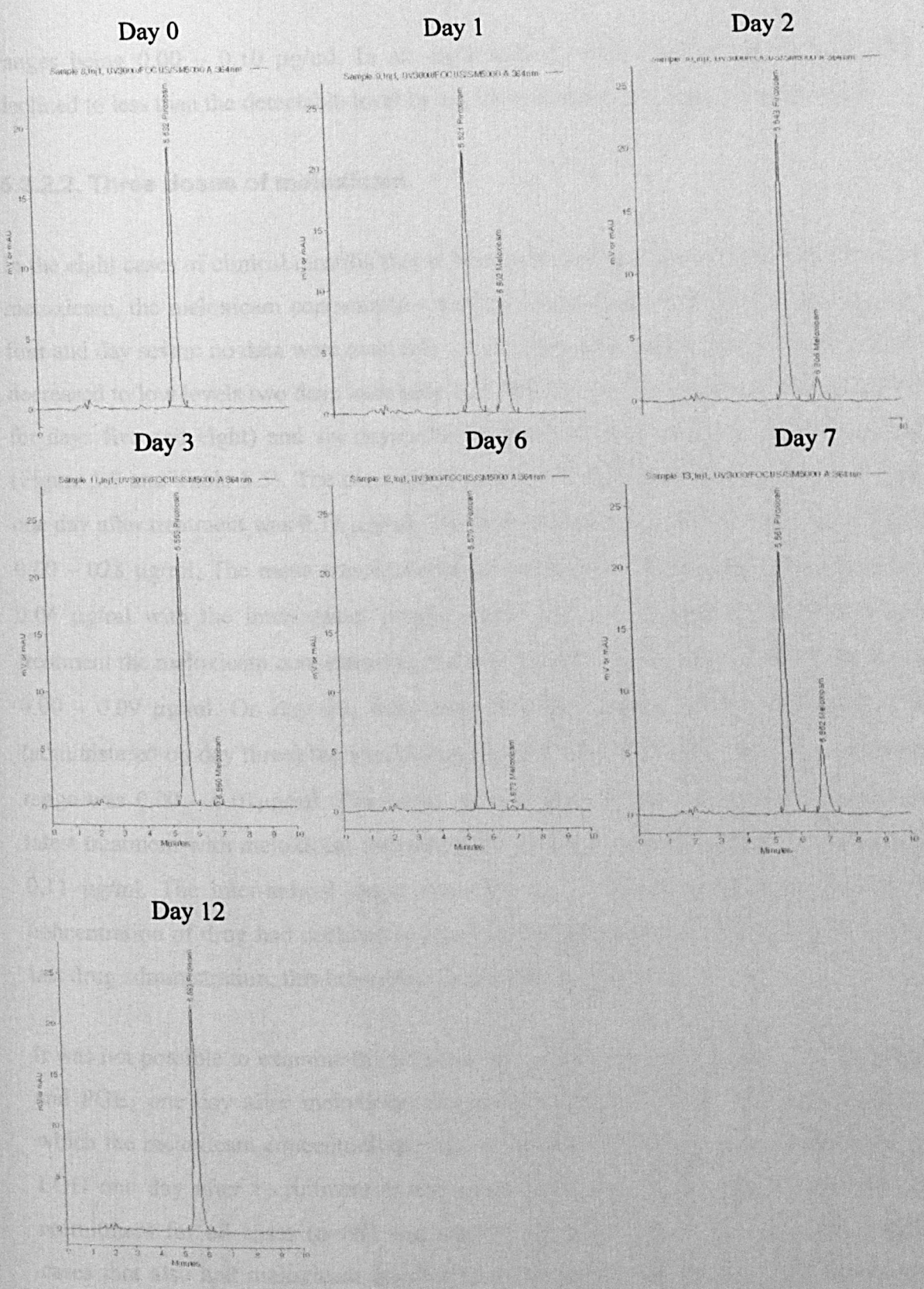


Figure 5.7: Typical chromatograms of meloxicam concentrations in the milk from the affected quarter of a cow (cow 14) treated with antibiotics and three doses of meloxicam (group 3) and spiked with piroxicam (0.5µg/ml) from day of recruitment to the study (day 0), day 1, day 2, day 3, day 6, day 7 and day 12 after recruitment

ranges being 0.00 – 0.10 μg/ml. In all eight animals the concentration of drug had declined to less than the detectable level by six days after the last drug administration.

5.3.2.2. Three doses of meloxicam

In the eight cases of clinical mastitis that were treated with antibiotics and three doses of meloxicam, the meloxicam concentration peaked one day after treatment (day one, day four and day seven: no data were available for day four after treatment) and subsequently decreased to low levels two days later (day two, day five and day eight: no data available for days five and eight) and six days after treatment concentrations were undetectable (Figure 5.9 and Table 5.5). The mean concentration of meloxicam measured in the milk one day after treatment was 0.11 μg/ml. The inter-animal range was quite variable, being 0.00 – 028 μg/ml. The mean concentration of meloxicam two days after treatment was 0.04 μg/ml with the inter-animal ranges being 0.01 – 0.12 μg/ml. Three days after treatment the meloxicam concentration was 0.02 μg/ml with the inter-animal range being 0.00 – 0.09 μg/ml. On day six, three days after the latest treatment with meloxicam (administered on day three) the mean concentration was 0.00 μg/ml and the inter-animal range was 0.00 – 0.01μg/ml. The mean concentration on day seven, one day after the latest treatment with meloxicam (administered on day six), was the same as for day one, 0.11 μg/ml. The inter-animal range was 0.05 – 0.25 μg/ml. In all eight animals the concentration of drug had declined to less than the detectable level by six days after the last drug administration, this being day 12 for cases in group 3.

It was not possible to examine the relationship between the concentrations of meloxicam and PGE_2 one day after meloxicam treatment because 87.5% (14/16) of the cases in which the meloxicam concentrations were measured had PGE_2 concentrations below the LOD one day after recruitment to the study. The PGE_2 concentrations one day after recruitment for all cases (n=69) was used to compare to the concentrations in the 16 cases that also had meloxicam concentrations measured. In the cases that were treated with antibiotics, 66.7% (14/21) had PGE_2 concentrations below the LOD one day after recruitment to the study, with the mean (SE) for the log PGE_2 concentrations one day after recruitment to the study being 0.05 (0.03). In cases that were treated with antibiotics and meloxicam, 72.9% (35/48) had PGE_2 concentrations below the LOD one day after

