Disease Investigation into Bovine Ischaemic Teat Necrosis: a severe, emerging disease of economic importance.

‘Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by *Hayley Ellen Crosby-Durrani*.’

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Abstract

Disease Investigations into Bovine Ischaemic Teat Necrosis: a severe, emerging disease of economic importance – Hayley E. Crosby-Durrani

Bovine ischaemic teat necrosis (ITN) is a recently emerging disease that has been reported on dairy farms in Great Britain (GB). The disease affects the teats of lactating animals and is of serious welfare and economic concern due to lesion severity. Lesions can progress rapidly, and cause discomfort to the affected animal and may lead to the removal of the teat either by the process of necrosis, self-trauma or due to surgical intervention to contain the disease. Until recently, only anecdotal information on aetiology, risk factors and prevalence were available to farmers and veterinary surgeons to aid treatment and control. The aims of this study were to expand the knowledge around ITN, using a multidisciplinary approach, to aid with diagnosis, control and treatment and to provide researchers with foundations for further studies.

This study covered several key areas: 1) reporting the GB incidence of disease and potential risk factors; 2) documenting the pathology; and 3) investigating the microbiological involvement.

A national, questionnaire-based, epidemiology study of 1855 GB dairy farms was undertaken with a useable response rate of 12.3%. The results showed that 51% of farmers had seen an ITN case on farm between 1985-2018. Rising numbers on farms indicated ITN is an emerging disease with 46.3% of farmers reporting their first case in 2015-2018. Univariable and multivariable models showed significant farm-level risk factors for having ITN on a farm, namely the presence of udder cleft dermatitis or chapped teats in milking cows. First lactation animals represented 47.3% of cases, and 78.9% of cases were in the first 90 days in milk. Only 20.8% of cases recovered and 22.8% of cases required culling. The remaining cases experienced complications such as loss of a teat and/or mastitis. From this data, the estimated cost of ITN, through production losses and expenditure, was estimated to be £1121 per farm per year. The cost was estimated at £720 and £860 for recovered and complicated cases respectively, with cases that required culling costing approximately £2133.

Documenting the different presentations of ITN was an important initiating process as this set the working definition and the inclusion requirements of cases for further investigation. Three main classifications of macroscopic lesions were devised and histopathology of the lesions documented. This process contributed to the suspicion of possible pathogenesis and aetiologies for further investigation. Due to a histopathological suspicion, several routes were taken to determine an association with a specific viral pathogen, including shotgun metagenomics analyses and a targeted PCR approach however no significant viral association was identified.

A selection of typical lesions were submitted for shotgun metagenomic analysis and 16S rRNA amplicon sequencing and compared with healthy teats. The main differences between the diseased samples and the healthy samples were that diseased samples contained significantly more Pasteurellaceae, especially *Mannheimia spp.*, than healthy samples while healthy samples contained significantly more commensal taxa, including Actinobacteria. These findings indicate a possible dysbiosis in the teat skin and a putative pathogen in the form of *Mannheimia spp*.

This study has significantly contributed to the characterisation of bovine ITN including pathological changes during disease development, emergence of the disease, associated risk factors and microbiological composition. Furthermore, several areas have been identified for further investigation to better underpin future control measures.

Abbreviations

AHBD	Agricultural and horticultural development board
BHM	Bovine herpes mammillitis
BoHV	Bovine Herpesvirus
bp	base pair
BSE	Bovine spongiform encephalopathy
CARD	Comprehensive Antibiotic Resistance Database
CGR	Centre for genomic research
CODD	Contagious ovine digital dermatitis
CPD	Continuous professional development
DD	Digital dermatitis
FCS	Foetal calf serum
FMD	Foot and mouth disease
FSA	Food standards agency
GB	Great Britain
H & E	Haematoxylin and eosin
IHC	Immunohistochemistry
IQR	interquartile range
ITN	Ischaemic teat necrosis
lci	Lower confidence interval
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis effect size
M	Molar as in concentration
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
ONT	Oxford Nanopore Technologies
OR	Odds ratio
OTEB	Oral treponeme enrichment broth
PCA	Principal component analysis
PCR	Polymerase chain reaction
RefSeq	NCBI Reference Sequence Database
RGI	Resistance gene identifier
ROC	Receiver operating characteristic
RUMA	Responsible use of medicines in agriculture alliance
sd	Standard deviation
TEM	Transmission electron microscopy
UCD	Udder cleft dermatitis
uci	Upper confidence interval
UK	United Kingdom
v/v	volume per volume
VS	Veterinary surgeon
WGS	Whole genome sequencing

