DETERMINING THE ORGANISMS
AND PATHWAYS OF INFECTION
LEADING TO MASTITIS IN EWES

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INTRODUCTION

*Mastitis: an overview*

Mastitis is an inflammation of the mammary gland primarily caused by bacterial infection that results in inflammation of the mammary tissue (Khan & Khan 2006). Mastitis can also be caused by viral infections, trauma, allergies, and physiological and metabolic changes (Bergonier et al. 2003). Mastitis can be classified as clinical, where an animal has overt clinical signs, or subclinical, where clinical signs are absent. Ewes that have developed mastitis may experience discomfort and pain in the udder, or be unwell overall or even die.

However, these are fairly rigid classifications of disease, and it can be argued that there is a single udder disease in sheep, caused by a variety of bacteria, with differences in clinical manifestation. It is thus important to consider mastitis with some flexibility of thought; it is likely that there is a continual shift in condition of the udder from health to disease and vice versa. There is no absolute point at which a mammary gland is diseased but rather, after colonisation of bacteria, a change in the ewe results in the development of disease. To date, much of the research on mastitis has tended to focus on causative species determined by culture and clinical signs, due to technical limitations. This has not allowed us to understand the dynamics of the disease in terms of when and how ewes’ udders become infected with potentially pathogenic bacterial strains.

Endemic diseases such as mastitis result in a direct and indirect economic loss for the industry. In both ewes kept to produce milk (hereafter dairy ewes) and in ewes kept to produce lambs for human consumption (hereafter suckler ewes), costs of diagnosis, treatments, preventive measures, labour, carcass disposal, and ewe replacements result in a substantial economic loss (Hogevseen et al. 2011; Pinzón-Sánchez et al. 2011). In dairy ewes, decreased milk yield (by up to 55%) (Saratsis et al. 1999), downgrading of milk due to high somatic cell counts (SCC) and higher bacterial counts would contribute to the economic loss attributed to mastitis (Fthenakis & Jones 1990). Milk bacterial counts are monitored due to the human health hazards posed. Milk is heated in order to minimise this risk, however in cases of cheese made with raw milk, and the possibility of thermostable toxins produced by mastitis causing pathogens surviving in pasteurised milk, controlling mastitis becomes a priority (Contreras et al. 2007). In meat producing sheep, costs occur through decreased live-weight gain of lambs, and loss of lambs that would have been reared by the affected ewe. In addition, lamb performance is decreased in lambs whose mothers have subclinical mastitis, due to decreased milk production (Fthenakis & Jones 1990; Keisler et al. 1992), and changes in suckling behaviour.
(Gougoulis et al. 2008), which is particularly important in suckler flocks. Improvement in management of mastitis will benefit the health and welfare of sheep and lambs and might help to reduce economic losses.

**Aetiology**

Over 130 different organisms have been associated with infection of the bovine mammary gland (Jeffrey L 1988), it is probable that a similar number would be found in the ovine mammary gland. The complexity of the infection is further highlighted when we consider not only species, but strain types.

In suckler ewes, bacteria commonly isolated from subclinical mastitis include coagulase-negative staphylococci (CNS), such as *Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus chromogenes, Staphylococcus xylosus* (Fthenakis 1994) and coagulase-positive staphylococci (CPS) such as *Staphylococcus aureus* (Bergonier et al. 2003; Kiossis et al. 2007; Kirk et al. 1996; Mork et al. 2007; Winter & Colditz 2002). In dairy ewes, CNS are a common cause of subclinical (undetected) mastitis (Kirk et al. 1996; Mork et al. 2007; Pengov 2001). Bacteria isolated from clinical mastitis include *Staphylococcus aureus, Mannheimia haemolytica, Escherichia coli, Mycoplasma* spp (Bergonier & Berthelot 2003) and *Streptococcus* spp. (Fragkou et al. 2011; Fragkou et al. 2007; Las Heras et al. 2002) including *Streptococcus uberis* (Marogna et al. 2010) and *Streptococcus agalactiae* (Lafi et al. 1998). Despite its renowned link with subclinical mastitis, CNS have also been shown to play a role in clinical mastitis in dairy ewes (Lafi et al. 1998).

*The defensive role of the udder*

There are three sources from which bacteria is likely to invade the mammary gland and cause infection: the lamb’s mouth, the ewe’s udder skin or the environment, including fields and bedding in housed sheep (Gougoulis et al. 2008; Piccinini et al. 2009). Pathogenic bacteria are likely to enter the udder half through the teat orifice, colonising the teat canal and cistern.