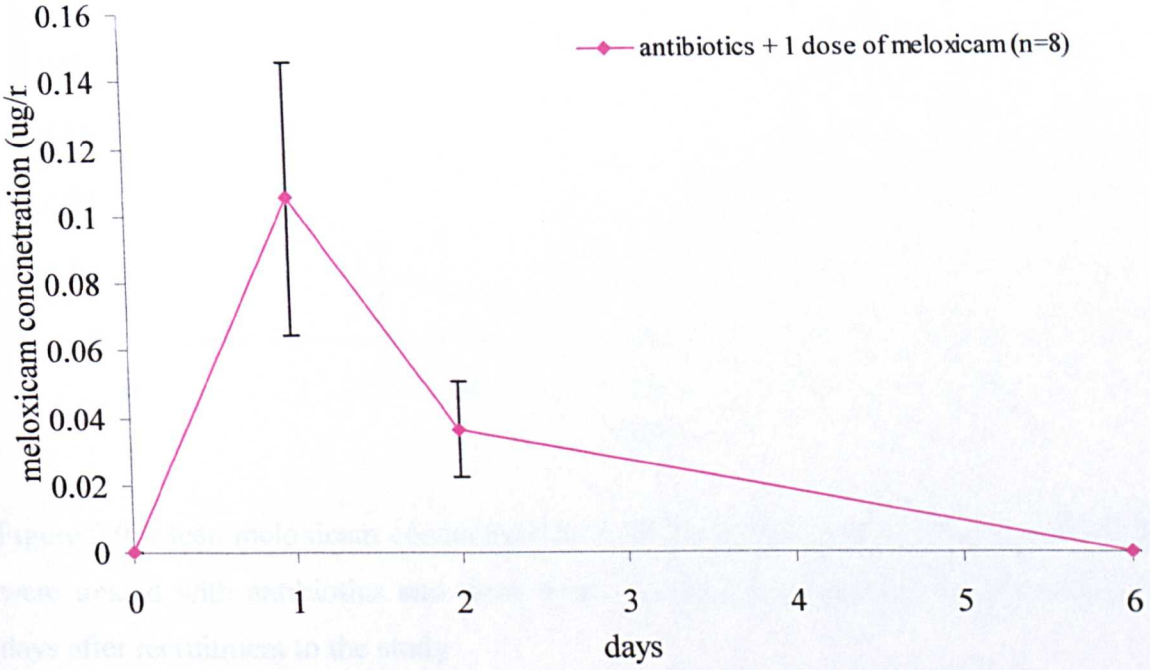


Figure 5.8: Mean meloxicam concentrations ± SE for eight cases of clinical mastitis that were treated with antibiotics and one dose of meloxicam (group 2) followed for six days after recruitment to the study

Day/Case ID	1	2	3	4	5	6	7	8	Mean ± SD
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00
1	0.10	0.08	0.38	0.10	0.07	0.05	0.03	0.03	0.11 ± 0.12
2	0.00	0.00	0.10	0.01	0.08	0.01	0.02	0.07	0.04 ± 0.04
6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00

Table 5.4: Meloxicam concentrations (µg/ml) in milk from eight cows treated with antibiotics and one dose of meloxicam (group 2) administered on day 0. Milk samples were taken prior to the administration of meloxicam

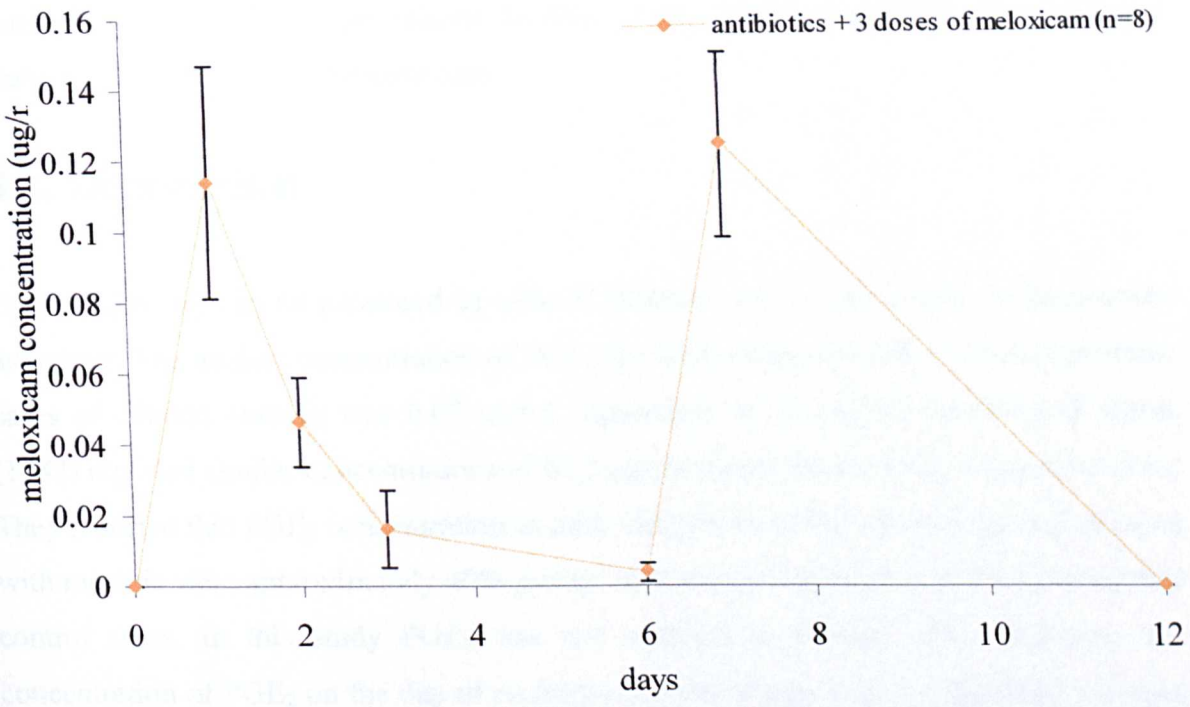


Figure 5.9: Mean meloxicam concentrations ± SE for eight cases of clinical mastitis that were treated with antibiotics and three doses of meloxicam (group 3) followed for 12 days after recruitment to the study

Day/Case ID	9	10	11	12	13	14	15	16	Mean ± SD
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00
1	0.14	0.08	0.19	0.08	0.01	0.28	0.13	0.00	0.11 ± 0.09
2	0.01	0.02	0.05	0.06	0.01	0.05	0.12	0.03	0.04 ± 0.04
3	0.00	0.01	0.00	0.00	0.00	0.01	0.09	0.02	0.02 ± 0.03
6	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00 ± 0.00
7	0.05	0.07	0.12	0.08	0.06	0.16	0.25	0.10	0.11 ± 0.07
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00 ± 0.00

Table 5.5: Meloxicam concentrations (µg/ml) in milk from eight cows treated with antibiotics and three doses of meloxicam (group 3) administered on day 0, 3 and 6. Milk samples were taken prior to administration of meloxicam

recruitment to the study, with the mean (SE) for the log PGE_2 concentrations one day after recruitment to the study being 0.04 (0.01). There was no significant difference between the log PGE_2 concentrations for cases treated with antibiotics only and those treated with antibiotics and meloxicam.

