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Determining the Organisms, Pathways of Infection and Risks for Ovine Mastitis

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Determining the Organisms, Pathways of Infection and Risks for Ovine Mastitis

Selin Cooper

University of Warwick

Supervisor: Prof Laura Green, Dr Kevin Purdy

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CONTENTS

1. Abstract

The aims of the first study were to estimate the incidence rate of clinical mastitis (IRCM) for suckler ewes (ewes rearing lambs for meat production) in England and to identify potential risk factors. A postal questionnaire was sent to 999 randomly selected English sheep farmers in 2010 to gather data on the IRCM and flock management practices for the calendar year 2009. The mean IRCM per flock was 1.2 / 100 ewes / flock / year (0.0-19.1). The IRCM was 2.0, 0.9 and 1.3 / 100 ewes / year for flocks that were always housed, always outdoors and a combination of both respectively. Six mixed effects over-dispersed Poisson regression models offset by flock size were developed to identify management practices associated with IRCM. Of particular interest were environmental factors, as indicated by the affect flooring and indoor versus outdoor rearing had on the IRCM, and host susceptibility as indicated by the association of IRCM with udder conformation (a potentially heritable trait). Based on these findings, the role of the environment was first considered by investigating the potential sources of mastitis-causing pathogens. Milk samples and udder skin swabs were taken from 27 pedigree Texel ewes with clinical mastitis and 3 ewes showing no clinical signs in order to identify whether udder skin could act as a bacterial reservoir for intramammary infections. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) was used to identify isolates to species level and compared to pulsed-field gel electrophoresis (PFGE) to assess the capabilities of MALDI-ToF-MS as a strain differentiation tool. MALDI-ToF-MS had good correspondence with PFGE for all species tested in this study including Staphylococcus warneri, Staphylococcus equorum, Rhodococcus corprophilus, Bacillus pumilus and Micrococcus luteus. The same strain type was found in mastitic milk and on ewe udder skin for Staphylococcus warneri and Rhodococcus corprophilus indicating a possible reservoir of bacteria that might enter the mammary gland. The third study built upon the findings from the second study by identifying the role of lamb mouths and ewe udder skin as potential transmission pathways of mastitis causing bacteria by sampling these environments and ewe milk over time in a longitudinal study of 21 ewes over 10 weeks. Twenty-six bacterial species were found in more than one location (lamb mouth, ewe udder skin and/or ewe milk), many of which have previously been associated with mastitis. Potential transmission events and persistence of the same bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time were identified for the first time in suckler ewes using MALDI-ToF-MS for a variety of bacterial species. Having identified udder conformation as the most significant factor associated with IRCM in the first study, the final study aimed to investigate whether udder and teat conformation are heritable traits that affect mastitis. A total of 968 records were collected from 10 Texel flocks over a period of 3 years (2012-2014) from England, Wales and Scotland to assess whether udder and teat conformation were heritable traits associated with higher levels of chronic mastitis. Univariate quantitative genetic parameters were estimated using individual

animal and sire models. The heritabilities for teat length and teat placement were greatest (0.42 and 0.35, respectively). The remaining traits (traits that generally describe the volume of the xv udder) were of moderate to low heritability. Univariable logistic regression was used to identify the phenotypic association between udder traits and chronic mastitis. The work in this thesis has addressed several gaps in the knowledge in mastitis epidemiology by providing the first estimate for the IRCM in suckler flocks in England, and generating hypotheses for factors that may affect the IRCM. The role of the ewe's environment and heritable traits that could potentially affect a ewe's likelihood of getting mastitis were subsequently investigated. The results have contributed to our understanding of the factors associated with the development of mastitis.

2. Introduction

Endemic diseases such as mastitis result in both 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 substantial economic loss (Hogeveen et al. 2011, PinzónSánchez 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 contribute to the economic losses attributed to mastitis (Fthenakis & Jones 1990a). In suckler ewes, 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 1990a, Keisler et al. 1992), and changes in suckling behaviour (Gougoulis et al. 2008a), which is particularly important in suckler flocks. In a recent study of 67 ewes followed through lactation, lower lamb growth rates were associated with an increase in the dams somatic cell count (>400,000 cells/ml) (Huntley et al. 2012). Improvement in the management and therefore prevalence of mastitis would benefit the health and welfare of sheep and lambs and help to reduce economic losses. An accurate estimate for the cost of mastitis to the UK sheep industry is not available, because costing farm animal disease is complex (Conington et al. 2008). Using computer models of flock dynamics, however, a 10% decrease in the risk of contracting mastitis (through control methods) has been estimated to be worth £8.40 per ewe in the Texel breed (Conington et al. 2008). In cows, the economic losses attributed to mastitis are estimated to be between €61 and €97 per cow-year based on dynamic programming models and herd-simulation models, respectively (Bar et al. 2008, Hagnestam-Nielsen & Østergaard 2009). There are, however, methods that can reduce the economic effect of mastitis on a flock, for example by supplementing 2 lamb feed in suckler flocks with mastitis in order to compensate for the reduction in milk yield. This would reduce the effect of mastitis on lamb performance (Keisler et al. 1992). 1.1.Mastitis: an overview Mastitis is defined as an infection of the mammary gland primarily caused by bacteria that result in inflammation of the mammary gland tissue (Khan & Khan 2006). Mastitis can also be caused by viral infections, trauma, allergies, and physiological and metabolic changes (Bergonier et al. 2003). Ewes that have mastitis may experience discomfort and pain in the udder, or be systemically sick or die. Mastitis has typically been categorised as clinical (CM), where an animal has overt clinical signs, or subclinical (SCM), where clinical signs are absent. Clinical mastitis is typically classified as per-acute, acute or chronic and may be severe or mild. It can result in death, visible swelling of the udder, abnormal discharge from or pain in the udder, milk composition modifications (Firat 1993, Gonzalez et al. 1990, Hovinen et al. 2008, Klaas et al. 2004), lethargy, weight loss and

increased rectal temperature (Arsenault et al. 2008). Farmers often diagnose acute clinical mastitis in sheep using whole animal signs such as lethargy or malaise or behavioural changes such as lameness, or kicking away sucking lambs. Typically, 3 clinically affected udder halves do not return to normal function (Mørk et al. 2007). Chronic mastitis is often detected when farmers palpate ewes' udders at weaning when deciding whether or not to retain a ewe for breeding. Subclinical mastitis can lead to a reduction in milk yield and altered milk composition (Bergonier & Berthelot 2003, Conington et al. 2008, Fthenakis & Jones 1990a). In sheep, there is no universally accepted definition of subclinical mastitis with no established 'normal' somatic cell count and as such different studies may define cases differently. Clinical and subclinical mastitis 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 different clinical manifestations. These categorisations depend very much on the degree of inspection given to an animal. In suckler ewes it is quite likely that changes in the milk alone are not detected by farmers and so these fall into the category of subclinical infection. In milk sheep and dairy cows such changes would be detected because the udder and milk are inspected twice each day. It is thus important to consider mastitis as a spectrum from infection to disease that shifts the status of the udder from healthy to diseased. To date, much of the research on mastitis has tended to focus on causative species and clinical signs, due to technical limitations in strain differentiation methods. Therefore we do not have a clear understanding of the dynamics of the disease, specifically how and when ewe's udders become infected with pathogenic strains. 1.1.1. Detection of mastitis The ability to detect cases, to identify accurately the causative bacteria to a suitable level, enables rapid intervention and the use of appropriate treatment (Ghebremedhin et al. 2008). Key methods of detection include visual observation of the ewe and udder, bacterial culture of milk samples, the California Mastitis Test (CMT), the Whiteside Test (WST), flow cytometry (FCM), and measurement of somatic cell count (SCC). Traditionally bacterial culture has been used to identify the causative agents of mastitis. Identification is normally based initially on fast and simple tests such as 4 Gram staining, catalase and oxidase tests, with secondary phenotypic tests used to complete the identification (Carbonnelle et al. 2011, Sintchenko et al. 2007). Bacterial culture was considered fast, accurate, and low cost for many decades; the gold standard for the identification of bacteria to at least genus if not species level (Ghorbanpoor et al. 2007). In recent years, ongoing revolution in bacteriology by means of molecular rather than phenotypic methods have deemed these tests labour intensive, slow, imprecise on occasion, and expensive (Carbonnelle et al. 2011, Ghorbanpoor et al. 2007). Somatic cell count is the number of somatic cells present per millilitre of milk (cells/ml) (Lafi 2006). Somatic cells are comprised of neutrophils, macrophages and ewe epithelial cells (Oviedo-Boyso et al. 2007). When the udder half is infected with bacteria, inflammation is triggered and the number of neutrophils recruited to the udder rises rapidly. This increases the somatic cell count which can be counted by Coulter or

Foss counters and is used to detect cases of subclinical or clinical mastitis in dairy cows. The somatic cell count gives an indication of whether an immune response has begun and when measured at regular intervals can indicate the timing and extent of this immune response and has been used in cows and sheep (GonzálezRodríGuez et al. 1995, Rupp & Boichard 2000). It should be noted that the somatic cell count is influenced by other factors aside from infection stage and pathogen such as physiological status of the animal, lactation stage, and milk yield. The distributions of somatic cell count between ewes with different udder health infection statuses overlap and therefore is it difficult to define a somatic cell count threshold for mastitis (Carta et al. 2009). However, a somatic cell count of >200,000 cells/ml is considered to be a rational threshold for the presence of bacterial infection in cows based on research on somatic cell counts of cows with and without mastitis (Dohoo & Meek 1982, Green et al. 2006, Madouasse et al. 2010). There is as yet no accepted somatic cell count threshold for the detection of infection in the udder of sheep. Whilst there is little regulation of the somatic cell count in sheep milk produced commercially, the EU legal limit for raw goat or sheep milk intended for manufacture of dairy produce is 500 x 103 cells/ml (European Commission 1992). 5 Although an increased somatic cell count can be used as a proxy for bacterial infection, there is some debate over how low somatic cell counts can affect an animal's susceptibility to mastitis. Research has indicated a non-linear relationship between somatic cell count and the odds for clinical mastitis in cows (Peeler et al. 2003). The importance of somatic cells in the protection of the udder against clinical mastitis has become apparent (Peeler et al. 2003) with low herd bulk milk somatic cell counts resulting in cows being at an increased risk of toxic mastitis (Green et al. 1996). Somatic cell count also varies depending on the bacterial species isolated from the infected udder half which makes using somatic cell count as a linear indicator of bacterial infection in the udder complicated (González-RodríGuez et al. 1995, Peeler et al. 2003). Ideally a combination of detection methods, for example somatic cell count and bacterial identification should be used in order to diagnose and effectively treat the infection. 1.1.2. Treatment and prevention of mastitis Early detection of mastitis is necessary to improve treatment success. If detection and therefore treatment is too late, it may not save the affected udder half or the ewe. Ewes with mastitis are usually treated or culled. Treatment for clinical mastitis is often an intramuscular antibiotic, although it is not used by all farmers due to the expense. Animals may also be re-infected after treatment and recovery. In order for antibiotics to be effective, information about the aetiology, history of clinical or subclinical mastitis cases and parity are useful, but are not always available (PinzónSánchez & Ruegg 2011). In addition, antibiotics are occasionally ineffective in controlling infections caused by certain bacteria resulting in reservoirs of bacteria within a herd or flock that results in chronic and/or persistent infections (Pellegrino et al. 2010). Prevention of mastitis altogether is unlikely; it would be unrealistic to expect a single prevention protocol to be effective for all mastitis cases, due to the multifactorial nature of the disease and the numerous causative

agents. However, advancing our understanding of mastitis, including identifying risk factors for mastitis, the methods by which causative agents gain access to the udder and the pathway of infection may 6 allow improvements in management practices, or preventative measures that can be used to reduce the levels of infection in a flock. Control strategies target sources and transmission. In order to control sources, existing infection must be removed through the culling of ewes with clinical mastitis, or through intramammary antibiotics during the dry period (dry-ewe therapy) for ewes with subclinical mastitis (Bergonier & Berthelot 2003, Gonzalo et al. 2005, Kiossis et al. 2007). Effective management practices can also be used to control disease transmission. Milking machine standards should be met; regular maintenance of milking machinery to reduce traumatic damage to the udder must be implemented. Gonzalo et al., (2005) found that udder health (by proxy of bulk tank somatic cell count) could be improved by optimising milking machine standards, for example by having reduced vacuum levels and elevated pulsation rate during machine milking (Gonzalo et al. 2005). Milking machinery and/or milkers' hands should also be cleaned thoroughly to reduce the likelihood of spread of contagious bacteria. Management practices such as teat dipping are regularly used in order to prevent new intramammary infections (Fox 1992, Neave et al. 1969, Philpot & Pankey Jr 1975). These management practices generally relate to dairy animals; there is a lack of information on effective management practices to control disease transmission in suckler ewes. In line with finding preventive measures to control mastitis, breeding for resistance is a long-term strategy that may reduce mastitis prevalence in sheep. Despite the accumulation of research on the genetic element of mastitis in cattle, little has been carried out on heritable traits that are associated with mastitis in sheep and even these focus on dairy rather than suckler ewes. This has resulted in a tendency to select towards traits that are profitable for dairy ewes if any, such as milk yield, rather than away from mastitis. Selecting for increased genetic resistance to mastitis tends to be indirectly through the prediction of health status of the udder based on inflammatory factors such as somatic cell count. Utilising direct selection- relating to the diagnosis of infection such as bacteriological examination and or observation of clinical cases is unusual in 7 genetics studies due to the large amount of records required for robust modelling. However, a genetic correlation between somatic cell count and udder infection status (using bacteriological analyses) was found (0.93) (Tolone et al. 2013). This would indicate that a reduction in somatic cell count could result in a reduction in mastitis incidence. This has been shown in a study of 2 lines of Lacaune ewes divergently selected for somatic cell count which showed that selecting for a reduction in somatic cell count lead to increased resilience to intramammary infections. This included lower incidence of clinical mastitis, prevalence of chronic (as indicated by mammary abscesses) and subclinical intramammary infections, and a better ability to recover from infections (Rupp et al. 2009). However it must be noted that selecting for low somatic cell count could damage the ability of the animal to initiate an appropriate immune response to pathogens and therefore resist infection.