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During this time, I have undergone many life changing events such as the birth of my wonderful but cheeky son, buying our first family home, sitting European board exams, getting my dream job and some not so good events such as being hospitalised with pneumonia and sepsis and shutdown of the laboratory facilities due to the Covid pandemic. All the way though I have had the unwavering support of my husband, Zeeshan, and my primary supervisors Dr. Nicholas Evans and Prof. Stuart Carter. Without them I would not have made it through my first year of this PhD. Particularly Nick and Stuart have been incredibly supportive and understanding when I've been an emotional wreck due to a combination of lack of sleep, hormones and unforeseen circumstances that have prevented me from being able to access the lab. They have also been patient when results caused a change in direction and guided me to a new path, for which I will be forever grateful.

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Chapter 1: Introduction

1.1 The importance of the bovine teats and udder in the dairy industry

The bovine teat and udder are a unique environment that undergo vast developmental changes and are the source of the main product in the dairy industry, milk. Anatomically, the udder is categorised as a modified sweat gland covered in haired skin changing to glabrous (non-haired) skin at the teat. The dairy cow (*Bos taurus*) is a remarkable animal that can cope with milking two to three times a day, often for over 300 days a year, and can produce an average of 8,000 (3,600-13,600) litres of milk/year for an all-year-round (AYR) calving Holstein/Friesian herd in Great Britain (GB) (AHDB, 2020a; Redman, 2020). Therefore, the dairy cow is an important animal in food security. As the teat is the location for milk to be removed from the udder, and the udder the site of milk production, any diseases affecting these locations could potentially impact on the food chain and food security through loss of milk production. This loss of milk production can also have a significant impact on the farm economics. For an average AYR calving Holstein/Friesian herd with milk priced at £0.28/litre the milk production output is £2,240 per cow per year (Redman, 2020), which reduces with any decrease in output. Such an economic impact increases if an animal has to be treated or culled due to a disease and subsequently replaced. The economic implications of teat lesions are not readily available; however, there have been estimates on the cost of clinical mastitis. In the UK dairy industry, over 20 years ago, clinical mastitis was estimated to cost £41.8 million per annum (Kossaibati and Esslemont, 2000) and these estimates will have substantially increased since then. These costs include: the loss of production from discarded milk; the price of veterinary treatments; the cost of additional labour; loss through reduced milk yields; and in severe cases the cost of replacing the animal due to culling or death (Green et al., 2009). In addition, more recent estimates from the dairy industry levy board for Great Britain (Agricultural and Horticultural Development Board (AHDB)) have the cost of a case of mastitis per animal at an average £250-

300 per animal (AHDB, 2022a). Although, average costs are highly variable and depend on individual farm circumstances (Green et al., 2009). Mastitis can often be induced by lesions to the teat allowing pathogens to enter the mammary gland or for teat lesions preventing the gland from being efficiently milked. Lewis et al. (2000) showed that only 24.7% of milking cows across 5 different herds had teats that were normal and without lesion. However, these were mostly mild teat end lesions as a response to machine milking. Nevertheless, this is particularly worrying as the teat is the site from which milk is harvested and potentially 75.3% of 1.85 million dairy cows in the milking herd (AHDB, 2022b), over 1.39 million dairy cows have a teat lesion of some sort. These lesions are of concern to the dairy industry in three areas: welfare of the animals, the farm economy and potentially for food security.

Animal welfare is an important area to consider with food producing species. There are five freedoms which are recognised as the gold standard for animal welfare and from which all animals should be free: 1. from hunger and thirst; 2. from discomfort; 3. from pain, injury and disease; 4. to express normal behaviour; and 5. from fear and distress (Farm Animal Welfare Council, 2009). Diseases that affect the bovine teat and udder can therefore impact on at least two of the five freedoms (freedom from discomfort and from pain, injury and disease) and require rapid control and treatment to allow the animal to return to a higher level of welfare. In the last few decades assessment of animal welfare has progressed to the current 2020 model of the five domains which include: 1. nutrition; 2. physical environment; 3. health; 4. behavioural interactions (including the animal's interaction with the environment, non-human animals and humans); and 5. mental state (Mellor et al., 2020). This move was to move away from good welfare being solely indicated by meeting the minimum of the animal's basic biological needs, towards ensuring the animal is able to have a life worth living and acknowledges the fact that animals are sentient and looks to enhance welfare positive behavioural effects (Mellor et al., 2020). The inclusion of mental state of the animal in the model was an important step as it

encompasses the other four domains. The animal will only have a good mental state provided the first four domains are met. As such, if there are teat lesions on the teats there may be an associated behavioural change in the animal, for instant showing signs of discomfort during milking.