5.4. Discussion

Prostaglandin E_2 can be measured in milk of mastitic cows using a radio-immunoassay technique. The median concentration of PGE_2 for affected quarters of mild and moderate cases of clinical mastitis was 0.07 ng/ml, equivalent to 70 pg/ml. Atroshi and others (1987) reported similar concentrations of 61.3 pg/ml (mean) in the milk of mastitic cows. They reported that PGE_2 concentration in milk samples from the affected quarter of cows with mastitis were approximately 40% greater than the concentrations of PGE_2 in normal control cows. In this study PGE_2 was not assessed in normal cows, however, the concentration of PGE_2 on the day of recruitment to the study was significantly increased in moderate cases of mastitis compared to mild cases. Atroshi and others (1987) reported a significant correlation between somatic cell count and PGE_2, however, the samples that were selected for measurement of PG concentration were selected based on having somatic cell count $<120 \times 10^3$/ml for 'normal' and somatic cell count $>500 \times 10^3$/ml for 'mastitic'. Other arachidonic acid metabolites, such as prostaglandin $F_{2\alpha}$ and thromboxane B_2, have been measured in milk of cows with clinical mastitis (Anderson and others 1985; Atroshi and others 1987). These mediators were also increased in cases of mastitis compared to normal cows, Anderson and others (1985) reported that the increase in the concentrations of prostaglandin $F_{2\alpha}$ and thromboxane B_2 were positively correlated with the clinical severity of mastitis.

The sensitivity of using PGE_2 concentrations to assess whether clinical mastitis was moderate in severity was very poor (36%) but the specificity was good (86%). From this study approximately four in ten cases of clinical were moderate rather than mild in severity. If the PGE_2 concentration detected the case as being moderate in severity, then it has a 63% chance of being truly moderate, and conversely if the PGE_2 concentration detected the case as not being moderate in severity it had a 66% chance of being truly moderate, thus the use of PGE_2 concentration has the potential to misclassify one third of cases.

The concentrations of PGE_2 decreased for mild and moderate cases over the two days following diagnosis and treatment of clinical mastitis. A treatment effect on the concentrations of PGE_2 was not detected in this study, but this may have been due to the frequency of sampling. A small experimental study by Banting and others (2000) reported a significant treatment effect of meloxicam on the concentrations of thromboxane B_2 in milk, where the concentrations of thromboxane B_2 in the meloxicam treated group were significantly lower than in the control group. Lees and others (1991) reported that the subcutaneous exudate concentration of PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 were significantly ($p<0.05$) reduced by meloxicam, in an experimental model in the horse. Both these studies monitored the concentrations of the eicosanoids every two hours after treatment administration. The PGs have very short half lives and some of the more potent PGs are very unstable (Higgins and Lees 1984). In experimental studies in dogs after injection of endotoxin the PGE_2 response was rapid, levels increased within two minutes in one study (Kessler and others 1973) and 30 minutes in another study (Fletcher and Ramwell 1977). In humans, injection of PGE_1 and PGE_2 induced local oedema and redness with the maximum reaction within 15-30 minutes and subsidence of the reaction after 1-2 hours (Crunkhorn and Willis 1969). The value of measuring PGE_2 concentration in milk of mastitic cows might be increased with more frequent sampling.

The different pathogenesis of microbiological causes of bovine clinical mastitis leads to differing degrees of invasion and destruction of the mammary gland depending on the causal agent and the strain of the pathogen. Approximately 80% of *Staph aureus* intramammary infections cause subclinical mastitis, and when they result in clinical infection the signs are mainly mild (Vestweber and Leipold 1993). In contrast, 80-90% of intramammary infections with *Escherichia coli* result in clinical mastitis (Smith and others 1985) with 10-15% causing severe toxic cases (Tadich and others 1998). This study did not include severe cases of clinical mastitis, however, it might be expected that those would have even greater concentrations of PGE_2 compared to cases of moderate clinical mastitis. The mean, median and range of concentrations of prostaglandin E_2 in clinical cases due to the environmental organisms, such as *S uberis*, *E coli* and *A pyogenes*, were greater than clinical cases due to *Staph aureus*. The log prostaglandin E_2 concentrations in mastitis due to *Staph aureus* were more similar, i.e. they had a narrower range and had lower mean and median values than the cases of mastitis with environmental organisms and cases from which no growth were detected. There was no

significant difference among the microbiological groupings for the mastitis organisms and the concentration of PGE_2 in this study but a trend was seen and perhaps with a larger dataset a significant result may be seen.

In this study, correlation with the concentrations of meloxicam and PGE_2 one day after treatment was administered was not possible due to the majority of cases having PGE_2 concentrations below the LOD. More frequent sampling might allow examination for a correlation between meloxicam concentrations and PGE_2 concentrations.

Meloxicam is reported to have a half-life of 17.5 hours after subcutaneous injection in lactating cattle (Anon 2003). Non-steroidal anti-inflammatory drugs are highly protein bound, >99% in some cases. This extent of protein binding explains the extended therapeutic activity in inflamed tissues which these drugs display relative to their plasma elimination half-life (Nolan 2000). An experimental study reported that the administration of meloxicam 50 hours before challenge with *E coli* endotoxin inhibited the rise in thromboxane A_2 and prevented severe adverse clinical signs of endotoxaemia, thus indicating a long duration of action. In this study the results of the meloxicam assays in treatment group 3 (antibiotics and three doses of meloxicam) demonstrated that the concentrations of meloxicam in milk were below the LOD in 50% (4/8 day three) to 88% (7/8 day six) of cases three days after treatment was administered.

Prostaglandin E_2 acts as a typical mediator of inflammation, and could, as this study and previous studies by Atroshi and others (1987) suggested, be used as a marker for mastitis and mastitis severity. Non-steriodal anti-inflammatory drugs could be targeted at the cases with higher concentrations of PGE_2. Further studies should be undertaken to assess the effect of meloxicam on the concentrations of PGE_2 in mastitic cows with more frequent sampling following the administration of the meloxicam.