Using repeatability test-day models, somatic cell count heritability estimates have ranged from 0.04 to 0.16 (Baro et al. 1994, El-Saied et al. 1998, Hamann et al. 2004, Othmane et al. 2002, Riggio et al. 2007, Serrano et al. 2003) and 0.11 to 0.18 for the average somatic cell count during lactation (Barillet et al. 2008, Barillet et al. 2001, Legarra & Ugarte 2005, Mavrogenis et al. 1999, Rupp et al. 2003). In parallel with cattle, the association between genetic and phenotypic factors (specifically udder conformation) and mastitis in dairy ewes (Casu et al. 2010, Legarra & Ugarte 2005, Marie-Etancelin et al. 2005) has been shown. In a study of 7558 records from 2262 primiparous ewes, the somatic cell count increased as cistern height increased and degree of separation and udder depth decreased. The genetic correlations between somatic cell count and udder depth, teat placement and degree of suspension were favourable (0.39-0.50) apart from for degree of separation (0.05). The heritability estimates for udder traits ranged from 0.32 to 0.50 (Casu et al. 2010). In a study of 82019 primiparous ewes from 352 flocks, the genetic correlations among udder traits were moderate to high (0.14-0.49) and between the estimated breeding values (EBVs) for somatic cell count and udder-type traits were weak but favourable (0.1-0.2). The heritability estimates were slightly lower (0.19- 0.33) (Marie-Etancelin et al. 2005) than those found by Casu et al., 2010. The same pattern was found in a study of Laxta sheep, whereby the genetic correlations among udder traits were moderate for all traits (0.34-0.58) apart from 8 teat size (0.05-0.31). Genetic correlations between somatic cell counts ranged from 0.01 for teat placement to 0.29 for teat size. The heritability estimates ranged from 0.26 to 0.40 (Legarra & Ugarte 2005). The effect of udder conformation traits on somatic cell count has been shown in a recent study cohort study of 67 suckler ewes, where pendulous udders and greater cross-sectional area of the teats were associated with an increase in somatic cell count (Huntley et al. 2012). Previous studies have indicated some udder conformation traits (in particular teat placement) have high repeatability within (de la Fuente et al. 2011, Fernández et al. 1995) and across lactation (de la Fuente et al. 2011) whilst others relating to udder size and therefore milk yield were affected by lactation and flock (Serrano et al. 2002). These traits also had lower heritability (Serrano et al. 2002). These traits are likely to be associated with mastitis due to affecting milk production, milk let down and suckling ease (Huntley et al. 2012). Based on the studies discussed, selection based on udder morphology traits could potentially result in improved resilience against mastitis. 1.2.Epidemiology of mastitis Several studies have identified a range of clinical and subclinical incidence and prevalence rates across the world (Table 1.1). The only estimate for the incidence of clinical mastitis in suckler ewes was 1.2% in Canada, whereas the prevalence of subclinical mastitis was between 9.2-50.0%. The only estimate for the prevalence of subclinical mastitis in suckler ewes in England is 11.7%. There is no incidence rate for clinical mastitis for suckler ewes in England. 9 Table 1.1: Worldwide incidence and prevalence rates of clinical and subclinical mastitis in flocks of sheep. Location Flock Size of study Mastitis classification Prevalence Incidence Reference Wales Suckler 8 flocks, 1093 ewes Subclinical

14.0% (Watson et al. 1990) England Suckler 7 flocks, 358 ewes Subclinical 11.7% (Watkins et al. 1991) USA Suckler 2 groups of 49 and 79 ewes Subclinical 17-50% depending on methods (Keisler et al. 1992) Spain Dairy 433 ewes Subclinical 36.7% (de la Cruz et al. 1994) Greece Dairy 760 ewes Subclinical 10.9 % (Fthenakis 1994) Greece Suckler 5 flocks Subclinical 29-43% (Stefanakis et al. 1995) USA Dairy 62 ewes Clinical 29% (Kirk et al. 1996) Jordan Suckler 12 flocks, 318 ewes Subclinical 18.3% (Al-Majali & Jawabreh 2003) Greece Experimental 2 flocks, 98 ewes Subclinical 8.3% (Fthenakis et al. 1998) 10 Location Flock Size of study Mastitis classification Prevalence Incidence Reference Jordan Mixed 46 flocks, 1736 ewes NA 29.8% (subclinical) 1.7 cases per 100 ewemonths (clinical) (Lafi et al. 1998) Spain Dairy 22 flocks, 564 ewes Subclinical 34% (Las Heras et al. 2002) Iran Dairy 178 ewes Subclinical 39.0% (Batavani et al. 2003) Greece Dairy 130 ewes Clinical and subclinical 94.0% (Fthenakis et al. 2004) Greece Dairy 140 ewes Subclinical 5.7-15.6% (control group) (Kiossis et al. 2007) Norway Mixed 353 flocks, 509 ewes Clinical (Mørk et al. 2007) Canada Suckler 30 flocks, 2792 ewes Clinical 1.2% (Arsenault et al. 2008) Azerbaijan Suckler 12 flocks, 260 ewes Subclinical 9.2% (Beheshti et al. 2010) Italy Dairy 2198 ewes Clinical 75.0% (clinical) (Marogna et al. 2010) 11 1.3.Risk factors associated with mastitis Time in lactation is associated with mastitis; Fthenakis (1994) found that the prevalence of subclinical mastitis increased as the lactation period proceeded. However, the prevalence of subclinical mastitis is particularly difficult to measure as it depends on the degree of inspection given to an animal. In suckler ewes, clinical cases often peak during the first week after lambing (Lafi 2006). In a study of 509 ewes in Norway, one third developed clinical mastitis in the first week. A second peak was also observed at three weeks (Mørk et al. 2007). Reviewers have suggested that the rationale behind these peaks in cases of mastitis include; 1. Milk accumulation in the cistern at peak lactation may act as a reservoir for pathogens (Winter 2001). 2. Rapid lamb growth may cause lambs to nurse aggressively, butting the udder and damaging teats, leaving them susceptible to bacterial infection (Winter 2001). Alternatively, the onset of lactation triggers mastitis because sheep may already have bacteria colonised in the udder, and when there is a change, either in the ewe or the environment, such as the onset of lactation, the infection becomes a detectable disease. It is also possible that functions of pregnancy and lactation are prioritised over the expression of immunity and hence reduced energy availability during these periods would compromise the immunity of the ewe (Coop & Kyriazakis 1999). Larsgard and Vaabenoe (1993) following 920 ewes over 6 years, identified several factors associated with mastitis in Norwegian sheep including the number of lambs born, breed, udder conformation, type of pasture and milk yield (shown through growth of lambs). Whilst age did not have an effect on the risk of mastitis, affected ewes were culled before the next season, which may have biased the results (Larsgard & Vaabenoe 1993). The two most recent articles to be published on risk factors for mastitis are also the most relevant, both focusing on suckler ewes. Despite the differences in country, 12 both produced similar results. Waage and Vatn (2008)

identified individual animal risk factors for ovine clinical mastitis in suckler ewes in Norway. In 1056 flocks, the total number of lambs born per ewe, ewe age, breed, whether assistance at lambing was required, mastitis in a previous lactation and the presence and degree of teat lesions were identified as risk factors for mastitis (Waage & Vatn 2008). In a study of 2792 ewes from 30 commercial suckler flocks in Canada, the risk factors for clinical mastitis were region, ewe age, body condition score, whether ewes had had mastitis previously and the number of lambs born per ewe (Arsenault et al. 2008). In agreement with these studies, it was suggested that ewes rearing more than one lamb are more likely to suffer from mastitis (Gross et al. 1978, Watkins et al. 1991). This may be a result of ewes rearing more than one lamb being more susceptible to lesions caused by lambs due to the cumulative stress on the mammary tissue (Lafi et al. 1998). Several previous studies have identified a link between age and mastitis, with older ewes being more susceptible (Arsenault et al. 2008, Gross et al. 1978, Watkins et al. 1991). Damage to the udder from previous lambs might also make older ewes more susceptible to mastitis (Lafi et al. 1998). It appears that there is a balance between ewe factors (predisposing conditions, existing trauma, lowered immunity and nutrition), the species of pathogen and the environment that affects the likelihood of the development of mastitis. 1.4.Aetiology of mastitis Over 130 different organisms have been associated with infection of the bovine mammary gland (Watts 1988). It is probable that a similar number would be found in the ovine mammary gland. Many of the bacterial species that cause bovine mastitis can also be isolated from ovine mastitis cases. The complexity of mastitis is further highlighted when we consider not only species, but strain type. Strain types within a species can vary in pathogenicity and transmission pathway (Zadoks & Fitzpatrick 2009). The organisms are likely to come from the ewes' environment, such as other infected udders, replacement animals (contagious) and/or bedding, soil, water and manure (environmental). 13 In suckler ewes, bacteria commonly isolated from subclinical mastitis include coagulase-negative staphylococci (CNS), such as Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus chromogenes, and Staphylococcus xylosus (Fthenakis 1994). Staphylococcus aureus is also typically isolated from cases of subclinical mastitis in suckler ewes (Bergonier et al. 2003, Kiossis et al. 2007, Kirk et al. 1996, Mørk et al. 2007, Winter & Colditz 2002). Coagulase-negative staphylococci are also a common cause of subclinical mastitis in dairy ewes (Kirk et al. 1996, Mørk et al. 2007, Pengov 2001). Bacteria isolated from clinical mastitis cases include Staphylococcus aureus, Mannheimia haemolytica, Escherichia coli, Mycoplasma spp. (Bergonier & Berthelot 2003) and Streptococcus spp. (Fragkou et al. 2011, Fragkou et al. 2007c, Fragkou et al. 2007d, Las Heras et al. 2002) such as Streptococcus uberis (Marogna et al. 2010). In addition, a variety of other bacterial species have been associated with mastitis including: Actinomyces pyrogenes (Saratsis et al. 1998), Arcanobacterium pyogenes (Fthenakis 1994, Fthenakis et al. 2004), Bacillus spp. (Batavani et al. 2003, Fthenakis 1994), Burkholderia cepacia (Berriatua et al. 2001), Clostridium perfringens