The mammary gland has a defensive role against pathogenic bacteria entering the teat duct. Sphincter muscles keep the teat canal tightly closed to prevent the entry of pathogens. The teat canal is lined with keratinocytes, considered to be the first line of physical defense for the udder (Forbes 1970). It hinders the movement of bacteria up the teat canal and also contains antimicrobial agents (Sordillo & Streicher 2002). In an experimental study in dairy cows, when the keratin was partially removal from the teat canal, the ability of the teat canal to act as a protective anatomical feature against bacterial pathogens from the external environment was compromised (Capuco et al. 1992). In addition, the presence of induced subepithelial lymphoid tissue between the teat
duct and teat cistern appear to have a role in the protection of the mammary gland against the early stages of bacterial infection (Fragkou et al. 2010; Fragkou et al. 2007).

Healthy mammary glands have bacteria colonised on the udder and teat skin (Fragkou et al. 2007), which may be a source of bacteria that would be transferred into the teat duct (Fragkou et al. 2011; Scott & Jones 1998). Interactions between the host’s mammary defense system and the virulence of the invading pathogenic bacteria determines the severity and extent of infection and disease. In order to understand the pathogenesis of a multifactorial microbial disease such as mastitis, it is important to consider the flora of the udder, as well as the bacterial content in the milk and in particular temporal interactions between them.

Entrance of a pathogen into the teat does not necessarily result in infection (Mavrogianni et al. 2006). For example, some bacteria may enter the teat canal but subsequently be withdrawn during suckling (Gougoulis et al. 2008). In an experimental study, *Mannheimia haemolytica* inoculated into the teat duct did not always cause clinical infection suggesting a protective role for the teat (Mavrogianni et al. 2005). In the sequel to that work, the same experimental design with ewes with natural or experimentally generated lesions on the surface of the teat had pathogenic bacteria deposited into the teat duct, resulting in the development of mastitis (Mavrogianni et al. 2006). It was postulated that *Mannheimia haemolytica* may form part of the teat duct flora without resulting in disease, suggesting that this bacteria does not necessarily always cause mastitis and for mastitis to occur, it requires either excessive accumulation of the bacteria (Fragkou et al. 2007) or injury to the teat (Mavrogianni et al. 2006). Indeed it appears that there is an increased risk in bacterial colonisation of the teat duct compared to the mammary gland of the same ewe (Mavrogianni et al. 2007).

*The protective role of udder skin microflora*

In addition, bacteria present on the udder skin may act as an inhibitor of major mastitis pathogens. For example, *Staphylococcus chromogenes* has been shown to protect udder quarters against elevated somatic cell counts post-partum. Further experimentation in vitro has revealed the in vitro inhibitory capability of *Staphylococcus chromogenes* (isolated from teat apices of heifers) against *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus uberis* using the cross-streaking method (De Vliegher et al. 2004). This also supports the earlier discussion of the inhibitory effect of CNS in the milk from protecting against major mastitis pathogens from causing severe infection (Woodward et al. 1987).

*Persistence of infections in the mammary gland*
Whilst a great deal of research has identified sources of infection, little research has been done on the persistence of particular strains of bacteria in milk throughout lactation in cows once bacteria have penetrated the mammary gland. Much of the treatment advice for persistent infections in sheep still leverages on bovine mastitis research. There is no research on the persistence of infections in sheep during the lactating or dry period. Different strains within a bacterial species can differ in pathogenicity and transmission routes, classification of isolates at the species level can incorrectly oversimplify control measures recommended, hence the need for longitudinal strain typing methods.