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Chapter 6

General discussion and conclusions

6.1. Overview

Mastitis is a common disease of dairy cows, with the UK national incidence estimated to be 35-40 cases per 100 cows per year (Booth 1997; Whitaker and others 2004). Mastitis causes a considerable economic impact (Wilson and Kingwill 1975; Esslemont and Peeler 1993; Hortet and Seegers 1998; Yalcin and others 1999) and has also significant welfare implications. It was reported by Esslemont and Kossaibati (1997) that mastitis accounted for over 10% of premature disposals from dairy farms in England and that mastitis was the most common cause of death in adult dairy cows (Menzies and others 1995; Esslemont and Kossaibati 1997). Over the last decade, partly as a consequence of the BSE crisis and foot and mouth outbreak, the Government has increasing concerns about the health and welfare of livestock. This has lead to the development of an 'Animal Health and Welfare Strategy', which aims to manage the impact of animal disease and improve the welfare of animals kept by man, whilst protecting the economic and social well-being of people and the environment. The framework of this initiative is to promote disease prevention and provide appropriate care to allow animals to produce higher yields and remain productive for a longer period of time. Traditional methods for disease detection rely on visual observation, however, increasing herd sizes and reduced manpower (Anon 2004) has resulted in a shortage of available skilled labour and increasing reliance on mechanised systems. Clinical mastitis is characterised by grossly abnormal milk and was traditionally detected by visual observation of the milk. Due to less time per individual cow being available, many farmers do not examine the foremilk despite this being a legal requirement (A.M. Biggs personal communication). The need for automated systems to monitor animal health and fertility has been recognised both for

economic and welfare reasons and there has been increasing interest in the use of biosensors (Mottram and others 2002).

6.2. Study objectives

This study was an intervention study following naturally occurring clinical mastitis cases over a six week period and aimed to assess the value of physiological and laboratory parameters as indicators of severity of clinical mastitis and pain possibly associated with the disease, and to examine the impact of the use of analgesic drugs in combination with intramammary antimicrobial infusion. The ability to assess clinical severity of mastitis may allow the use of more appropriate treatment for mastitis, while the use of some physiological and laboratory parameters as indicators of the presence of disease could have value in automated milking systems.

6.3. Study design

One hundred and seventeen cases of clinical mastitis were recruited into the study, however, only 70 cases were followed for the complete six week period. Forty seven cases were dropped from the study due to the re-occurrence of mastitis in the same quarter, development of mastitis in another quarter, or the development of lameness, which again highlights the prevalence of mastitis in dairy cows.

Ideally the trial would have been double-blinded to minimise observer and reporter bias, however, owing to the considerable difference between the regimens an open design was unavoidable. Random allocation of treatment groups and random selection of the initial leg for the measurement of mechanical threshold helped to minimise bias.

The antimicrobial product used in the trial, cefquinome (Cephaguard LC intramammary, Intervet UK limited, Milton Keynes), is a third generation cephalosporin preparation. This product was selected for its broad spectrum of activity and due to the absence of any corticosteroid. Corticosteroids have anti-inflammatory properties and are often used in intramammary preparations to reduce the inflammation in the udder tissue (McKellar and others 1991). A preparation containing corticosteriod would confound the effect of NSAID. A parenteral preparation of NSAID was selected as a previous study by

Fitzpatrick and others (1998) reported that a single intravenous dose of NSAID reduced the alterations in nociceptive pathways to mechanical stimulation but a specially prepared intramammary infusion containing NSAID was not effective in reducing these alterations in cows with clinical mastitis. Repeat dosing of NSAID to give a longer duration of analgesia was also carried out in this study as Fitzpatrick and others (1998) reported that the single intravenous dose of NSAID reduced the alterations in pain processing but only over a one day period after drug administration.

Although all cases were observed walking by the researcher on every sampling day, bilateral subclinical lameness could have been overlooked and could have interfered with clinical and laboratory results. In future projects lifting each hindleg in turn and paring the top surface of the solar horn off to allow examination for lesions could reduce the risk of recruiting cases of clinical mastitis that also had bilateral subclinical lameness.

In this current study it was not possible to assess if there was a breed effect on any of the physiological or laboratory parameters, as most farms consisted principally of one breed and breeds differed among farms. It was likely that management differences rather than the breed could explain any variation in physiological and laboratory parameters among farm.

The basis of the pain assessment was the use of mechanical pain threshold testing. Pain threshold testing is the application of a noxious stimulus such that physiological pain is induced and the response to the stimulation can be measured and quantified. This technique has been used reliably in lameness studies in sheep (Nolan and others 1987; Ley and others 1989) and cattle (Whay and others 1996) and in a previous study of mastitis in dairy cattle (Fitzpatrick and others 1998).

6.4. Physiological parameters

The heart rate, respiratory rate and rectal temperature of cases with moderate clinical mastitis were significantly higher than those in mild cases of clinical mastitis. These parameters were assessed for usefulness as predictors of severity of clinical mastitis and the sensitivities and specificities of categorising clinical mastitis as moderate in severity using the heart rate, respiratory rate and rectal temperature were 48%, 66% and 59%, and

73%, 68% and 68%, respectively. As predictors of disease these parameters had similar sensitivities of 60%, 46% and 50%, respectively. These values would mean that approximately 40-50% of cases would be misclassified. The hindleg stance of cows with clinical mastitis was different from normal cows ($p<0.001$). Cases of clinical mastitis were shown to stand with their hindlegs further apart than normal cows and as a predictor of clinical mastitis, hock-to-hock distance had a sensitivity of 64% and specificity of 84%. These physiological parameters may act as additional tools with the aim of aiding in the decision to administer anti-inflammatory and/or analgesics to the cases with more severe mastitis.

The results from the mechanical threshold testing demonstrated that alterations in the response to mechanical stimulation were a feature of acute clinical mastitis in dairy cows. There was no evidence of adaptation to repeated use of the mechanical threshold device, as demonstrated by the normal animals, which reacted at similar thresholds (26.5±1.60 kPa) for the six week study duration. Whay and others (1996) also reported no effect of time on the response of normal cattle to a mechanical nociceptive stimulus.

Whay and others (1997) reported that lame cows demonstrated increased sensitisation to mechanical stimulation with increasing severity of lameness, as judged by a numerical rating scale. In the current study the threshold difference on the day of recruitment to the study was higher in moderate cases of mastitis compared to mild cases, but was not significant. In a larger dataset this trend might become significant, such that an alteration in the nociceptive pathways in response to noxious stimulation might be seen with increasing severities of mastitis. Whay and others (1998) demonstrated that the duration of hypersensitivity was different for different types of lesions in dairy cattle lameness. They reported that cows with solar ulcers and white line disease displayed hypersensitivity for a minimum of 28 days. In this study alteration in response to mechanical stimulation lasted for between six and 45 days; we could not demonstrate that alteration in response to mechanical stimulation lasts for different durations depending on the causal pathogen or duration of infection, but this may be due to the limited power of the study.

Normal cows reacted at similar threshold levels to the ipsi-lateral leg in cases of mastitis and the threshold measurements of the contra-lateral leg in the cases of mastitis were

significantly higher than in normal cows. This may be because the threshold to stimulation is truly higher in the contra-lateral leg, or because cows are reluctant to shift weight onto the ipsi-lateral leg. If the threshold to stimulation is higher in the contra-lateral leg than in normal animals, it would be more accurate to describe the phenomenon seen as hypoalgesia or hyposensitivity of the contra-lateral leg rather than hyperalgesia or hypersensitivity of the ipsi-lateral leg. This phenomenon is likely to be due to the complexity of the innervation of the mammary gland and indicates that the alterations in pain processing in mastitis is harder to explain than those seen in lame animals.