(Osman et al. 2009), Corynebacterium spp. (Mavrogenis et al. 1995), in particular Corynebacterium bovis (Beheshti et al. 2010), Klebsiella pneumoniae (Bergonier & Berthelot 2003, Fthenakis & Jones 1990b), Listeria monocytogenes (Fthenakis et al. 1998), Pseudomonas aeruginos (Bergonier & Berthelot 2003) and Serratia marcescens (Tzora & Fthenakis 1998). The differences in isolated mastitis causing pathogens could be due to differences in management practices between dairy and suckler ewes. 1.4.1. Environmental and contagious pathogens Traditionally, mastitis pathogens have been classified as either environmental or contagious. However, the availability and reduced cost of molecular and DNA-based methods in mastitis research has resulted in an increased understanding of the transmission, reservoirs and persistence of bacterial strains that cause intramammary infections. Much like the classifications of clinical and subclinical mastitis, these classifications are too rigid and not perfect. Some pathogens, traditionally thought of only as 14 contagious, can also be environmental in origin. These infections would not be affected by the implementation of a control and prevention program aimed at contagious pathogens, indicating the importance of strain typing studies (Zadoks & Fitzpatrick 2009). Different strains may differ in their ability to infect an udder, spread from ewe to ewe, alter somatic cell count, impact milk yield and respond to antimicrobial treatments. 1.5. The defensive role of the mammary gland The mammary gland has anatomical features have could reduce the likelihood of pathogenic bacteria entering the teat duct. Sphincter muscles keep the teat canal tightly closed in order to prevent the entry of pathogens. The teat canal is lined with keratin, considered to be the first line of physical defence 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 of dairy cows, where 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 appeared 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. 2007b). Although not playing a defensive role, udder conformation can increase or decrease a ewes susceptibility to mastitis based on reducing damage to the udder. In a study of 2251 ewes, udder conformation was associated with the risk of mastitis and increased somatic cell count. As the cistern height increased and the degree of udder suspension (the weakness of the suspensory ligament and how loose and pendulous the udder was) and udder depth (the depth of the udder from the abdominal wall to the udder cleft when viewed from behind) decreased, the risk of mastitis increased (Casu et al. 2010). Non-diseased mammary glands have bacteria colonising the udder and teat skin (Fragkou et al. 2007a), which may be a source of bacteria that could be transferred into the teat duct (Fragkou et al. 2011, Scott & Jones 1998). Interactions between the host's mammary defence system and the virulence of the invading pathogenic 15 bacteria determine

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 microbiota of the udder skin, as well as the bacterial content in the milk and in particular temporal interactions between them. It has been postulated that entrance of a pathogen into the teat would not necessarily result in infection (Mavrogianni et al. 2006a). For example, some bacteria may enter the teat canal but subsequently be withdrawn during suckling (Gougoulis et al. 2008b). In an experimental study, Mannheimia haemolytica inoculated into the teat duct did not always cause clinical infection suggesting the protective role of the teat (Mavrogianni et al. 2005). In the sequel to that work, using the same experimental design including ewes with natural or experimentally generated lesions had pathogenic bacteria deposited into the teat duct, which resulted in the development of mastitis, unlike those ewes in the previous study without lesions (Mavrogianni et al. 2006b). It was postulated that Mannheimia haemolytica may form part of the teat duct bacterial community without resulting in infection, 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. 2007a) or previous injury to the teat, lowering the teats bacterial defences in sheep (Mavrogianni et al. 2006b). 1.6.Sources & transmission pathways of pathogenic bacteria There are three possible routes for bacteria to invade the mammary gland and cause infection: galactogenic route of entry (that is through the teat canal, with ascending infection), haemotogenous spread which would diffuse through mammary paranchyma, and percutaneous entry (wounds) (Meador 1988). There are also several reservoirs of bacteria that could cause mastitis, such as the lamb mouth, the ewe's udder skin or the environment, such as the bedding (Gougoulis et al. 2008b, Piccinini et al. 2009). Pathogenic bacteria are most likely to enter the udder half through the teat orifice, colonising the teat canal and cistern. Previous research on the transmission and persistence of mastitis causing bacteria from different environments of ewes is lacking. 16 1.6.1. Mouth & nasal cavities Mannheimia haemolytica has been isolated from the mouths of ewes and lambs, and the teat skin of pregnant and lactating ewes, which may invade the mammary gland during suckling (Fragkou et al. 2011, Scott & Jones 1998) and has been indicated as being transmitted to the teat duct during suckling, along with staphylococci in a study on the microbiota of the teat before and after suckling in ewes (Gougoulis et al. 2008b). Although healthy teat ducts provide an effective defence for bacteria transmitted from the lamb's mouth to the teat duct of the ewe, suckling is likely to increase the risk of infection by increasing the teats exposure to bacteria (Gougoulis et al. 2008b). Despite this indication, strain typing methods were not used in these studies and therefore transmission could not be confirmed. In addition, providing evidence for the transmission of an isolate from one environment to another is difficult; there is the possibility for a strain to mutate as it moves which could only be detected by single nucleotide polymorphisms (SNPs) rather than methods typically used for strain typing. Therefore whilst you would expect isolates involved in transmission to be

indistinguishable using strain typing methods, there is the possibility that they would not be, complicating the confirmation of a transmission pathway. In sheep, identical Staphylococcus aureus pulsotypes (using pulsed-field gel electrophoresis as the strain typing method) were identified from body sites and in the milk. In particular the majority of pairs of isolates from the nasal cavity of ewes and their lambs, twins and repeated sampling provided identical pulsotypes indicating transmission of Staphylococcus aureus between the dam and her lambs (Mørk et al. 2012b). However, this could also be due to the predominance of a particular strain type within these flocks, and therefore does not necessarily indicate that a transmission event occurred. This is the only study indicating a transmission route of mastitis causing bacteria between the dam and her lambs using a strain typing method in suckler ewes. 1.6.2. The milking environment Improper milking techniques can increase the likelihood of mastitis, due to the increased teat canal dilation time which allows bacteria to invade the teat (Gougoulis 17 et al. 2008b). Staphylococci have been thought to originate from milkers' hands or animal teat skin. Hand milking has been shown to increase the risk of bacterial colonisation of the teat, although only a small and non-significant increase in the risk of intramammary infection associated with hand milking has been shown (Mavrogianni et al. 2006c). 1.6.3. Bedding and dirt Bedding type has an effect on the number of coliforms and Klebsiella spp. found on teat ends (Zdanowicz et al. 2004), which is to be expected as cows lie down on the bedding, allowing bacteria to be transferred from the bedding material to the teat skin. In addition, it has been suggested that pathogens could be transmitted to milk via dirty udders and teats in cattle (Vissers et al. 2007). 1.6.4. Udder skin In dairy cows, potential reservoirs of Staphylococcus aureus have been identified; heifers with teat skin colonised by Staphylococcus aureus at parturition were more likely to have intramammary infections caused by Staphylococcus aureus at parturition (Roberson et al. 1994). In addition, using pulsed-field gel electrophoresis, the same pulsotypes of Staphylococcus aureus were identified from teat skin as in the milk in several studies (da Costa et al. 2014, Haveri et al. 2008, Zadoks et al. 2002). Pulsed-field gel electrophoresis has also been used to identify the same Staphylococcus aureus pulsotypes from body sites (particularly the hock skin), the immediate environment of lactating cows and milk samples previously (Capurro et al. 2010). A study with extramammary sites including dairy cow teat skin, teat canals, skin lesions, milking liners and the hands and nostrils of milking personnel in two dairy herds found indistinguishable Staphylococcus aureus pulsotypes for isolates infecting the mammary gland and extramammary sites, again indicating potential reservoirs of intramammary infections (Haveri et al. 2008). However, the direction of transmission could not be ascertained. 18 1.6.5. Teat lesions Teat bites could predispose intramammary infections due to removal of the acid mantle on the teat skin which could aid bacterial attachment (Sieber & Farnsworth 1984). In addition teat lesions could harbour bacteria, which could be transmitted up the teat canal during suckling. Authors have identified an association between Arcanobacterium pyogenes, Streptococcus dysgalactiae and teat lesions in

cows (Ericsson Unnerstad et al. 2009). 1.6.6. Teat apices Bacteria present on teat apices may have a different natural community to that of the rest of the udder due to suckling calves or lambs. The bacteria present on the teat duct may be advanced up the teat canal during suckling, unlike bacteria present on the udder. The teat duct could harbour bacteria, which could invade the mammary parenchyma, causing mastitis (Mavrogianni et al. 2006a). Klebsiella, streptococci and staphylococci have been isolated from the teat ends of dairy cows (Rendos et al. 1975). Lactococcus spp., yeasts and Pseudomonas spp. have also been isolated from dairy cow teats and udders, although there was no correlation between the level of Lactococcus spp. and yeasts in milk and on the teats and udders (Desmasures et al. 1997). Supré et al identified the same Staphylococcus devriesei strain from teat apices and the milk of dairy cows (Supré et al. 2010). In a study of 11 ewes, Mannheimia haemolytica was isolated from the teat duct only after suckling, indicating it had been transmitted to the teat duct during suckling by the lamb (Gougoulis et al. 2008b). Bacteria such as Staphylococcus chromogenes, Staphylococcus hyicus, and Staphylococcus aureus have been isolated from teat canal keratin and milk secretions. In addition, quarters from cows with teat canal colonisations of bacteria without a clinical intramammary infection had a higher somatic cell count than quarters from cows without teat canal colonisation and intramammary infections, suggesting the importance of teat canal colonisation on the health of the mammary gland (Trinidad et al. 1990). 19 1.6.7. Other potential reservoirs of mastitis-causing pathogens Strains of Staphylococcus aureus predominant on some farms were isolated from flies collected in the barns of lactating cows and milking equipment (Capurro et al. 2010). The infected udder gland is also a reservoir for transmission between individual udder halves (Mørk et al. 2007, Vautor et al. 2003). Although these studies provide accumulating evidence for reservoirs and transmission routes of pathogens associated with intramammary infections, molecular methods at the subspecies level over time are required in order to ascertain whether the isolates are the same strain type, therefore proving a transmission event has occurred. 1.7.The protective role of bacterial species In recent years, the potential protective role of coagulase-negative staphyloccci in mastitis in dairy cows has been a subject of debate, with contradictory results having been published. Some authors suggest that quarters infected with minor pathogens, such as coagulase-negative staphylococci are preventive for natural infections by major pathogens. (Rainard & Poutrel 1988, White et al. 2001). For example, in a challenge study, protective effects against Staphylococcus aureus infection were found using Staphylococcus chromogenes (Matthews et al. 1990). In vitro studies have supported this, showing the inhibition of Staphylococcus aureus and streptococci growth by Staphylococcus chromogenes (De Vliegher et al. 2004). Furthermore, some strains of coagulase-negative staphylococci were shown to inhibit the growth of Streptococcus agalactiae, possibly due to increased somatic cell count in the milk or antibacterial peptides produced by the bacteria (dos Santos Nascimento et al. 2005, Pyörälä & Taponen 2009). Conversely, studies have found that

heifers infected with coagulase-negative staphylococci pre-calving increased the risk for post calving infection with coagulase-negative staphylococci, Staphylococcus aureus or Streptococcus uberis (Compton et al. 2007, Parker et al. 2007). Alternatively, one study has shown that quarters infected with Staphylococcus spp. were less susceptible to experimental 20 challenge with certain species, such as Staphylococcus aureus, but more susceptible to Streptococcus agalactiae (Nickerson & Boddie 1994). Finally, some studies have shown no protective or predisposing role of coagulasenegative staphylococci in mastitis caused by Staphyloccocus aureus, or Streptococcus uberis (Hogan et al. 1988, Zadoks et al. 2001). 1.8.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 in the milk throughout lactation in cows once bacteria have penetrated the mammary gland. In addition, much of the research on ovine mastitis 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, and therefore classification of isolates at the species level can incorrectly oversimplify control measures recommended, hence the need for longitudinal strain typing methods. 1.8.1. Infection persistence during lactation Recent studies have used strain typing techniques such as pulsed-field gel electrophoresis to isolate the same strain of bacteria from the same mammary quarter on more than one occasion indicating the persistence of the same infection over time with coagulase-negative staphylococci based on a study of 12412 milk samples from 3 dairy research herds (Gillespie et al. 2009). In fact, coagulase-negative staphylococci were shown to persist 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% (Döpfer et al. 1999, Lam et al. 1996). In addition the occurrence of recurrent episodes of the same strain in any quarter in a cow is 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 21 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 such as Listeria moncytogenes, are able to persist in milking parlor environments which could be a source of reinfection (Ho et al. 2007). The ability of Staphylococcus aureus to produce biofilm could be why mastitis associated with this species of bacteria so often becomes chronic and persists. The role of exopolysaccharride (slime) in the colonization and virulence in bovine mastitis has been shown