**Infection persistence during lactation**

Recent studies have used strain typing techniques such as pulse field gel electrophoresis to test whether the same strain of bacteria from the same mammary quarter is present over time indicating persistence with CNS based on a study of 12,412 milk samples from 3 dairy research herds (Gillespie et al. 2009). In fact, CNS persisted for up to 10 months (Gillespie et al. 2009). *Escherichia coli* has also been shown to persist in the bovine mammary gland, in a study of 300 dairy cows, with an estimated occurrence of between 4.8% and 9.1% of the herd population (Döpfer et al. 1999; Lam et al. 1996). However this was thought to be an underestimation. In addition the occurrence of recurrent episodes of the same strain in more than one quarter in a cow was high (Döpfer et al. 1999), suggesting transmission between quarters, which could increase persistence of the strain. Lipman et al., (1995) also found persistence of the same *Escherichia coli* serotypes in the bovine mammary gland, although different methods (serotyping and DNA polymorphism patterns) were used. In addition, infection with *Escherichia coli* more than once in a lactation was infrequent (Lipman et al. 1995). Conversely, in a study of 503 cows from 5 herds, quarters were often infected with multiple *Streptococcus uberis* strain types, despite the ability of *Streptococcus uberis* to persist in the udder (McDougall et al. 2004). This suggests that some pathogens have a superior mechanism of bacterial persistence than others. In fact, some pathogens subtypes, such as *Listeria monocytogenes*, are able to persist in the milking parlour which could be a source of reinfection (Ho et al. 2007).

**The role of the dry period**

The bovine mammary gland is thought to be particularly susceptible to new environmental coliform and streptococcal infections during the dry period (Larry Smith et al. 1985; Oliver & Mitchell 1983; Todhunter et al. 1995), including *Streptococcus uberis* (Todhunter et al. 1995) and *Escherichia coli* (Bradley & Green 2001; Döpfer et al. 1999; Lipman et al. 1995). Indeed, experimental studies have shown the ability of pathogens to remain within the udder, causing clinical disease after the onset of lactation (AJ 2002; McDonald & Anderson 1981).
Two studies in particular highlight the significance of the dry period in the persistence of mastitis causing pathogens.

In a study of 629 cows from 6 commercial herds, samples were collected during the dry period and from clinical quarters of these cows during the subsequent lactation allowing comparisons to be made between these time periods. DNA fingerprinting showed the persistence of enterobacterial organisms acquired during the dry period, causing disease after the onset of lactation (Bradley & Green 2000). One quarter remained persistently infected for >200 days before resulting in severe clinical mastitis (A.J 2002). Of all the enterobacterial mastitis occurring in the first 100 days of lactation, 52.6% arose in quarters previously colonized with the same strain of bacteria during the dry period (Bradley & Green 2000).

Bradley and Green (2001), observed 6 commercial herds over a period of 12 months in order to identify changes in the behavior of *Escherichia coli* as a mastitis pathogen. DNA fingerprinting allowed the identification of the genotypes of strains involved in recurrent cases of clinical *E.coli* mastitis. In the majority of cases, the same genotype was implicated as the cause of disease in recurrent cases and often the same genotype was identified in different quarters of the same cow suggesting that the same genotype may persist in the mammary environment (for more than 100 days), causing recurring infections and that bacteria may be spread between quarters (Bradley & Green 2001).

PCR based DNA fingerprinting identified the same *Streptococcus uberis* and *Streptococcus dysgalactiae* subtypes from some infected mammary glands from one lactation to the next, highlighting the persistence of these organisms through the dry period and during lactation (Oliver et al. 1998).

Statistical modelling has been used to identify the relationship between intramammary infection during the dry period, and clinical mastitis in the next lactation. The probability of an udder quarter developing clinical mastitis increased when *Streptococcus dysgalactiae, Streptococcus faecalis, Escherichia coli,* or *Enterobacter* spp. were cultured at drying off. In addition, the risk of clinical mastitis for specific pathogens increased if they were cultured in 2 or more late dry and post calving samples. Interestingly, the time that an isolate was identified was important in whether it increased or decreased the risk of clinical mastitis development; *Corynebacterium* spp., when isolated at drying off were associated with an increase, but when isolated in the late dry or post calving samples was associated with a reduction in the risk of clinical mastitis (Green et al. 2002). Although this study did not use strain typing to prove the persistence of certain strains in the mammary gland, it provides evidence for the significance of the dry period on clinical mastitis development.
AIMS, OBJECTIVES AND HYPOTHESES

The overall aim of this PhD is to contribute to the understanding of the pathways of infection in the udder. Ultimately this will assist in identifying when and how ewes’ udders become infected and whether certain strains of bacteria are responsible for infection.

Specific aims:

• Investigate the development of intramammary infection, from colonisation with an infecting strain to development of disease
• Determine links between strains of bacteria found on udder skin and the intramammary infections they cause
• Identify management and environmental factors that are associated with a ewe’s risk of developing intramammary infection

Objectives:

Stored samples of milk and udder swabs will be used, and new data will be collected during field work on sheep farms in England. Analysis will integrate results from culture, molecular analysis and statistical modelling. All samples will be analysed using MALDI-TOF MS. A subset of samples will be validated using other techniques such as PFGE and MLST alongside traditional culturing methods.