Non-steroidal anti-inflammatory drugs are the most widely available analgesics for cattle in the UK and are used for their anti-inflammatory, antipyretic, anti-endotoxic and analgesic properties. The use of meloxicam, a NSAID, had a significant beneficial effect on the pain threshold levels in cows with mild and moderate clinical mastitis. We did not demonstrate a significant positive effect of NSAID treatment on the milk yield of cases of clinical mastitis, however the limited results from this study did suggest a potential yield benefit. This is in contrast to a study by Shpigel and others (1994) who showed a significant effect of the use of NSAID on the milk yield.

6.5. Laboratory parameters

The microbiological results from the present study showed that the most prevalent isolates from clinical mastitis cases were *S uberis* (30%), *E coli* (22%), *Staph aureus* (11%), other microbiological isolates (6%), *A pyogenes* (4%) and *S dysgalactiae* (3%). No growth was seen in 24% of submitted samples. These results are similar to those of other studies (Anon 1982; Wilesmith and others 1986; Erskine and others 1988; Anon 1989; Anon 1993; Booth 1993; Anon 2000; Bradley and Green 2001; Milne and others 2002), that environmental pathogens, including *S uberis* and *E coli*, are the most commonly isolated pathogens from clinical cases of mastitis in dairy cows. Morin and others (1998) consider that clinical signs alone are inadequate to differentiate between clinical mastitis caused by Gram-positive and Gram-negative bacteria, however, Milne and others (2003) reported that certain clinical signs were associated with specific pathogen types. This study reports significant associations between microbiological results and physiological and laboratory parameters. The isolation of *Staph aureus* was more likely in mild cases, no growth more likely in cases with relatively low IQSCC, *S*

uberis isolation more likely in moderate cases with relatively high IQSCC and *E coli* more likely in cases with higher heart rates.

Somatic cell counts may be used to estimate the degree of inflammation within the udder of a cow and hence the presence of mastitis (Dohoo and Meek 1982; Cullor and Tyler 1996; Sears and others 1993). Data from quarter samples provides the most accurate assessment of the prevalence of infection in the herd, as IQSCC will not be affected by the dilution of low SCC milk from non-infected quarters of the same cow in an ICSCC or of all cows in a BMSCC (Reneau 1986). In this study IQSCC were significantly increased in the affected quarters and the contra-lateral quarters of cases of mild and moderate clinical mastitis on the day of recruitment into the study compared to normal cows. Hillerton (1997) reported that the IQSCC for clinical infections with *Staph aureus* could be 4 million cells/ml and for clinical infections with *S uberis* or coliforms the IQSCC could be 20 million cells/ml. In this study the median IQSCC for the affected quarter in mild and moderate cases of clinical mastitis was 10 million cells/ml. The IQSCC for the affected and diagonally opposite quarters of cases with mild or moderate clinical mastitis decreased over the 45 day study period, however, they were still significantly higher than the normal cows at the end of the 45 day study period. There was no significant difference between the affected quarters and the diagonally opposite quarters of mild or moderate cases of clinical mastitis. This suggests that IQSCC for the affected quarters reached 'normal' levels for that cow, although the IQSCC for the affected quarter of cases of clinical mastitis were still significantly different to normal animals by day 45 after recruitment to the study. There was no information on the IQSCC or ICSCC prior to recruitment into the study and it is possible that normal cows could have had lower ICSCC than the cows that subsequently developed mastitis.

The milk composition was altered due to clinical mastitis, protein content was increased and lactose content was decreased. Other studies have shown decreases in fat (Houben and others 1993) and protein content (Myllys and Rautala 1995), therefore suggesting that the milk composition is not a reliable indicator of mastitis.

The differential cell count of the milk samples did vary considerably over the 45 day study period, however there was no difference in the cell proportions between mild and moderate cases and between the cases that completed and those that did not complete the

study. In cases of clinical mastitis the predominant cell type was PMNs and over the 45 day period the proportion decreased with the proportion of macrophages and lymphocytes doubling. The differential cell counts were not useful predictors of severity of disease or likely outcome.

In contrast APP concentrations have been shown to be useful indicators of mastitis. The concentration of Hp in milk increased with increased severity of clinical mastitis - a phenomenon that was also reported in respiratory disease of calves (Godson and others 1996). Mammary-associated serum amyloid A was significantly increased in clinical mastitis and its local production (Vreugdenhill and others 1999; McDonald and others 2001) makes it a very specific indicator of mastitis. The concentration of M-SAA, however, did not increase with increasing severity of mastitis on the day of diagnosis and many cases of mastitis had low concentrations of M-SAA on the day of recruitment to the study. This result contradicts the findings of Nielsen and others (2004), who reported that M-SAA concentrations increased with increasing CMT score. In this study the ability of Hp and M-SAA to differentiate between mild and moderate severities was investigated. The sensitivity and specificity of differentiating mild clinical mastitis from moderate clinical mastitis using Hp were 68% and 68%, respectively and using M-SAA concentrations the sensitivity and specificity were 63% and 36%, respectively. These results confirm that in this dataset Hp concentrations were more useful than M-SAA concentrations in distinguishing between mild and moderate clinical mastitis.

Prostaglandins are recognised mediators of inflammation that are released via the arachidonic acid pathway as a consequence of tissue damage and/or inflammation (Xie and others 1992). Previous studies have demonstrated an increase in eicosanoid concentration in bovine mastitis (Anderson and others 1985; Atroshi and others 1987). In this study PGE_2 concentrations were measured in milk with concentrations ranging from 0 – 10.2 ng/ml for mild and moderate cases of clinical mastitis on the day of recruitment to the study. The concentrations significantly increased with increasing severity of the mastitis. The sensitivity and specificity of differentiating between mild and moderate cases of clinical mastitis using PGE_2 concentration were 85% and 36%, respectively.

Meloxicam concentrations were measured over a six day period in eight cows that were treated with meloxicam on the day of recruitment and over a 12 day period in another

eight cows that were treated with meloxicam on the day of recruitment, three and six days after recruitment. The kinetics of meloxicam followed a similar pattern for all 16 cows; the peak concentration occurred one day after administration and there was a rapid decline to less than half the peak concentration by two days after administration. The current milk withdrawal period following parenteral administration is five days, however, the results from this study suggest that concentrations of meloxicam were below the limit of detection in 50-88% of the cows three days after administration.

6.6. Future direction

A non-invasive method to detect the daily variations in body temperature of a cow and the assessment of the hock-to-hock distance by use of laser or infra-red technology would be useful tools for automated systems. These technologies could be used to recognise early signs of disease and improve the welfare of dairy cows. They could also be used to tailor and monitor therapy.