in cows (Cucarella et al. 2004). It is thought to be involved in the enhancement of initial attachment to epithelial cells and in the subsequent formation of micro-colonies, as has been shown for Escherichia coli strains (Chan et al. 1982). 1.8.2. The role of the dry period The bovine mammary gland is thought to be particularly susceptible to 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). Experimental studies have shown the ability of pathogens to remain within the udder, causing clinical disease after the onset of lactation (Bradley 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 Enterobacteriaceae 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 (Bradley 2002). Of all the enterobacterial mastitis occurring in the first 100 days of lactation, 52.6% arose in quarters previously colonised with the same strain of 22 bacteria during the dry period (Bradley & Green 2000). This suggests that chronic infections should be considered and environmental management during the dry period is imperative in the control of environmental mastitis. Bradley and Green (2001) observed 6 commercial herds over a period of 12 months in order to identify changes in the behaviour of Escherichia coli. DNA fingerprinting allowed the identification of the genotypes of strains involved in recurrent cases of clinical Escherichia 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 in excess of 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 strain types from the same 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 infections during the dry period, and clinical mastitis in the next lactation. The probability of an udder quarter developing clinical mastitis increased when Streptococcus dysgalactiae, Enterococcus faecalis, Escherichia coli, or Enterobacter spp. were cultured at drying off. In addition, the risk of clinical mastitis caused by 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 confirm the persistence of certain strains in the mammary gland, it provides evidence for the significance of the dry period on clinical mastitis development. 23 1.9.The importance of strain typing Throughout this review, strain typing has been shown to be important in studies of reservoirs, transmission and persistence. However the importance of strain typing is also highlighted when host responses are considered. Host responses to intramammary infections differ between pathogen species and strains (Zadoks & Fitzpatrick 2009). In particular, Staphylococcus aureus is particularly difficult to control due to its wide pattern of virulence factors such as leukotoxins, which can selectively destroy host polymorphonuclear leukocytes (PMN) and monocytes and secretion of exopolysaccharides and thermostable toxins (Craven & Williams 1985). As many as 30 extracellular products are produced by Staphylococcus aureus (Rogolsky 1979). This includes several toxins that contribute to the pathogenesis of mastitis (Rogolsky 1979). There also appears to be differences between the toxins produced by strains of Staphylococcus aureus that are isolated from ewes with subclinical mastitis and clinical mastitis. For example, toxins produced from Staphylococcus aureus isolates from sheep with subclinical mastitis were less enterotoxigenic than those from acute clinical cases (de Santis et al. 2005, Hamann 2005). 1.10.Methods used to understand mastitis 1.10.1. Molecular epidemiology In order to study disease in populations and identify factors that determine its occurrence, the integration of molecular biological techniques and epidemiologic research is required. Epidemiological research identifies incidence, prevalence and patterns of disease at the population level. A longitudinal study can be used to investigate an outcome and exposure variables over a period of time. Such studies are particularly useful to study the progression to an outcome of interest over time, providing temporal associations between the outcome and exposure variables. Causal associations can be deduced more robustly in a longitudinal study, with statistical evidence for these associations (Hill 1965). Such studies are also flexible and can test a variety of hypotheses simultaneously. However, longitudinal studies are expensive and time-consuming. In 24 addition, data that are collected repeatedly over time from the same individual result in clustered data that need to be managed during analysis. Different strains within a bacterial species can vary in terms of reservoir, transmission pathway, persistence and pathogenicity. Strain typing is therefore necessary to identify sources and transmission routes of mastitis-causing bacteria and to choose appropriate prevention and treatment measures. Although contributing to our understanding of mastitis, molecular epidemiological studies have not yet reached a stage where strain typing is time and cost effective enough to be used in real time to contribute to outbreak analysis and prevention and/or treatment options. Ideally, a method of strain typing that is rapid and cost effective is required in order for large numbers of samples from numerous animals to be analysed. 1.10.2.

Laboratory based techniques Culturing of bacteria There are a variety of methods used to identify mastitis causing pathogens. Traditional culture techniques are often the first step in identification of an isolate, in order to culture pure growth. Biochemical tests and Gram stains are often used to identify bacteria to the genus or species level. Polymerase Chain Reaction (PCR) amplification In the past, commercial kits differentiated species of bacteria based on specific biochemical reaction characteristics of each. These methods, however, were based on phenotypic reactions and often failed in the identification of different species of coagulase-negative staphylococci (Heikens et al. 2005). In order to overcome these issues, alternative identification methods based on molecular rather than phenotypic characteristics were explored. Several genomic targets have been used for the molecular identification of different bacterial species, most commonly the genes for 16s rRNA (Onni et al. 2010). The majority of mastitis causing pathogens in cows can now be detected based on PCR which detect the main species-specific DNA sequences. Techniques have been developed to identity a single species (uniplex) or multiple species simultaneously 25 (multiplex) (Gillespie & Oliver 2005, Phuektes et al. 2001, Riffon et al. 2001). Recent advances have resulted in a multiplex PCR to be developed for the simultaneous detection of up to 10 bovine mastitis pathogens (Shome et al. 2011) and is now being used to detect pathogens found in bulk tank milk samples (Boss et al. 2011). In sheep, coagulase-negative staphylococci have been identified from ovine milk samples via PCR amplification of the staphylococcal 16S rRNA and gap genes (Onni et al. 2010) whilst Staphylococcus aureus has been identified via PCRRestriction Fragment Length Polymorphism Analysis of the aroA Gene (Marcos et al. 1999). Both the biochemical and PCR methods described above can identify isolates to species level in some cases. However in order for multiple isolates to be identified to strain level, the following methods are most often used. Pulsed-field gel electrophoresis (PFGE) Pulsed-field gel electrophoresis is a method of typing an organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The bacterial genome is digested with a restriction enzyme that has relatively few recognition sites; generating fragments from 10-2000kb on the agarose gel unlike traditional gel electrophoresis whereby fragments of 0.5-25kb are resolved. Pulsed-field gel electrophoresis makes use of a current being applied in three directions to three electrodes, resulting in a higher level of fragment resolution (Vautor et al. 2005b). Multilocus sequence typing (MLST) Multilocus sequence typing (MLST) involves sequencing fragments of housekeeping genes (most commonly 7 housekeeping genes) from different strains of the same organism and comparing them. For each gene, a sequence of approximately 450bp is amplified (PCR) and then sequenced, which is presented in a dendrogram. Alleles at each locus based on nucleotide sequence are assigned. Multilocus sequence typing could be used to track a virulent strain of a bacterial pathogen as it moves through a population (Vautor et al. 2005b). 26 Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a relatively novel

ionization technique which allows the analysis of biomolecules. Co-precipitation of a UV-light absorbing matrix and a biomolecule is vaporized by a laser pulse. The matrix becomes ionized when it absorbs most of the laser energy (preventing unwanted fragmentation of the biomolecule). The energy is 'passed from the matrix' to the vaporized protein molecules which are accelerated in an electric field, drawn into the evaporation chamber into the mass spectrometer and are separated according to their mass to charge ratio by reaching the detector at different times. Each molecule yields a distinct signal (De Bruyne et al. 2011). MALDI-ToF-MS, due to its speed and low running costs, has the potential to replace conventional phenotypic identification. In addition, several recent studies have indicated MALDI-ToF-MS could be used for rapid strain differentiation and identification for Escherichia coli (Christner et al. 2014, Karger et al. 2011), Yersinia enterocolitica (Stephan et al. 2011), Salmonella enterica (Dieckmann & Malorny 2011), Clostridium difficile (Reil et al. 2011), Staphylococcus aureus (Boggs et al. 2012, Wolters et al. 2011), Propionibacterium acnes (Nagy et al. 2013), Listeria spp. (Barbuddhe et al. 2008), Leptospira spp. (Rettinger et al. 2012), Saccharomyces cerevisiae (Moothoo-Padayachie et al. 2013), Mycobacteria spp. (Hettick et al. 2006), and Arthrobacter spp. (Vargha et al. 2006). Due to the application of this method to strain typing being very recent, it is not typically used for strain typing studies and has not been used to support epidemiological studies. 27 1.10.3. Epidemiological techniques 'Epidemiology is the study of disease in populations and of factors that determine its occurrence' (Thrusfield 2013). Cross-sectional studies Cross-sectional studies allow the examination of the relationship between disease prevalence and exposure at a single point in time in a given population. The crosssectional study in this thesis utilises a questionnaire. Questionnaires are particularly useful when there is no previous information readily available on a subject. They are designed to record information in a standardised format and the aim is to achieve a high response rate. The advantages of using a postal questionnaire include a wide coverage of a population and the fact that they are quick to administer and avoid interview bias. However, there is no way to check if a question is misunderstood. A pilot study can ensure that questions are understood, and if not feedback can expose defects in the questionnaire that can be amended (Thrusfield 2013). The advantages of using a cross-sectional study to examine the risk factors of mastitis include the large population size. As the questionnaire is sent to a large number of farms with different management practices, a whole array of data can be obtained. The large sample, and the large amount of data obtained from this population can produce a wealth of information on the study of risk factors. However, often questionnaires, as in the current study, collect information retrospectively. This can result in less accurate information as respondents will not always remember management practices/detailed information about their farm and flocks over a year ago. However, these studies are hypothesis generating rather than testing cause and effect associations. Longitudinal studies A longitudinal study can utilise a group of individuals exposed to a particular disease compared with an unexposed group by observing

each for a new disease, in this case an episode of mastitis. In this thesis, a cohort of ewes is followed and samples taken over time. Despite the potentially long duration for follow-ups and the expense, 28 cohort studies are flexible and can be used to test a variety of hypotheses simultaneously. 1.11.Thesis aims Based on the findings of the literature, the overall aim of this thesis was to improve our understanding of the development and persistence of intramammary infections. In order to address this aim, the following objectives were implemented: 1. To identify management practices that might act as risk factors for clinical mastitis (Chapter 2) 2. To investigate the use of MALDI-ToF-MS as a diagnostic tool for species identification and strain differentiation in order to: a. Identify reservoirs of mastitis causing pathogens on the skin of ewes (Chapter 3) b. Observe the carriage of these bacteria into the udder, which might lead to mastitis and identify whether bacteria persist over time in the mammary gland (Chapter 4) 3. To investigate whether udder and teat conformation are heritable traits that affect mastitis (Chapter 5)

3. Materials and methods

Chapter 2 methods

The number of sheep holdings in England in the 2003 census was 45,801 (DEFRA 2003). Based on this, the sample size required was 385, assuming at least 50% of flocks had at least one case of clinical mastitis, with 95% confidence and 90% power (Win-Episcope-2 2010). Assuming a response rate of 30% (Kaler & Green 2008), 999 farmers whose details were provided by EBLEX, the levy body for English sheep and beef farmers, were contacted in January 2010.

1.1.1. Design of the questionnaire

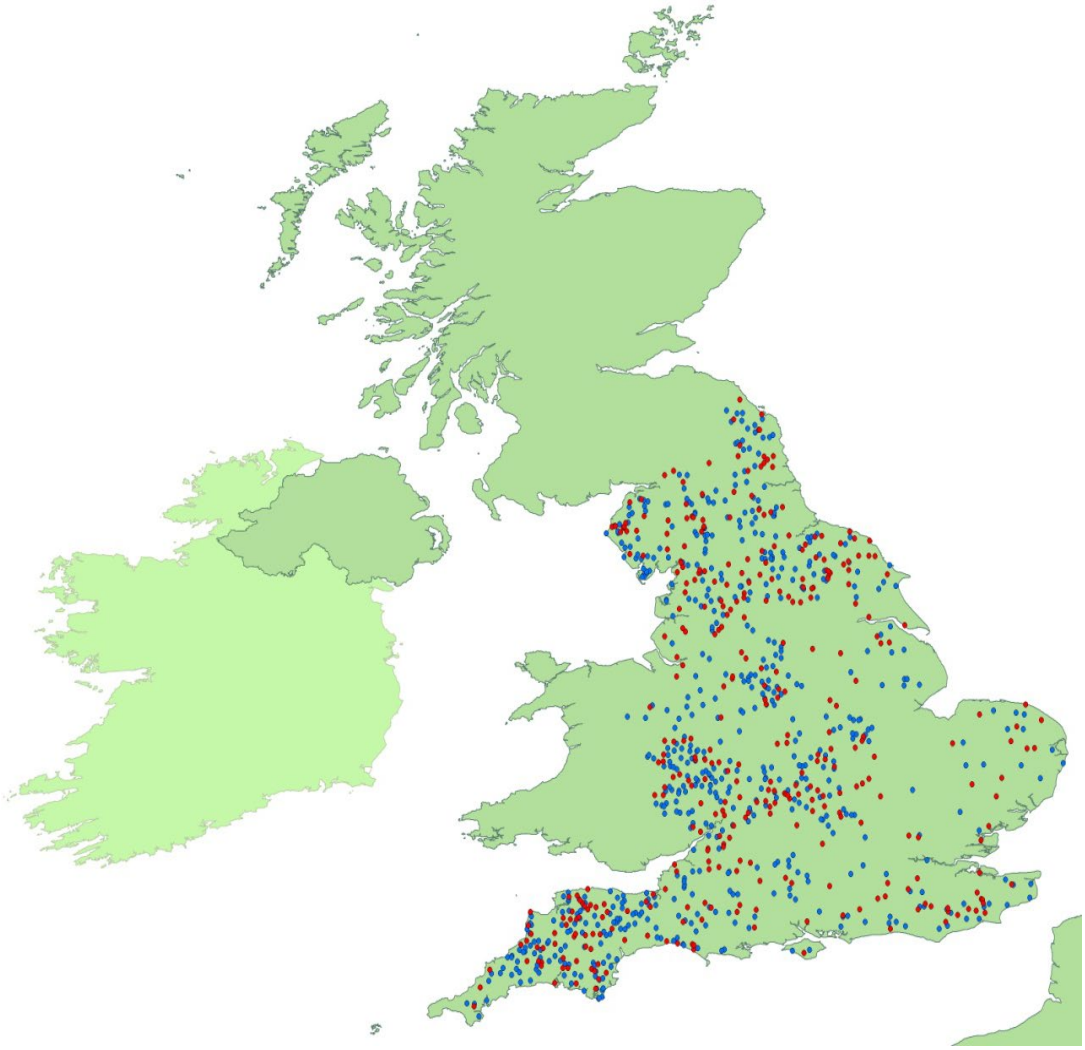
Published literature and veterinary expertise on risk factors for mastitis in sheep and cattle was used to design the postal questionnaire (Cooper 2011). Questions were based on the farm, flock, ewes, management regimes, mammary gland health, nutrition and housing. There were a total of 114 questions. The majority of questions were closed or semi-closed, however, there were some open questions, these included whether farmers thought that their ewes got clinical mastitis in certain fields, whether the farmer had changed farm management between 2008 and 2009 and opinions on causes of mastitis and preventive actions.