Hypotheses:

1. Development of intramammary infection is as a result of colonisation of the udder with certain strains of bacteria.
2. Ewes with intramammary infection have certain bacterial strains in their milk in the weeks after the same bacterial strains have been isolated from the environment (lambs mouths, bedding, teat skin/teat lesions).
The general research plan up to 2 years is shown in appendix 1.

**Year 1**
- The model on risk factors for intramammary infection from the cross-sectional study is ongoing.
- Texel flock samples used as a pilot to test and parameterise all laboratory techniques, as well as producing results on this sample flock.

**Year 2**
- All of the samples (both milk and swabs) from the longitudinal studies LS_2010 and LS_2011 are analysed and bacterial strains identified. Statistical comparisons made between the milk and swabs for each ewe and other data could also be combined to produce a multilevel model.
- Field-work studies have been designed in order to collect the remaining data required.
- The model on risk factors for intramammary infection from the cross-sectional study finished.

**Year 3**
- Field-work on farms in England.
- Lab analysis continues on samples on days that there are no farm visits allowing the studies to progress quickly.

**Year 4**
- Statistical analysis and modelling in order to explore patterns within the datasets thus contributing to the understanding of the pathways of infection in the udder.
- By the end of year 4, all samples would be analysed and statistical analysis would be completed. Thesis submission.
RESEARCH PLAN YEAR 2 (DETAILED)

Texel flock

By the beginning of my second year, I hope to have begun PFGE on selected Texel isolates. Ideally, this would only continue a few months into my second year, and so would give me the opportunity to write this chapter up in the early stages of year 2.

Cross sectional study

The majority of the statistical analyses for this study will be done in year 2. I intend on using R for this study and thus extra time must be allowed for this (10 months in total). I may have an undergraduate or MSc student working on this part of the project with me. I hope to complete this as a chapter in my second year.

LS_2010 and LS_2011

These two studies will begin in my second year. They will require a large amount of time in the lab and I will also need to work for a few months in Somerset in order to use the MALDI-TOF if we do not have this here. I have given myself a year for both studies running alongside one another. Although I anticipate that the chapters will not be completed until my third year.

New field work study set-up

I will be able to assess my progress towards the end of my second year and decide whether I need more samples, and if I do then I can design a study that will directly address my remaining aims.

Year 3

- Set up new study to address remaining aims
  - Field work, laboratory analyses and statistical analyses
- Begin statistical analysis (cross-classified mixed effect model)
- Decide on remaining studies
CURRENT WORK

Study Texel flock: June-Present

**Aim:** Determine links between environmental strains of bacteria and the intramammary infections they cause

**Overview:** This study is a pilot and a standalone project that will investigate bacterial species found on udder skin and in ewe milk. Comparisons can be made between these for ewes with and without clinical intramammary infection.

**Data collection:**

*Flock*
- 100 pedigree Texel ewes

*Number of samples*
- 28 milk samples from ewes with clinical intramammary infection
- 5 milk samples from ewes without clinical intramammary infection
- 14 swabs

*Sample storage*
- Milk stored at -80°C with glycerol
- Swabs stored at -80°C in BHI and glycerol

**Analysis:**
- ✓ Culture and sterile streak all isolates identified
- ✓ Use MALDI-TOF-MS to identify species from the majority of samples
- ✓ Use species information to decide on which isolates to strain type
  - Use PFGE to strain type
  - Use statistical analyses to investigate links between isolates

**Results:** In total the MALDI-TOF identified **515/753** isolates. Am now starting to PFGE these isolates.
Alongside my PhD, I am also doing a postgraduate award in transferable skills, which has given me the opportunity to produce my own research webpage:

http://www2.warwick.ac.uk/study/csde/gsp/eportfolio/directory/pg/lsrgbd/

I have worked at Quality Milk Management Services (QMMS), allowing me to collaborate with other researchers in the veterinary epidemiology field.

My research will also be shown at the Warwick Postgraduate Symposium and the Society for Veterinary Epidemiology and Preventive Medicine, which will allow me to represent Warwick and EBLEX.

I have frequently presented my research in my lab meetings at Warwick University and will also be presenting in the microbiology seminar at Warwick in the next few months.
### Appendix 1 Gantt chart up to year 2.5 of PhD

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*Note: Colors represent different stages of work.*
REFERENCES