The theory that cows with unilateral mastitis are reluctant to shift weight onto the ipsi-lateral leg could be tested using pressure plates placed underneath the feet of the cow. The use of pressure plates could well be easier to perform routinely and be more user friendly than the current mechanical threshold device. Pressure plate devices could be adapted for use in milking parlour and would especially be suited to robotic milking systems.

The mechanism behind the alterations in the threshold measurements in cows with clinical mastitis needs to be examined further to explain the findings of hypoalgesia in the contra-lateral leg and the fact that the ipsi-lateral leg responds at a similar threshold level to normal animals.

Further studies examining the effect on milk yield could be undertaken. In addition, the monitoring of feed intake might also serve as a good indicator of a positive effect of NSAID.

Investigations into the cell type that is responsible for the local production of M-SAA could be performed.

6.7. Conclusions

Reduced manpower on UK farms often means that dairymen have less time to spend on individual cows in the parlour and there is increasing reliance on automated systems to detect mastitis. It is necessary to have a rapid and accurate means of detection clinical mastitis, as the milking of mastitic milk into the bulk milk tank will result in wide fluctuations in the total bacterial count (TBC) or bactoscan (Davidson and Jackson 1984) and could lead to the BMSCC exceeding >400,000 cells/ml and this has financial implications. The European Community Health and Hygiene Directive 92/46, detailing standards for the hygienic quality of milk intended for human consumption, requires bulk milk to have a TBC < 100,000 bacteria/ml and SCC < 400,000 cells/ml.

The ability to differentiate between mild and moderate cases of clinical mastitis is improved when the physiological parameters, heart rate, respiratory rate and rectal temperature, were combined with the laboratory parameters, Hp and PGE_2. There is approximately a thirteen times increased likelihood that the clinical mastitis is mild in severity if lower values and concentrations for these parameters are present. There is approximately a five times increased likelihood that the clinical mastitis is mild in severity when using low values of rectal temperature, and low concentrations of PGE_2 and Hp as predictors of severity. The development of non-invasive techniques to assess body temperature and concentrations of PGE_2 and Hp that could be adopted into automated systems would be useful to allow more severe cases of clinical mastitis to receive more specific treatment.

In summary, the combined use of physiological, mechanical threshold stimulation testing and laboratory parameters has been shown to useful in differentiating between clinical cases of mastitis and normal animals, and mild and moderate clinical mastitis. These parameters could have potential value as indicators to allow therapy to be targeted as appropriate to the severity of the mastitis. Some of these parameters will also increase the available armoury to identify disease and monitor the health and welfare of dairy cows in automated milking systems. Importantly, the use of meloxicam produced a demonstrable reduction in the duration of the alterations in response to mechanical stimulation in cases of mild and moderate clinical mastitis. The routine use of NSAID in cases of clinical

mastitis would serve to increase animal comfort and welfare and increased usage should be encouraged.

Appendix 1

Exclusion criteria

A case of clinical mastitis will be excluded from the project if;

- The cow is systemically ill on any of the sampling days, or
- The cow is lame during the sampling period
- Has a concurrent inflammatory condition, e.g. metritis, endometritis, retained foetal membranes, septic arthritis, pneumonia, endocarditis, gastro-enteritis, other any other focal sepsis or inflammation including ear tagging, tattooing or freeze branding, on any of the sampling days
- Has had previous incidents of mastitis in the same quarter in the same lactation period that have not responded to treatment
- The cow has mastitis in two or more quarters
- The cow is to be 'dried off' in less than eight weeks time
- Mastitis recurs in the affected quarter or any other quarter of the cow during the sampling period
- The cow displays an adverse reaction to the treatments
- The cow is difficult to handle
- The cow is sold or dies during the sampling period
- Dry cow therapy is administered to the cow during the sampling period

Appendix 2

Random numbers – treatment allocation

Tx 1	Tx 2	Tx 3
2	3	1
9	4	5
10	7	6
16	12	8
17	14	11
21	19	13
22	25	15
23	27	18
26	31	20
28	34	24
30	40	29
32	41	35
33	42	39
36	47	43
37	48	44
38	49	45
52	51	46
53	56	50
54	59	55
60	61	57
63	65	58
64	73	62
66	75	68
67	80	69
79	81	70
82	85	71
86	88	72
90	89	74
91	95	76
93	96	77
94	99	78
97	103	83
98	104	84
101	106	87
102	107	92
105	109	100
111	110	108
	112	

Appendix 3

Aseptic milk collection- instructions for farmers

It is essential that **clean** milk samples are obtained to ensure that the correct cause of mastitis is identified in the laboratory.

Milk samples for diagnosis of mastitis must be taken before any treatment is given.

Please ensure that the person who takes the milk sample follows the procedure below.

1. Prepare all teats as for routine milking (e.g. wash, dry).

2. Use cotton wool soaked in disinfectant to clean all teats. Use a clean piece of wool for each teat. Concentrate especially on the teat end – make sure it is as clean as possible.

3. Draw out a few steaks of milk and discard these.

4. Use another clean piece of cotton wool soaked in disinfectant to rub **only** the teat end.

5. Put on gloves.

6. Draw milk into the container. Hold the container at an angle so that dust/dirt does not fall into it. Only fill the container to the half-way mark. Do not touch the end of the contained with your fingers. Keep the cap of the container clean. Replace the cap.

7. Label the tube with the date, cow number and quarter affected.

Appendix 4

Bovine Haptoglobin Assay (Tridelta Development Ltd, Ireland)

Assay principle

The haptoglobin assay is designed to detect the acute phase protein in milk and serum. The haptoglobin assay kit is a solid phase Enzyme Linked Immuno Sorbent Assay (ELISA). In this assay haemoglobin is coated onto microtitre plates which binds the free haptoglobin in the well. Samples, including standards of known haptoglobin content, are added into the wells and allowed to incubate. Any haptoglobin present in the well is captured onto the plate by the haemoglobin. The microwells are then washed to remove any unbound haptoglobin. A HRP-labelled anti- haptoglobin monoclonal antibody is then added to the well, which will bind to any haptoglobin present, and allowed to incubate. After washing for a second time TMB substrate solution is added. The intensity of the colour produced is proportional to the concentration of haptoglobin present in the original specimen.

Sample and reagent preparation

Samples – Milk

Milk samples should be collected into suitable vials. Samples should be diluted 1/20 in 1× calibrator/sample diluent prior to testing. Vortex samples prior to dilution.

All samples can be stored for up to 24 hours at 2-8°C. For longer periods, storage at -20°C is recommended where samples are not for immediate assay. If specimens have levels of haptoglobin greater then the top standard, they should be diluted out further in the calibrator/sample diluent and retested.

Wash concentrate (20×)

Dilute 1 volume of wash buffer concentrate (20×) in 19 volumes of distilled water. Store both the wash buffer and working buffer (1×) in the refrigerator. Diluted wash buffer is stable for up to 2 weeks when stored at 4°C.