1.1.2. Pilot study

The pilot questionnaire was sent to 12 convenience selected farmers in the north of England that included commercial and pedigree flocks situated in lowland, hill and upland areas with between 50 and 1000 sheep. As a result of the feedback from the pilot study several additional questions were added to the questionnaire, and questions that had poor response rates or were answered incorrectly were re-designed.

1.1.3. Data collection & storage

The final questionnaire was sent out on 8th January 2010, a reminder was sent to non-respondents on 10th February 2010 and a second reminder and a second copy of the questionnaire were sent to non-respondents on 21st April 2010.
A database was designed in Microsoft Access 2007. Data were entered using multiple choice drop down boxes and checked. The postcodes from the 999 farmers were transformed into X and Y co-ordinates and inputted into ArcView with the worldwide shapefile from the Economic and Social Research Institute (ESRI) to create a map of respondents and non-respondents (Figure 2.1).



1.1.4. Data Analysis

Measures of dispersion and central tendency were used to investigate the data (R Core Team 2013). Normality was tested using Shapiro-wilks test and the arithmetic or geometric mean was calculated for variables in R. Obvious errors were corrected, and categories within variables with <5 responses were merged where logical. Queries were used to select and link data from related databases in Microsoft Access to select data for statistical analysis. Respondents with ≤20 ewes

in their flock were removed from the analysis (*n*=4). Analysis of variance (ANOVA) was used to test the differences between group means in R.

The incidence rate of clinical mastitis (IRCM) per flock was calculated. The variance was greater than the mean and so over-dispersed Poisson regression models, offset by flock size, were used to investigate factors associated with IRCM. A total of 144 variables were used to investigate management from 8 weeks before lambing, during lambing and during lactation. Farmers managed sheep either wholly indoors or outdoors or a combination of both, as a consequence 6 separate models were necessary. Model 1 included all respondents and covered general information about the farm, flock, lambing, mastitis, health management and nutrition. Model 2 included flocks housed in barns from up to 8 weeks before lambing to lambing. Model 3 included flocks housed during lambing, and Model 4 included flocks housed after lambing. Model 5 included flocks outdoors during lambing, and Model 6 included flocks outdoors after lambing. Outliers were assessed to determine their impact on the coefficients.

The models took the following general form:

$$g(E(Y) = \beta_0 + \sum \beta_m x_m - \log(O_i)$$

...where g is the log-link function, E(Y) the expected values of the outcome variable Y (the number of ewes with clinical mastitis in 2009), β_0 the intercept and βm the regression coefficients (expressing effects of the included predictor variables x_m), O_i as the offset (the number of ewes in the breeding flock in 2009).

Risk ratios were exp(β). To estimate the 95% confidence interval for exp(β), the standard error (S.E.) was calculated as the two-sided confidence coefficient assuming a normal distribution and multiplied by the standard error from the model: exp(β) ± 1.96 × S.E.

Chapter 3 methods

1.1.5. Flock and animals

A flock of approximately 100 pedigree Texel suckler ewes was selected. Twenty-eight milk samples from 27 ewes with clinical mastitis were collected by a veterinarian in August 2010. In October 2010, a further 5 milk samples were taken from 3 ewes with no signs of clinical mastitis and 14 udder skin swabs were collected from 10 ewes (8 from 7 clinically infected ewes and 6 from 3 clinically uninfected ewes).

1.1.6. Sampling

Milk

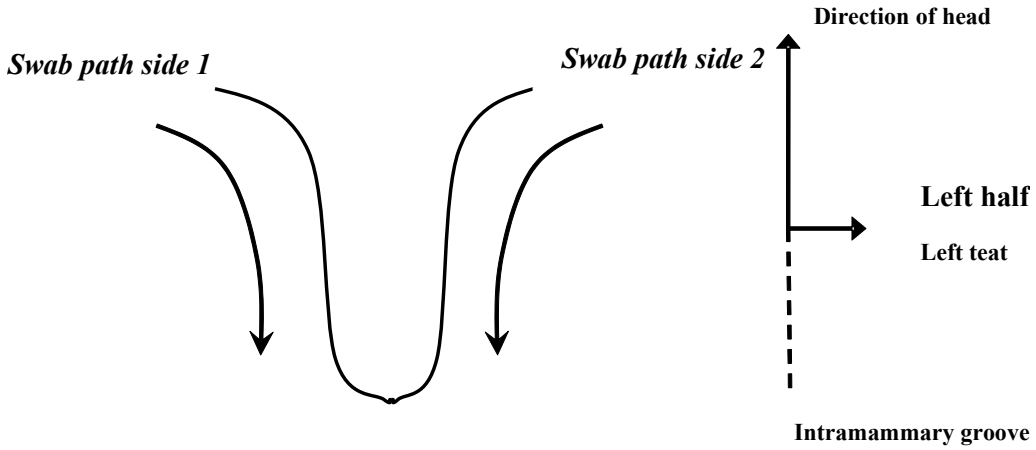
Milk samples (approx 5 ml) were taken in August 2010 by a veterinarian into a sterile universal and transported to the University of Warwick on ice. Milk samples were taken from the affected half in

ewes with clinical mastitis. In one case, both udder halves were affected and so two milk samples were taken. For those milk samples collected in October 2010, the teats were disinfected with 70% ethanol wipes and the first streams of milk discarded. Udder half milk samples (approx 5 ml) were aseptically collected into pre-labelled universal tubes and stored with glycerol (10% v/v). In 2 of 3 ewes without signs of clinical mastitis, milk samples from both halves were taken.

Udder skin swabs

The ewe to be tested was turned over and swabs were placed in 1 ml of Phosphate buffered saline (PBS) (to facilitate collection of viable bacteria from the dry surface of the teat) and then run along the outside of the teat base to tip, twisted and then run along the inside of the teat (Figure 3.1). The tip of the teat was not tested as this was the area held to keep the teat steady. Swabs were immediately broken off into 1 ml of Brain Heart Infusion media (BHI) (3.7% w/v Sigma-Aldrich) and glycerol (10% v/v) into a cryovial tube. Udder skin samples were taken from both halves with the separate swabs.

Figure 3.1: The technique used to take a teat skin sample with a swab.



1.1.7. Swab and milk sample storage

Milk (with glycerol added to 10% v/v) and skin swab samples (in BHI and 10% v/v glycerol) were stored and transported on ice to the laboratory (University of Warwick). They were then stored at -20°C until analysis.

1.1.8. Growth and selection of isolates from agar plates

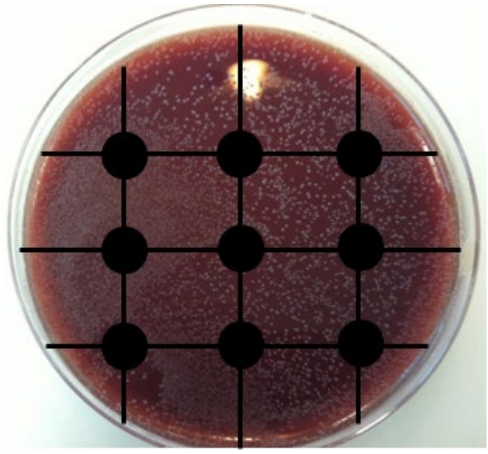
Milk

In July 2011, milk samples were thawed at room temperature and 100µl of milk were spread across a sheep's blood agar (SBA) plate (3.7% w/v BHI and 1.5% w/v Bacto Agar (Sigma-Aldrich 2014))

containing 5% (v/v) sterile defibrinated sheep's blood (Oxoid 2014). Plates were incubated inverted at 37°C and checked at 24 and 48 hours.

At least one of each morphologically unique isolate was selected for further analysis. For plates with heavy growth of morphologically identical isolates, up to 9 of each morphologically unique isolate were picked from 9 locations on the plate where 3 gridlines vertically and horizontally met (Figure 3.2).

Figure 3.2: Location of isolates from milk picked where there was heavy growth.

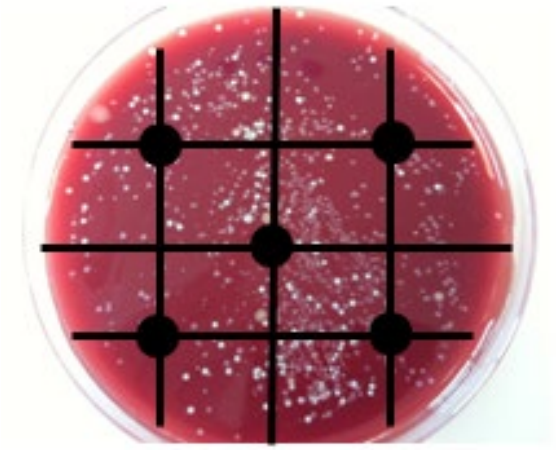


Udder skin swabs

In July 2011, udder skin swab samples in 1 ml of BHI and glycerol (10% v/v) were thawed at room temperature. Once thawed, the cryovial tube was mixed gently by pipetting and 100µl was spread across a sheep's blood agar (SBA) plate containing 5% (v/v) sterile sheep's blood. Plates were incubated inverted at 37°C and checked at 24 and 48 hours. At 24 hours (and 48 hours if new colonies had grown between this time), individual colony types were selected for further analysis.

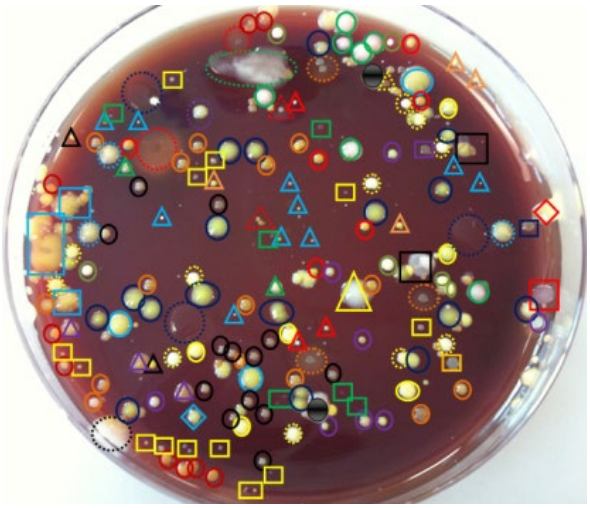
At least one of each morphologically unique isolate was selected for further analysis. For plates with heavy growth of morphologically identical isolates, up to 5 of each morphologically different isolate were picked from 5 locations on the plate (Figure 3.3).

Figure 3.3: Location of isolates picked from udder skin swabs where there was heavy growth.



For plates with >3 of a variety of morphologically unique isolates (example shown in Figure 3.4), plates were photographed and annotated in Microsoft Word, with morphologically identical isolates highlighted with the same colour and shape (Figure 3.4). Isolates of all distinct morphological types were selected after plate annotation.

Figure 3.4: Example of annotation of morphologically similar isolates.



Contrary to dairy industry standards, which assume milk sample plates with >3 morphologically distinct colonies are contaminated, there was no number of distinct colonies types that were defined as “contamination”. This was particularly relevant to the highly diverse skin swabs.