There are many diseases that affect the bovine teat and udder in dairy cattle that often have a complex aetiology, and as highlighted above can have food security, economic and welfare implications. Also, as milk enters the food chain, milk can act as a potential source for zoonotic diseases, such as Tuberculosis, caused by the bacterium *Mycobacterium bovis*. Tuberculosis was the main driver towards pasteurisation of milk in many countries to control disease in the human population (O'Reilly and Daborn, 1995). As such, diseases in food producing species can change human practices and require monitoring to protect human health.

1.2 Skin diseases affecting the bovine teat and udder

There are a number of diseases that can adversely affect the skin of the udder and teat in dairy cows (Table 1.2.1). These diseases can be separated based on the anatomical location affected, such as either only affecting the teats or the udder, or the potential to involve both the teats and udder and as such can affect both glabrous and haired skin. Skin lesions on the bovine teat and udder can also lead to other issues such as mastitis, as eluded to earlier, and may present as part of a systemic disease, for example in Foot and Mouth disease (FMD)(Wellenberg et al., 2002). The loss of skin integrity can allow for bacterial entry and has the potential to lead to septicaemia and death as can occur with *Escherichia coli* mastitis (Burvenich et al., 2003). In addition, many bovine udder skin diseases are zoonotic, e.g. Pseudocowpox, and therefore have implications for human health. Furthermore, diseases such as FMD and vesicular stomatitis

can produce teat lesions and are notifiable diseases (Gibbs, 1984). Both of these diseases (FMD and vesicular stomatitis) are currently not present in the United Kingdom (UK).

Table 1.2.1 List of important skin diseases that affect the bovine teats and udder separated by known aetiological agent category.

Bacterial	Viral	Milking machine induced	Other
Blackspot	Bovine herpes mammillitis	Teat end hyperkeratosis	Ringworm
Udder acne (*Staphylococcus* dermatitis)	Pseudocowpox	Teat oedema (blueing of the teat)	Photosensitivity
Udder cleft dermatitis	Cowpox	Congestion	Udder oedema
	Bovine papilloma (warts)	Haemorrhage	Teat sunburn
	FMD	Wedging/ringing of the teat	Trauma
	Vesicular stomatitis		

An entity that has appeared to be of increasing importance on GB dairy herds is bovine ischaemic teat necrosis (ITN) (Fig 1.1). Bovine ischaemic teat necrosis (ITN) is a relatively new disease, first reported in 2004 (Blowey, 2004). There are few previous descriptions and reports of ITN (Blowey, 2004; Andrews et al., 2008; Blowey and Edmondson, 2010; Mauldin and Peters-Kennedy, 2015; Clegg et al., 2016b). In some literature, ITN has been referred to as summer sores and teat eczema (Blowey and Weaver, 2003). In all previous reports, the macroscopic and histological descriptions of ITN are either inadequate or completely lacking. This disease affects the teats of dairy cows and can be striking in appearance with affected

animals frequently self-traumatising their own teats and has been considered a psychogenic disease with potential roles for udder oedema and histamine in the pathogenesis (Mauldin and Peters-Kennedy, 2007). Many of the affected animals have to be culled on welfare grounds causing economic loss for the farmer and attempts at treatments are often futile (Clegg et al., 2016b). Currently, little is known around the incidence of the disease in GB, the animals most at risk, farm level risk factors, clinical presentations of the disease, histopathology and most importantly potential aetiological agents. As such, treatments currently are non-specific, supportive, and more often than not ineffective leading to the premature culling of the affected animal. A pilot study investigating a limited number of aetiological agents in ITN cases from 12 affected cow found a possible association with digital dermatitis associated treponemal bacteria; however, all farms had cases of Digital Dermatitis (DD) lameness in the milking herd (Clegg et al., 2016b).