Diluent buffer (10×)

Dilute 1 volume of diluent buffer concentrate (10×) in 9 volumes of distilled water. Store both the diluent buffer concentrate and working buffer (1×) in the refrigerator. Diluted diluent buffer is stable for up to 24 hours when stored at 4°C.

Calibrator

Dilute calibrator 1/1000 in calibrator sample diluent (1×) 10µl in 10ml. This is solution A.

Further dilute this to a final concentration of 1/4000 by adding 1ml of solution A to 3ml of calibrator/sample diluent. This is solution B.

It is recommended that either solution A or solution B is aliquoted and stored frozen for future use.

Set up the standard curve as follows:

Tube No.	Volume Calibrator	Volume Diluent	Tube Concentration
C1	250µl Solution B	-	500ng/ml
C2	250µl Solution B	250µl	250ng/ml
C3	250µl C2	250µl	125ng/ml
C4	250µl C3	250µl	62.5ng/ml
C5	250µl C4	250µl	31.3ng/ml
C6	250µl C5	250µl	15.6ng/ml
C7	-	250µl	0mg/ml

Prepared calibrators are stable for 8 hours when stored at room temperature (20-25°C).

Procedure

1. Determine the number of 8-well strips needed for the assay. Re-bag extra strips, seal bag and store in refrigerator.
2. Vortex the serum or milk samples to be examined in the test. Dilute the serum or milk as directed in the samples preparation. Add 100µl, in duplicate, of diluted sample or standard to each well.
3. Cover the plate with dust cover and incubate plate for **1 hour at 37°C.**
4. After incubation aspirate or decant and wash the plate four times with diluted wash buffer. After the last wash, tap the plate dry on absorbant paper.
5. Add 100µl of conjugate to each well.
6. Cover the plate with dust cover and incubate for **1 hour at 37°C.**
7. After incubation aspirate or decant and wash the plate four times with diluted wash buffer. After the last wash, tap the plate dry on absorbant paper.
8. Add 100µl of TMB substrate.
9. Cover the plate and incubate in the dark at **room temperature for 15 minutes.**
10. Add 100µl of the stop solution.
11. Read the absorbance of each well at 450nm using 630nm as reference, if available. Alternatively blank the plate reader against a TMB blank composed of 100µl of TMB substrate and 100µl of stop solution.

Interpretation of test results

1. Calculate the mean absorbance for each sample, control or standard.
2. Plot the absorbance of the standard concentrations on standard graph paper. (If necessary, the background absorbance for the 0ng/ml may be subtracted from each of the data points, including the standards, unknowns and controls prior to plotting). Draw the best smooth curve through these points to construct the standard curve.
3. Determine the concentrations of the test samples and controls from the standard curve by multiplying the interpolated value by the appropriate dilution factor (eg. milk samples diluted 1/20 should be multiplied by 20). Samples that have a signal greater than the highest standard should be further diluted in diluent buffer and re-analysed.

Phase™ range Serum Amyloid A Assay (Tridelta Development Ltd, Ireland)

Assay principle
The Serum Amyloid A assay is designed to detect the acute phase protein in serum and plasma. The test can also be used to detect SAA in milk, cell culture medium, and in other biological fluids. The SAA assay kit is a solid phase sandwich Enzyme Linked Immuno Sorbent Assay (ELISA). A monoclonal antibody specific for SAA has been coated onto the wells of the microtitre strips provided. Samples, including standards of known SAA content, are added into microwells along with biotinylated ant- SAA monoclonal natibody. Any SAA present in the well is both captured on the plate by the immobilized antibody and labelled with the conjugate antibody in a one step procedure. After washing to remove all of the unbound material, Streptavidin-Horse Radish Peroxidase conjugate is added to the well and incubated. Following the second incubation, TMB substrate solution is added. The intensity of the colour produced is proportional to the concentration of SAA present in the original specimen.

Sample and reagent preparation
Samples – Milk
Milk samples should be collected into suitable vials. Samples should be vigorously vortexed to assure accurate determination of SAA concentration. Samples should be diluted 1/50 in diluent buffer prior to testing.
All samples can be stored for up to 2 days at 4°C or stored frozen at -20°C for longer periods. If specimens have levels of haptoglobin greater then the top standard, they should be diluted out further in the calibrator/sample diluent and retested.

Diluent buffer (10×)
Dilute 1 volume of diluent buffer concentrate (10×) in 9 volumes of distilled water. Prepared reagent is stable only for one day at room temperature and should not be stored for extended periods.

Wash buffer (20×)
Dilute 1 volume of wash buffer concentrate (20×) in 19 volumes of distilled water. Store both the wash buffer and working buffer (1×) in the refrigerator. Diluted wash buffer is stable for up to 2 weeks when stored at 4°C.

NOTE: Ensure that any crystals that may have developed in the wash buffer or diluent have been completely dissolved prior to dilution for use.

Biotinylated anti-SAA (All biological fluids)
Dilute 1/100 in 1× diluent buffer. Prepare appropriate volume of the diluted conjugate depending on the number of strips used:
50µl of conjugate is used for each well (400µl/strip). Discared any excess diluted conjugate.

Streptavidin-HRP

Dilute the streptavidin peroxidase to a 1/4000 final dilution by adding 5µl of the stock solution to 20ml of 1× diluent buffer. At 100µl per well this is sufficient for twelve 8-well strips. Dilute only as much conjugate a will be used in the assay and return the excess streptavidin-HRP stock to the refrigerator. Discard any excess diluted conjugate.

Dilution of SAA standards

1. Reconstitute the standards by adding 200µl of distilled water to the vial. Vortex vigorously to dissolve completely.
2. Label 6 tubes C1 to C6. Add diluent to each tube as directed in Table 1. To tube C1 add 60µl of reconstituted standard from step 1 to 240µl diluent (see table 1). This is further diluted two-fold serially 4 times (tubes C2 to C5) to provide the working standards as indicated in table 1 below. Tube C6 contains diluent only. Note: The top standard and subsequent values will vary depending on the species under investigation. The working ranges for each species are provided in Table 2. Excess reconstituted standard can be stored at –20 °C.

Tube No.	Volume of calibrator	Volume of diluent	Serial dilution
C1	60µl	240µl	-
C2	-	150µl	150µl C1
C3	-	150µl	150µl C2
C4	-	150µl	150µl C3
C5	-	150µl	150µl C4
C6	-	150µl	-

Table 1: Preparation of working standard curves

Standards	Bovine (ng/ml)	Porcine (ng/ml)	Canine (ng/ml)	Human (ng/ml)
C1	300	2000	160	200
C2	150	1000	80	100
C3	75	500	40	50
C4	37.5	250	20	25
C5	18.8	125	10	12.5
C6	0	0	0	0

Table 2: Concentration of calibrators or serum, plasma or milk

Procedure

Allow test reagents and samples to reach room temperature before use.