1.1.9. Isolate culturing and storage

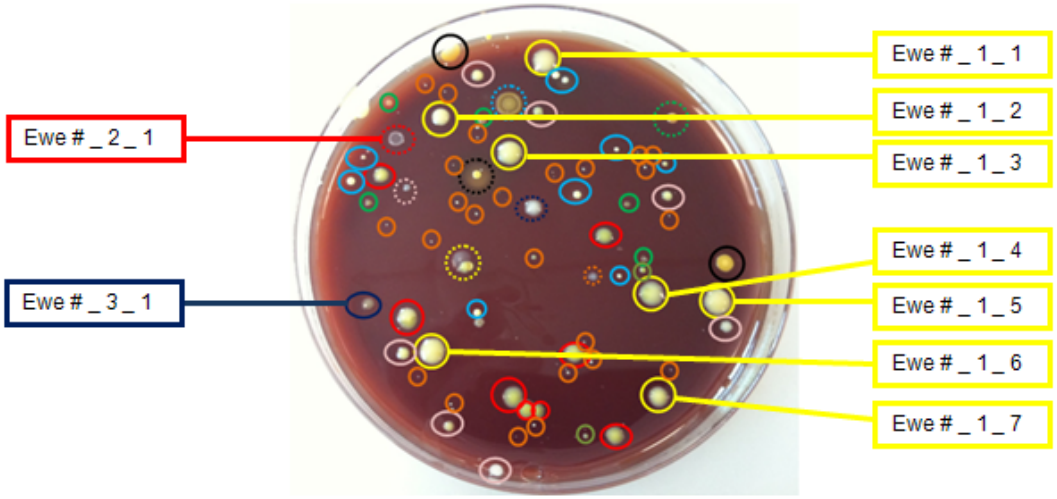
Once selected, individual colony types from milk and udder skin samples were sterile streaked using a sterile loop on a brain heart infusion plate (BHI) and incubated inverted at 37°C and

checked at 24 and 48 hours. A single colony was then picked and sub-cultured onto one quadrant of a BHI plate to provide sufficient culture for further testing (Figure 3.5).

Figure 3.5: Workflow from sample plate, to sterile streaking to quarter plating.

Plates were labelled with ewe name, udder half (if applicable) and sample type (udder skin swab or milk). For a single plate, isolates were identified by a system highlighted in Figure 3.6. Therefore, **ewe** = the ewe number, morphologically unique **isolate** = the second number in the series, which would indicate a morphologically unique isolate and **colony** = isolates with the same morphology but which had separate colonies.

Figure 3.6: Example of the annotation and labelling of a single milk or skin sample from one ewe.



A 1µl loop of growth from each quadrant was placed in 5 ml of BHI and glycerol (10% v/v) in a glass universal and incubated at 37°C with vigorous shaking at 200 revolutions per minute (rpm) until turgid (usually at least 24 hours). A 1 ml aliquot of the incubated culture was transferred into a cryovial tube, labelled, snap frozen in dry ice and stored at -80°C.

1.1.10. *Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry* analysis of isolates

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) allows the analysis of biomolecules by vaporizing protein molecules with laser energy, yielding

distinct signals (mass spectra) for each sample which can be compared to a database to identify bacteria to the species level.

Fresh growth of isolates was prepared prior to MALDI-ToF-MS analysis at QMMS, Somerset.

Sample preparation

Direct transfer

A cocktail stick was dabbed into a distinct single colony and zig-zagged across two sequential spots on a Bruker 96 well sample plate. Matrix (1µl) was placed on top of each sample. Matrix was made up using 1 tube of Bruker "HCCA matrix portioned" (Bruker Daltonics) and 250 µl of an “organic solvent” (50% acetonitrile (AN), 2.5% trifluoracetic acid (TFA) and 47.5% deionised H_2O). The prepared matrix was stored in the dark at room temperature and used within 2 weeks.

The bacterial test standard (BTS) (Bruker Daltonics) was used as a control. BTS was made up using Bruker BTS portioned sample, and 50µl of organic solvent. The direct transfer method was used initially for each isolate.

Extraction method

For some isolates, analysis using the direct transfer method did not produce suitable spectra (usually characterised by a high background and low peak intensity). In these cases the extraction method was used to prepare a sample for MALDI-ToF-MS analysis and so obtain a clearer spectrum. 300 µl of distilled H_2O was aliquoted into an Eppendorf tube. A single colony was picked from a quadrant streaked plate using a 1 µl sterile loop and placed into the tube. 900 µl of ethanol was added and mixed thoroughly. This solution as then centrifuged at 13,000 rpm for 4 mins and the supernatant discarded. This was repeated to remove residual Ethanol and the pellet was left to air-dry for up to 30 minutes. 50 µl of 70% formic acid and 50 µl of pure acetonitrile was added to the pellet in a fume hood and pipette mixed. This was centrifuged at 13,000 rpm for 2 mins. The supernatant (1µl) was placed onto the target plate in duplicate and allowed to air dry. This was overlaid with 1 µl of matrix solution and air dried.

Species identification

Sample plates were then analysed using MALDI-ToF-MS following the manufacturers instructions. The spectra produced by MALDI-ToF-MS were automatically compared to every spectra in the Bruker database. A species name for each isolate and a ‘confidence score’ were assigned to each isolate. The score value for each isolate was automatically calculated by MALDI Biotyper 3.0 (utilising Matlab), using a matching algorithm that computes different characteristics of two spectra

to compare patterns within the spectra. Between two spectra, the number of signals that have a closely matching partner, and the symmetry of the matching signal pairs are determined. If the number of signals and the intensity of signals of each spectrum correspond, this results in a high matching value. Bruker Daltonics suggests a conservative score of 2.0+ to be used for species identification. In this study, a score of 1.7+ was used as a cut-off point due to the additional confirmation of species identification through the number of technical repeats, culture morphology, Gram staining results and use of Pulse Field Gel Electrophoresis.

The results were saved in HTML format and transferred into Microsoft Access. Queries were used to merge technical repeats, allowing the top score for each colony to be identified. Isolates with at least one score of ≥1.7 were included in analysis. The species level identification was also compared to previous laboratory analyses (culture morphology, and Gram stain) to ensure accuracy.

Strain differentiation

Data analyses were carried out in MALDI Biotyper software 3.0, ClinProTools, and Flex Analysis. Several parameters for dendrogram and correlation coefficient indices (CCI) production in MALDI Biotyper 3.0 were tested, and the literature was reviewed. Dendrograms (Distance measure: correlation, Linkage: average, Score) were used to cluster isolates into groups and CCI (Mass 3000-12000, Resolution: 4) were used to compare isolates of interest. Ultimately only CCI images were used as values were absolute, whereas dendrogram distances changed depending on the isolates inputted into the analysis. CCI matrices were colour coded according to Microsoft Excel conditional formatting to easily view isolates that were very similar (high CCI estimates in red) or very different (low CCI estimates in green).

Isolates from species found in abundance on both udder skin and in milk were taken forward for further analysis (Pulsed field gel electrophoresis) including isolates identified as *Staphylococcus xylosus, Staphylococcus equorum, Staphylococcus warneri, Rhodococcus corprophilus, Micrococcus luteus,* and *Bacillus pumilus.*

1.1.11. Pulsed field gel electrophoresis

The typing method is considered the gold standard for analysing a number of organisms. The protocol was based on McDougall *et al.,* (2003). In brief, cells grown to a specific optical density were immobilised in low melting point agarose gel plugs and the immobilised cells were enzymatically lysed within the gel matrix. Cell debris was then washed from the plugs leaving behind essentially intact DNA molecules in the plug. The method was specifically optimised for all species found on both udder skin and in milk to enable high quality PFGE analysis in future studies.

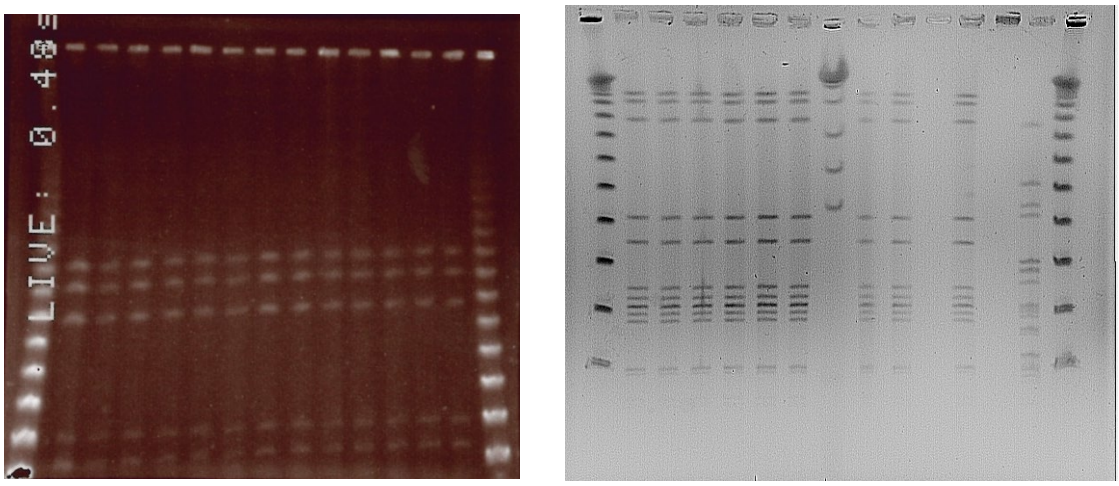
Several parts of the method, based on McDougall *et al.,* 2003, were optimised in order to improve PFGE gel quality and subsequent analysis (Table 3.1). An example of the original versus optimised gel image show tighter bands, suitable run time, and optimal image acquisition (Figure 3.7).

Table 3.1: Optimisation parameters compared to original method McDougal et al., 2003 and the improvements these optimisations had.

PFGE stage	PFGE Parameter		Original	Final	Reason
Plug production	Growth media		5 ml BHI	5 ml BHI and glycerol (10% v/v)	Improved growth of all isolates
	Cell concentration		Abs 0.9-1.1 (OD_{610})	Abs 0.4-1.1 (OD_{610})	Altered depending on genus, as too high an OD meant too many cells were in the plugs which hindered lysis, or too low an OD meant bands would not be visible
	Plug agarose concentration		1.8%	1.2%	Improved final gel image (possibly due to lysis and restriction digest being better with a less concentrated agarose)
	Equilibration of culture and agarose		10 mins at 37°C	25 mins at 50°C	Improved lysis, and ensured cell suspension was at a similar temperature to agarose improving mixing and encouraging uniform plugs.
Lysis	Addition to 300µl cell suspension	Proteinase K (20mg/ml)	None added	25µl (all species apart from staphylococci)	Improved lysis and therefore final gel image for all but staphylococcal species.
		Sodium dodecyl sulfate (SDS) (10% w/v)	None added	25µl (all species apart from staphylococci)	Improved lysis and therefore final gel image for all but staphylococcal species.
		Lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate)	4µl	3µl (staphylococcal spp. only)	3µl was a suitable amount to ensure cell lysis
		Lysozyme (20mg/ml)	NA	20µl (all species apart from staphylococci)	Improved lysis and therefore final gel image
	Addition to 3ml lysis buffer	Proteinase K (20mg/ml) and Lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate)	None added	Added	Addition of proteinase K and lysozyme in the lysis buffer further improved lysis.
	Lysis incubation time		4 hr	Overnight	Improved lysis

PFGE stage	PFGE Parameter	Original	Final	Reason
Wash	Number of washes	4	6	Additional washes were required for ensure Proteinase K and SDS were removed
	Composition	TE only	TE X 4, ultrapure sterile water x 2	Ultrapure sterile water washes reduced the smearing of gel images.
Restriction digestion	BSA	NA	1%	Improved restriction digest substantially reducing time needed to achieve complete digestion
	Digestion temp	25°C	30°C for staphylococcal species, 37°C for all others	Allowed complete restriction digest
	Digestion incubation length	2-3hr	Overnight for staphylococcal species. 4 hours for all other species.	Allowed complete restriction digest
Running parameters	Gel concentration	1%	1.2%	Encouraged clear band separation (especially for bands that were extremely close) and reduced smearing.

Figure 3.7: Original versus optimised PFGE gels



Original **Optimised**

Final method

One colony was transferred from a quadrant streaked plate to 5 ml BHI and glycerol (10% v/v) liquid broth in a glass universal. This was incubated for varying times and temperatures in a shaking incubator (170 rpm) depending on the genus of the isolate (Table 3.2).

Table 3.2: Incubation time and temperatures by genus.

Genus	Temperature	Time
Bacillus	25°C	24 hours
Micrococcus	25°C	24 hours
Rhodococcus	25°C	48 hours
Staphylococcus	37°C	24 hours

Plug production

200 μl of liquid culture was transferred to a fresh glass universal containing 5ml BHI and glycerol (10% v/v) for the same time, at the same temperature. The OD_{610} of the cell suspension was adjusted with PBS (Table 3.3).