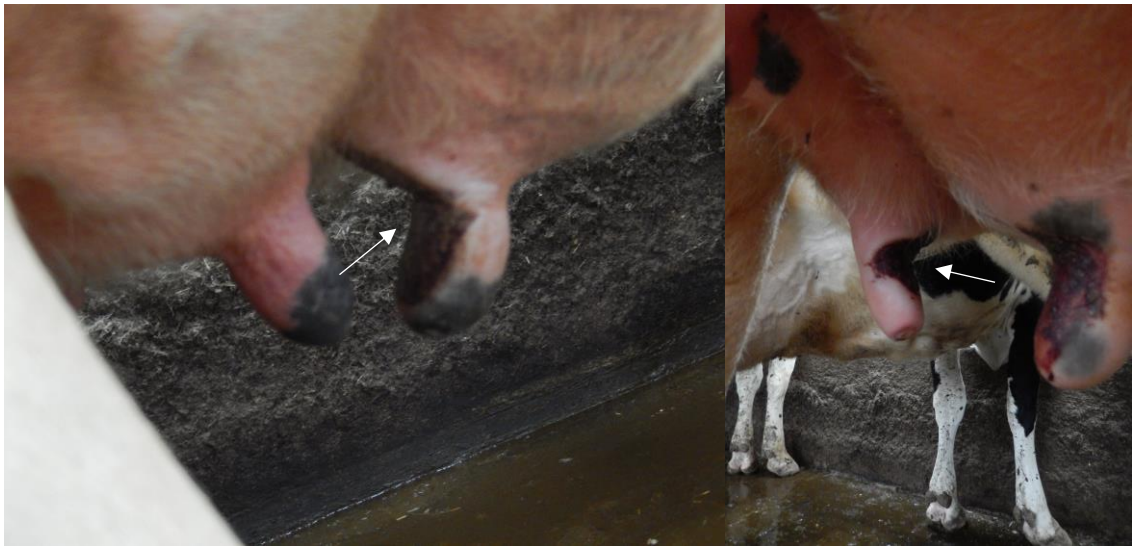


Figure 1.1 Classical presentation of an ischaemic teat necrosis lesion (arrows) on the teats of two dairy cows. Lesions are on the medial aspect of the teat and have a well demarcated, dark red to black, dry area of necrosis at the base of the teat. The lesion may extend down the length of the teat or up onto the skin of the udder.

There are many differential diagnoses for ITN. However, the appearance and clinical scenarios are drastically different from other well documented skin diseases of the bovine teat and udder.

1.2.1 Viral diseases affecting the teat and udder skin

One of the major differential diagnoses for ITN is bovine herpes mammillitis (BHM) (Fig 1.2). These two diseases may be differentiated based on their clinical presentations as ITN presents as a focal dry red to black area of necrosis on one or more teats (Blowey and Edmondson, 2010) compared with the exudative lesion produced by BHM that can affect one teat or involve the entire udder (Gibbs, 1984; Shearer et al., 2008). Another different clinical presentation between the diseases is that ITN cases can be highly pruritic in nature (Mauldin and Peters-Kennedy, 2015), which is not a reported sign of BHM, which is caused mostly by Bovine Herpes Virus 2 (BoHV 2) and to a lesser extent Bovine Herpes Virus 4 (BoHV4) (Gibbs, 1984; Shearer et al., 2008). The BHM affected cows may be febrile and commonly resist milking (Gibbs, 1984). Clinical signs for BHM can be widely variable in terms of severity and extent, from subclinical to severe cases that affect multiple teats and coalesce on the skin of the udder. BoHV 2, an alpha herpes virus, primarily infects and causes clinical BHM in heifers and young 1st lactation cows in the postpartum period, particularly if they had severe udder oedema present (Gibbs, 1984). BoHV 4 is a gamma herpes virus that targets the bovine endothelial cells and can cause similar BHM lesions to BoHV 2 (Wellenberg et al., 2002). As BHM is widespread throughout the UK dairy industry, many cows have been exposed with older cows tending to be seropositive and largely subclinical, unless close to calving (Sieber and Farnsworth, 1984). A multiplex PCR has been developed by Cargnelutti et al., (2017) to allow for rapid detection of these herpesvirus to diagnose BHM. However, as herpesviruses are able to undergo latency in the body and a positive PCR result does not necessary identify the cause of disease unless

reported with consistent clinical signs. BHM often causes lesions in animals that have dry chapped skin on their teats and once traumatised by some event, clinical signs can emerge or re-emerge with widespread ulceration and formation of scabs. An atypical case of BHM, observed affecting both cows and calves, was documented in 2008 and described as "extensive vesicular lesion across the skin of the udder and on to the teats with vast sloughing of the epithelial surface" (Kemp et al., 2008). This report was sloughing of the epithelium and not the whole teat as can occur with ITN. BHM is also considered to be a seasonal disease with cases more common in the colder months (Gibbs, 1984) and can also exhibit similar lesions to those caused by Pseudocowpox. Consequently, Pseudocowpox is another major differential diagnosis for ITN.