1. Determine the number of 8-well strips needed for the assay. Re-bag extra strips, seal bag and store in refrigerator.
2. Add 50µl of diluted Biotinylated anti-SAA to each well.
3. Vortex the serum, plasma or milk samples to be examined in the test. Dilute the serum and plasma 1/500 and milk samples 1/50 in 1× diluent buffer. Add 50µl, in duplicate, of diluted sample or standard to each well. Tap sides of the plate gently to mix.
4. Cover the plate with dust cover and incubate plate for **1 hour at 37°C.**
5. After incubation aspirate or decant and wash the plate four times with diluted wash buffer. After the last wash, tap the plate dry on absorbant paper.
6. Add 100µl of streptavidin-peroxidase to each of the wells.

7. Cover the plate with dust cover and incubate in the dark at **room temperature for 30 minutes.**
8. After incubation aspirate or decant and wash the plate four times with diluted wash buffer. After the last wash, tap the plate dry on absorbant paper.
9. Add 100µl of TMB substrate.
10. Cover the plate and incubate in the dark at **room temperature for 30 minutes.**
11. Add 50µl of the stop solution.
12. Read the absorbance of each well at 450nm using 630nm as reference, if available. Alternatively blank the plate reader against a chromogen blank composed of 100µl of TMB substrate and 50µl of stop solution.

Interpretation of test results

4. Calculate the mean absorbance for each sample, control or standard.
5. Plot the absorbance of the standard concentrations on semi-logarithmic or standard graph paper. (If necessary, the background absorbance for the 0ng/ml may be subtracted from each of the data points, including the standards, unknowns and controls prior to plotting). Draw the best smooth curve through these points to construct the standard curve.
6. Determine the concentrations of the test samples and controls from the standard curve by multiplying the interpolated value by the appropriate dilution factor (eg. serum and plasma diluted by 1/500 should be multiplied by 500). Samples that have a signal greater than the highest standard should be further diluted in diluent buffer and re-analysed.

Appendix 5

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	Mild (n=56)	Range	Moderate (n=61)	Range
Age (Years)	5.95 (0.22)	3-10	5.40 (0.27)	2-11
Parity	3.82 (0.23)	1-7	3.25 (0.24)	1-9
Days calved	109 (38-163)	0-438	79 (11-159)	3-376
Milk yield (litres)[#]	31.1 (1.70) (n = 25)	15-48	33.9 (1.85) (n = 18)	22-55
Body condition score	2.0 (1.5-2.5)	1.0-4.0	2.0 (1.5-2.5)	0.5-5.0
Heart rate (beats/minute)	74.1 (0.97)	64-104	78.5 (1.07)	64-96
Respiratory rate (breaths/minute)	21.1 (0.37)	16-28	24.1 (0.58)	16-40
Rectal temperature (°C)	38.2 (0.06)	37.1-39.2	38.9 (0.13)	37.2-41.5
Hock-hock distance (mm)	272 (6.65)	170-410	271 (6.15)	160-410
Udder temperature difference (°C)	0.10 (0.15)	-3.33-2.17	0.48 (0.18)	-4.17-3.33
Threshold difference (kPa)	12.9 (2.39)	-25.5-56.5	15.5 (2.50)	-53.1-53.8

Table A5.1: Mean/median (SE/IQR) and ranges for clinical parameters of mild (n=56) and moderate (n=41) cases of clinical mastitis on day of recruitment to study (day 0)

	Complete (n=70)	Range	Non-complete (n=47)	Range
Age (Years)	5.51 (0.20)	3-10	5.89 (0.31)	2-11
Parity	3.4 (0.20)	1-7	3.72 (0.29)	1-9
Days calved	112.5 (27-174)	0-438	75 (28-136)	4-376
Milk yield (litres)[#]	29.5 (8.23) (n = 26)	15-48	36.5 (6.43) (n = 17)	25-55
Body condition score	2.0 (1.5-2.5)	1.0-4.0	1.75 (1.5-2.0)	0.5-5.0
Heart rate (beats/minute)	75.8 (0.88)	64-104	77.3 (1.33)	64-96
Respiratory rate (breaths/minute)	22.3 (0.40)	16-32	23.1 (0.73)	16-40
Rectal temperature (°C)	38.4 (0.09)	37.1-40.3	38.8 (0.14)	37.2-41.5
Hock-hock distance (mm)	272 (5.65)	170-390	270 (7.46)	160-410
Udder temperature difference (°C)	0.24 (0.14)	-4.17-3.17	0.41 (0.20)	-2.5-3.3
Threshold difference (kPa)	13.4 (2.23)	-46.9-56.5	15.6 (2.77)	-53.1-51.7

Table A5.2: Mean/median (SE/IQR) and ranges for clinical parameters of cases that completed the 45 day study (n=70) and cases that did not complete the 45 day study (n=47) on day of recruitment to study (day 0)

	Normal (n=45)	Range	Mild (n=41)	Range	Moderate (n=29)	Range
Age (Years)	5.62 (0.37)	2-14	5.95 (0.25)	3-10	4.96 (0.31)	3-9
Parity	3.36 (0.32)	1-10	3.83 (0.26)	1-7	2.82 (0.28)	1-6
Days calved	117 (73-163)	17-305	128 (81.5-198)	0-438	70 (6.5-140)	3-288
Milk yield (litres)[#]	32.7 (1.71) (n = 24)	15-44.3	29.3 (2.18) (n = 18)	15-48	30 (2.07) (n = 8)	22-39
Body condition score	2.0 (2.0-2.5)	1.5-3.5	2.0 (1.5-2.5)	1.0-4.0	2.0 (1.5-2.5)	1.0-4.0
Heart rate (beats/minute)	71 (0.66)	60-80	71.2 (1.18)	64-104	77.4 (1.25)	68-92
Respiratory rate (breaths/minute)	22 (0.43)	16-28	21 (0.41)	16-28	24 (0.67)	20-32
Rectal temperature (°C)	38.1 (0.07)	37.0-39.0	38.2 (0.07)	37.1-39.2	38.8 (0.16)	37.5-40.3
Hock-hock distance (mm)	226 (4.13)	170-310	270 (7.84)	170-380	274(8.1)	200-390
Udder temperature difference (°C)	-0.23 (0.11)	-1.7-1.7	-0.07 (0.16)	-2.2-3.3	-0.47 (0.26)	-3.2-4.2
Threshold difference (kPa)	-0.58 (0.71)	-17.9-17.9	11.8 (2.73)	-25.5-56.5	17.2 (3.77)	-46.9-53.8

Table A5.3: Mean/median (SE/IQR) and ranges for clinical parameters of normal animals (n=45), mild (n=41) and moderate (n=29) cases of clinical mastitis on day of recruitment to study (day 0)

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