Table 3.3: Final concentration of cell suspension range by genus.

Bacterial genus	Concentration of cell suspension @610nm
Bacillus	0.800-1.000
Micrococcus	0.800-1.000
Rhodococcus	0.400-0.700
Staphylococcus	0.900-1.100

The cell suspension (200 μl) was centrifuged at 12,000 rpm for 3 minutes. The supernatant was aspirated and the pellet resuspended in 300μl of 1 ml Tris-EDTA (TE) buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]).

Preparation of bacterial cell agarose plugs and subsequent cell lysis

Staphylococcal species: 4μl of conventional lysostaphin stock solution and 296μl of molten 1.2% SeaKemGold agarose in TE buffer [pH 8] were added to the cell suspension, gently mixed and dispensed into the wells of a plug mould.

All other species: 20μl of lysozyme (20mg/ml), 3μl of lyzosyme (1mg/ml), 25μl of 10% Sodium dodecyl sulfate (SDS), and 25μl of Proteinase K (20mg/ml) were added to the cell suspension and incubated at 50°C for 25 minutes. 227μl of 1.2% SeaKemGold agarose in TE buffer [pH 8] were added to the incubated cell suspension and gently mixed, and dispensed into the wells of a plug mould.

Plugs were left to solidify at room temperature for 10-15 minutes and then removed from the plug molds and placed in a sterile plastic bijoux containing 3ml of lysis buffer (6 mM Tris HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroylsarcosine, 0.83mg/ml Proteinase K, 0.83mg/ml lysozyme). The tubes were then incubated overnight at 37°C in a shaking incubator (190 rpm). The plugs were transferred to a sterile plastic bijoux containing 3ml of TE wash and incubated in a shaking incubator at 37°C for an hour. This was repeated four times. The plugs were washed in the same manner with ultrapure sterile water twice. Digested plugs were stored in fresh TE at 4°C.

Restriction digestion of extracted DNA in agarose plugs

A whole plug slice was then transferred to a sterile eppendorf containing the restriction enzyme, restriction buffer and BSA for varying lengths of time depending on the genus, and restriction enzyme (Table 3.4).

Gel preparation and agarose plug loading

In order to prepare the gel, SeaKemGold agarose was prepared in 0.5X TBE (1.2%) and poured into the gel mold, which was left to solidify for an hour. The comb was removed and plug slices were washed in ultrapure sterile water, dried and loaded into wells.

PFGE running parameters

PFGE was performed using a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF Mapper, Bio-Rad, USA). Running parameters were as follows: 200 V (6 V/cm); temperature, 14°C; initial switch, 5 s; final switch, 40 s; which changed over the entire run and time, 21 h. Gels were stained in 0.5mg/ml ethidium bromide solution for 30 minutes in a covered container and then destained in distilled water for 1 hour. The water was changed twice during this time. Gels were viewed by UV transillumination and the images stored for further analysis.

Table 3.4: Restriction digest enzymes, amounts, temperatures and lengths by genus.

Bacterial genus	Enzyme	Amount (µl)				Temperature (°C)	Time (hours)
		Restriction buffer 10X	H_2O	BSA (1%)	Restriction enzyme		
Staphylococcus	SmaI	20	180	6	3	30	18hr
Rhodococcus	AseI	20	180	5	5	37	4h
Bacillus	ApaI	20	180	5	5	37	4h
Micrococcus	XbaI	20	180	5	4	37	4hr

1.1.12. Data management

GelCompar II (Applied Maths, Belgium) was used to cluster isolates. Gel images were imported into the software, and edited to select lanes, bands, curves and to normalise between gels using ladders on either side of the gel. Clustering (Band based: different bands, Dendrogram type: UPGMA (unweighted pair group mean average)) was carried out to cluster isolates of the same or different PFGE types. Isolates were considered to be different strain types if they had ≥1 band difference (dendrograms did not have 100% similarity for those isolates). This is because isolates that are indistinguishable by PFGE are likely to have been derived from a common parent (Tenover *et al.* 1995), which is necessary in terms of outbreak, reservoir and transmission analyses.

Dendrograms produced from GelCompar II were compared to CCI indices from MALDI Biotyper 3.0 to assess the use of MALDI-ToF-MS as a strain clustering method. PFGE images in GelCompar II also used to identify paired strain types in milk and on udder skin. Conditional formatting in Microsoft Excel was used to produce a colour scale for each CCI matrix.

Chapter 4 methods

1.1.13. Flock and animals

A farm in Shropshire, England was selected as the study farm. Forty-six ewes comprising 20 3 year old ewes and 23 6 year old and 3 2 year old ewes were randomly selected for milk sampling at time of enrolment and the following time points: 1-4 days of lambing, at approximately 4 weeks and approximately 8 weeks into lactation.

1.1.14. Sampling

Milk samples for bacteriology were aseptically collected by Selene Huntley according to the methodology described in Chapter 3. Swab samples were taken according to the methodology described in Chapter 3. Both milk and udder skin swabs were taken from all ewes at 1-4 days after lambing, and approximately 4 weeks and 8 weeks into lactation.

For lamb mouth samples, swabs were inserted to the back of the lambs' throat and twisted on each side at the first observation only (1-4 days of lambing).

Milk (with glycerol added to 20% v/v), udder skin and lamb mouth swab samples were stored and transported on ice to the laboratory (University of Warwick). Swabs were immediately broken off into 1ml of Brain Heart Infusion agar (BHI) and glycerol (at 10% v/v) into a cryovial tube. They were then stored at -20°C until analysis.

1.1.15. Growth and selection of isolates from agar plates

A random selection of these samples were taken for laboratory analysis across all three age groups: 8 3 year old ewes and 8 6 year old and 3 2 year old ewes.

Each sample was thawed at room temperature and plated onto three different types of agar using aseptic techniques; a sterile 10µl loop was used to sterile streak onto half a BHI + sheep's blood agar (SBA) plate containing 5% (v/v) sterile sheep's blood and a new sterile 10µl loop for half an Edwards agar plate (a selective medium for isolation of *Streptocococi* typically involved in bovine mastitis). In addition, 100µl was pipetted onto a MacConkey agar plate (a selective medium for isolation of Gram-negative and enteric bacilli) and spread. The plates were incubated inverted at 37°C. Plates were checked at 24, 48 and 72 hours, and colonies picked as described below.

Milk

Due to the low numbers of isolates cultured from milk samples, the majority of isolates were picked under the supervision of an experienced microbiologist, and in particular those typically morphologically associated with mastitis. Morphologically identical isolates were not found frequently as in Chapter 3, apart from where there was pure growth of one species. However, in all cases, up to 5 of each morphologically unique isolate was picked for further analysis.

Udder skin swabs

The altered plating technique (using three plates, two of which are selective media and sterile streaking onto SBA plates) provided a more efficient method for picking off isolates of interest. Under the supervision of an experienced microbiologist, isolates that were morphologically similar to isolates picked from their milk and/or lamb mouth counterparts were selected for further analysis.

Lamb mouth swabs

As growth was limited to a single morphologically distinct colony type from most lamb mouth swab samples, up to 5 of each morphologically unique isolate were picked for further analysis regardless of whether these were also identified in the milk or on udder skin.

Isolate storage

Once selected, isolates were quarter streaked as in Chapter 3.However, plates were labelled with sample number (a unique 5 digit number for each udder half), the sample type (S-Skin, Blank-Milk, L-Lamb), the media in which they were isolated from (MC-MacConkey, ED-Edwards, BD- SBA) and a unique letter (A-P). For example a *Staphylococcus aureus* isolate from an SBA plate might be labelled 43472 BD A. As there was no milk sample number pre-lambing, samples were labelled with

the Ewe number, lamb number (if the sample came from a lamb mouth), the media in which they were isolated from as above, and a unique letter as above.

In order to reduce spectral variability, they were only passaged once before MALDI-ToF-MS analysis. This quadrant was also used to take culture for long-term storage in BHI and glycerol (at 10% v/v) at -80°C as in Chapter 3.

1.1.16. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

Optimisation of MALDI-ToF-MS sample preparation

Substantial efforts went into assessing and minimising spectral variability between technical replicates and media types to ensure strain differentiation would be accurate. Technical replicate variability were tested using *Staphylococcus aureus, Streptococcus dysglactiae* and *Streptococcus uberis*.

The media types tested were SBA (the standard agar used for MALDI-ToF-MS), MacConkey and Edwards agar, using coagulase-negative staphylococci for SBA and MacConkey agar and *Streptococcus uberis* for Edwards agar as these were the media types that would be used in this study.

MALDI-ToF-MS protocol

The reagent and sample set up was as described in Chapter 3. The bacterial test standard (BTS) (Bruker) was used as a control. Extracted isolates were spotted in duplicate on a 96 well Bruker target plate and run through MALDI-ToF-MS. Spectra were compared to the Biotyper 3.0 database and a species name and score were assigned (as in chapter 3).

Spectral analysis

Species found in abundance on udder skin, in lambs mouths and ewe milk were taken forward for further software analysis (and therefore strain differentiation) using Biotyper 3.0, FlexAnalysis and ClinProTools. The results were saved in html format and transferred into Microsoft Access.

1.1.17. Data management

Two databases were constructed; one containing ewe data such as observation date, ewe ID, body condition score, lambing date, lamb ID, sex, and litter size. The second database contained the results from MALDI-ToF-MS analysis in order to select isolates of interest for potential transmission events.

These included isolates where the same species were identified in:

1) The same location for the same ewe over 2 or more observations (Potential bacterial persistence)

2) Two different environments for the same ewe (ewe milk, udder skin and/or the ewe's lambs mouth) at the same time point (Potential reservoir or transmission pathway of unknown direction)

3) Two different environments for the same ewe (ewe milk, udder skin and/or the ewe's lambs mouth) over 2 or more observations (Potential transmission pathway)

Queries were used to merge technical replicates (Microsoft Access), allowing the top score for each isolate to be selected. Score values calculations are defined in chapter 3. Queries were used to identify ewes where the same species were present on the udder skin and/or lamb mouths and at the next observation in the milk, or vise versa or where species persisted in the milk or on the skin over time.

Composite correlation indices (CCI) were calculated for spectra of interest using Biotyper 3.0 (described in chapter 3) in order to select isolates that were likely to be the same strain types within samples from the same ewe and spectra from these isolates were manually compared using FlexAnalysis and ClinProTools. CCI matrices were exported to Micrososft Excel and conditional formatting was applied to produce a heat map for easy viewing.

1.1.18. Flock & Ewe Selection

Ten performance recorded pedigree Texel flocks with individual ewe identification were convenience selected based on recommendations and farmer interest. Flocks included representatives from England, Scotland and Wales (Figure 5.1).

Figure 5.1: A map of study flock locations.

1.1.19. Data collection

All Texel ewes from each flock were included in the study, which took place between 2012 and 2014. Two flocks were visited over several years. All ewes lambed between January and May of each year. Data collection occurred mid-lactation and included 10 udder and teat conformation traits, and individual ewe and lamb IDs.

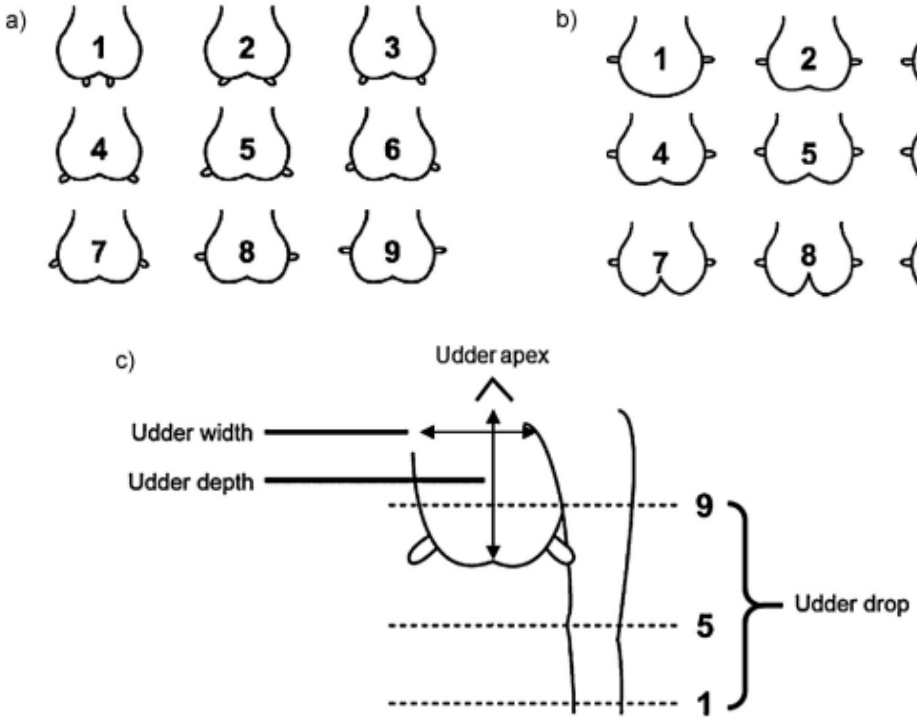
Variable definitions

Udder traits

All ewes were scored for 10 udder and teat traits using a combination of linear scores, measurements, udder palpation and visual examination. Linear scores were used to characterise teat placement, udder drop, and the degree of separation of the udder halves as described previously (Casu *et al.* 2006, Marie-Etancelin *et al.* 2005) (Figure 5.2). A tape measure was used to record

udder width from the rear at the widest point and the left teat length only because the initial study indicated a strong positive correlation between left and right udder teat length and width. Udder masses were detected by manual palpation of each udder half, and teat lesions were observed by visual inspection. All measurements were performed by two researchers and data recorded on custom-designed data recording sheets.

Figure 5.2: Scoring methods for a) teat placement, b) degree of separation and c) udder drop and udder width.



Binary variables

Chronic mastitis was defined as a palpable mass in either udder half.

Teat lesions (left and/or right) were defined as any lesion present on the teat at the observation. These could include bites, tears and grazes, which tended to be attributed to suckling; or spots, warts and/or proliferative scabs on the teat.

1.1.20. Data summary and analyses

All data were entered into Microsoft Excel spreadsheets. Individual ewe data were matched to flock book numbers via BASCO or by the farmers. Flock level pedigree data including sire and dam IDs were obtained from the BASCO database (BASCO). Data were moved into Microsoft Access. Queries were used to select and link data from Microsoft Access for statistical analysis. Ewe age in years at the time of observation was calculated as the difference between the date of birth and the observation date. Number of days in milk was calculated as the difference between the ewes' lambing date and the observation date.

Measures of dispersion and central tendency were used to investigate the data (R Core Team 2013). Obvious errors were corrected where possible.

4. Results

Please see chapter 2 results from thesis
Please see chapter 3 results from thesis
Please see chapter 4 results from thesis
Please see chapter 5 results from thesis

5. Discussion

The aim of this thesis was to contribute to the understanding of the development and persistence of mastitis in suckler ewes. The likelihood of a ewe developing mastitis is influenced by a farmer's management practices, the environment of the ewe, the ewe's susceptibility to developing mastitis and the bacterial strains in the environment and mammary gland. Due to the multifactorial nature of these infections, several research approaches were used in this thesis. The initial study estimated an incidence rate of clinical mastitis (IRCM) of 1.2 ewes/100 ewes/year, the first estimate that indicates the impact of mastitis on suckler flocks in England. The estimate varied depending on management system, with ewes that always reared lambs outdoors having a lower IRCM than those that always reared lambs indoors. In addition, the risk for an increase in IRCM varied by management system and temporally. This indicated that different management systems expose

ewes to different risks and therefore need to be treated independently when considering risk factors for the development of mastitis. Whilst the IRCM obtained is the same as the only other IRCM in suckler ewes (Arsenault et al. 2008), there were some respondents that never checked their ewes for mastitis. Only 88% of respondents checked ewes' mammary glands for disease at lambing and the regularity of checks decreased over time after lambing (Chapter 2), therefore this IRCM may be an underestimate. In addition, ewe movement was significantly associated with IRCM: flocks that were kept in the same barns during and after lambing, or in the same field before and after lambing had a decreased IRCM. A likely explanation for this is that when ewes are moved they are exposed to new potential pathogens and so suffer more mastitis. The potential importance of the microbial environment was also highlighted by the association between floor type and IRCM, which is likely to be linked to the how easy it is for a flooring material to be cleaned and/or the effect of flooring type on how saturated bedding becomes. Hard flooring, associated with a decrease in IRCM, is uneven which could improve fluid drainage and so impede bacterial colonisation. In order to ascertain this link, this hypothesis requires further investigation. 161 Finally, poor udder conformation was significantly positively correlated with IRCM. This questionnaire-based study helped generate the hypothesis that udder conformation is significantly associated with chronic mastitis This hypothesis was subsequently tested in Chapter 5, where udder conformation traits were found to be significantly associated with chronic mastitis. This highlights the usefulness of questionnaire-based studies for hypothesis generation and subsequent testing in observational studies. The role of the environment was first considered by investigating the potential sources of mastitis-causing pathogens (Chapter 3). In order to effectively link an environmental organism to a case of mastitis, it is necessary to identify organisms to at least the strain level. The current gold standard strain typing methods (MLST, genotyping and PFGE) can be extremely time-consuming and expensive, particularly for epidemiological studies where samples need to be taken from multiple ewes over time. MALDI-ToF-MS offered a quicker, cheaper alternative to these methods and therefore was compared to PFGE in order to investigate whether this method could be used as a strain typing tool. The results from the study showed that MALDI-ToF-MS had good correspondence with PFGE when utilising composite correlation indices (CCI) for Staphylococcus warneri, Staphylococcus equorum, Rhodococcus corprophilus, Bacillus pumilus and Micrococcus luteus to varying levels – most species had distinct strain level cut-off points for CCI estimates, whereas the threshold for Staphylococcus warneri was more ambiguous. For example, MALDI-ToF-MS analysis, supported by PFGE, indicated that many different strain types of Staphylococcus equorum were isolated from different samples. These data suggest there are many different sources of Staphylococcus equorum which fits the traditional view of this organism having an "environmental" origin. The "environmental" versus "contagious" classifications, although rigid, does provide useful information as to potential transmission pathways of bacterial species in mastitis. The same species and strain types for Rhodococcus corprophilus, and Staphylococcus warneri were identified on udder skin and in ewe milk, indicating

162 the udder skin as a possible reservoir of mastitis causing pathogens. However as the study in Chapter 3 was not a longitudinal study, the direction of transmission (if any) could not be ascertained. In Chapter 3 only blood agar was used to culture bacteria from udder skin and milk samples which could have limited the variety of bacterial species isolated and then identified. In addition, isolates were prepared for MALDI-ToF-MS using the direct smear method which results in increased spectral variability and signal to noise ratio and therefore greater technical CCI variability. The study design was improved in a larger, longitudinal study (Chapter 4) with varied media and sampling which provided the ability to investigate potential transmission pathways. As udder skin was identified as a potential reservoir of pathogenic bacteria (Chapter 3), this chapter focussed on potential transmission pathways via lamb mouths and udder skin by sampling these environments and ewe milk over time. To maximise the success rate of finding potential transmission pathways of mastitis causing bacteria, a variety of selective media were used in the study. In addition, a formic acid extraction method was used instead of the direct smear method to reduce spectral variability. Despite only sampling from ewes showing no clinical signs, a variety of mastitiscausing pathogens were isolated from ewe milk, udder skin and lamb mouths. This differs to chapter 3 where only a few mastitis-causing pathogens were identified despite ewes showing clinical signs of mastitis. This could be due to the increased number of samples collected in chapter 4. Alternatively, the use of selective media in this study may have enhanced the growth of these species, highlighting the importance of media selection to ensure optimal conditions for bacterial isolation. Twenty-six species were found in more than one location (lamb mouth, ewe udder skin and/or ewe milk). The fact that some bacterial species were found in all 3 locations and some in only 2 of 3 locations may give some indication as to their transmission pathways. For example, species only present in lamb mouths and milk may be directly transmitted between lamb and ewe. Alternatively, isolates only identified on ewe skin and in the milk may be transferred from the ewe's environment to the skin and then the milk rather than via her lamb. 163 To the authors knowledge, Mannheimia haemolytica is the only mastitis-causing pathogen isolated from lamb mouths previously (Fragkou et al. 2011). In this study, a variety of bacteria associated with mastitis were isolated from lamb mouths, including: Aerococcus viridans, Bacillus licheniformis, Escherichia coli, Enterococcus faecalis, Enterococcus faecium and coagulase-negative staphylococci, providing evidence for the role of lambs in the transmission of mastitis. Potential transmission events over time were observed between lamb mouths, ewe udder skin and milk. Most notably, the same Escherichia coli strain type was isolated from lamb mouths and udder skin at the first observation after lambing but not on udder skin pre-lambing or in ewe milk. In addition, the same strain types of several coagulase-negative staphylococci were isolated at different sampling time points in ewe milk and on udder skin, including initial detection on udder skin followed by subsequent detection in the milk at the next observation. This represents evidence of transmission from the udder skin to the milk of a potential pathogen. There were also several cases of colonisation of the same strain type on udder skin and in ewe milk at the same observation. Whilst

these data suggest passage between udder skin and ewe milk it is not possible to determine the direction of transmission. The persistent isolation of the same strain types were found over time for Bacillis licheniformis, Staphylococcus hominis, and Staphylococcus warneri for ewe milk and Staphylococcus equorum and Staphylococcus aureus on ewe udder skin. Due to the long intervals between sampling points, these may represent re-colonisations by the same strain rather than persistence. However, these persistent isolations occurred in a large number of ewes, therefore it seems reasonable to assume these were persistent colonisations, particularly in the case of ewe milk. These results indicate the capacity of MALDI-ToF-MS as a rapid strain identification method to determine potential transmission events and persistence of bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time. The identification of these potential transmission pathways are particularly important as they highlight potential routes for infection for other 164 mastitis causing pathogens as well and so may inform general treatment and control strategies for mastitis. Having investigated farmer management practices and the environment of the ewe in the role of mastitis, the ewe's susceptibility to developing mastitis also needed to be investigated. In particular, having identified udder conformation as the most significant factor associated with IRCM in Chapter 2, the final study aimed to investigate whether udder and teat conformation are heritable traits that affect mastitis. In this study (Chapter 5), all udder traits (bar teat placement) were associated with the number of days in lactation, indicating that these traits were correlated to milk production. Ewe age was also correlated with all udder traits. It was therefore important to account for ewe age and days in lactation in the association between udder traits and chronic mastitis. Even after accounting for these factors, there were phenotypic association between udder traits and chronic mastitis. This supports the hypothesis generated in Chapter 2, where the IRCM was positively associated with the percent of flock with poor udder conformation and highlights the importance of this factor. Interestingly, the presence of a teat lesion on either half also increased the risk of a ewe having chronic mastitis. Although this was a tested factor in chapter 2, it was not significantly associated with IRCM. In this study (Chapter 5) researchers did thorough checks on all the mammary glands on ewes in the study whilst the questionnaire based study in Chapter 2 indicated that many farmers only check their animals irregularly. As teat lesions are fairly transient in nature they may well have been missed with irregular checking, which may explain the discrepancy between these two studies. The udder conformation traits showed good levels of heritability for all but udder width, and for chronic mastitis and teat lesions on the right udder half indicating that selection on these traits could be feasible, provided there was sufficient data recording. Although these analyses provided good estimated of the heritabilities of udder traits, knowledge of genetic correlations between these traits and others is required to determine the genetic progress that would be made if these traits were included in a selection program. 165 The work in this thesis has addressed several gaps in the knowledge in mastitis epidemiology as outlined in Chapter 1. Namely by providing the first estimate for the IRCM in suckler flocks in England, and generating hypotheses for factors that may affect

the IRCM. One of the associated factors, udder conformation, was investigated in a cohort study as to whether these traits are heritable and whether they influence chronic mastitis cases. Finally, the role of the ewe's environment was investigated by identifying the potential sources and transmission pathways for a variety of mastitis causing bacteria. This thesis highlights the value of a multidisciplinary approach utilising microbiology and epidemiology whereby a cohort of ewes could be investigated over time using microbiological techniques in order to improve our understanding of the development of mastitis. In conclusion, the results from these studies have contributed to our understanding of influences on the development of mastitis and generated hypotheses for future studies to reduce IRCM in suckler flocks.

6. Industry messages

Further work could include testing the hypotheses linked to the ewe's microbial environment generated in Chapter 1, in particular the effect of flooring type on the IRCM and whether this is linked to bedding saturation and therefor microbial colonisation. The effect of ewe movement between environments on the IRCM was another interesting hypothesis that could be tested further by investigating bacterial communities in different environments for example individual lambing pens, group lambing pens and each field. Further evidence for the potential transmission pathways highlighted within this thesis could be gathered using shorter sampling periods using single nucleotide polymorphisms (SNP). In addition, the somatic cell count could be compared to this dataset in order to observe whether somatic cell count (indicating an immune response) increases after a potential transmission event.

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