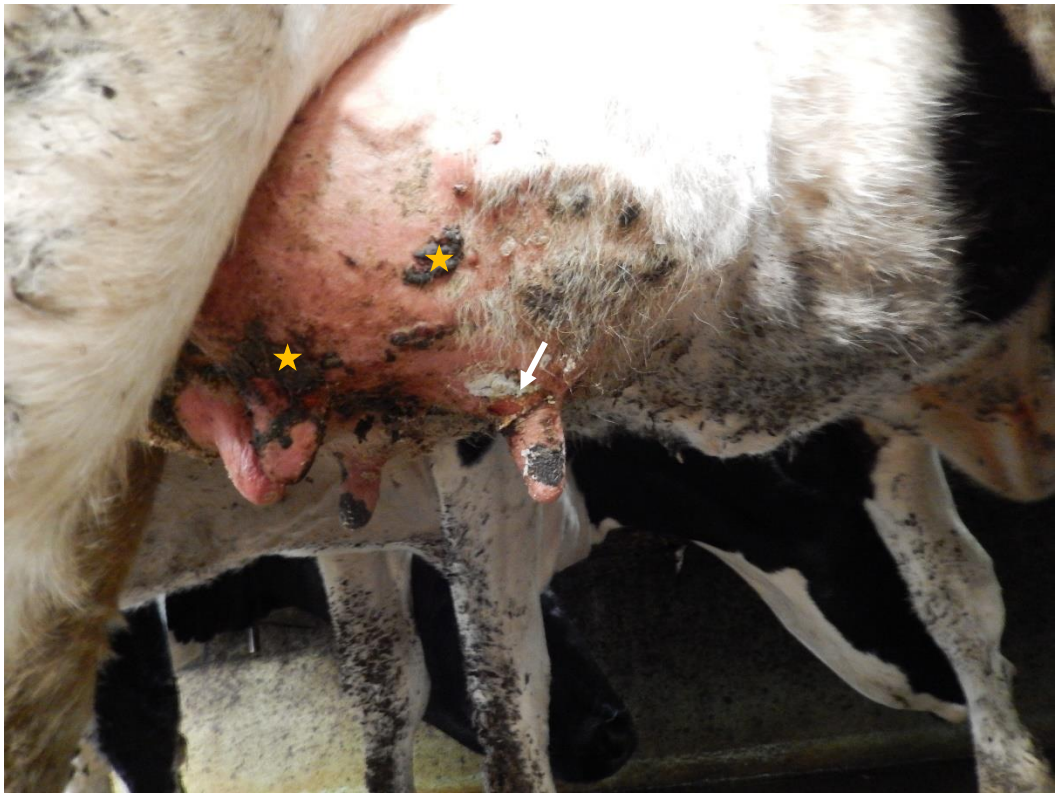


Figure 1.2 Udder from a cow with a confirmed case of bovine herpes mammillitis. The vesicles have scabbed (stars) over and there is a focal area of sloughed epidermis at the teat base (arrow).

Pseudocowpox is caused by parapoxvirus that commonly affects the teat skin and can be transmitted to calves causing lesions on the mouth and muzzle. In addition, pseudocowpox is zoonotic causing Milker's nodules on human hands (Gibbs, 1984). Pseudocowpox often starts with crusting lesions with scab formation that may slough between 7-12 days, giving rise to a ring or horseshoe shaped red lesion on the skin of the teat (Shearer et al., 2008). As both pseudocowpox and BHM affect the teat skin, Cargnelutti et al., (2017) developed a PCR to distinguish the two entities in difficult cases. Pseudocowpox does not tend to affect solely the teat base; however, this disease when extensive can easily be confused for ITN lesions and needs to be ruled out.

Other diseases that are less similar but for completeness are also on the differential diagnosis list for ITN include, but are not limited to, cowpox, bovine papilloma (warts), blackspot and ringworm. Cowpox is an orthopoxvirus that despite the name, now rarely causes lesions in cattle. However, it is becoming increasingly common as a cause of skin lesions in cats (O'Halloran et al., 2016). Another orthopoxvirus that may affect the bovine udder is the vaccinia virus. This virus was made famous due to its use in the development of the smallpox vaccine and again vaccinia virus is now rarely seen in UK cattle but is re-emerging in Brazil (Matos et al., 2018). There are many bovine papilloma viruses that have been shown to give rise to papillomas or warts on the teats (Gibbs, 1984) (Fig 1.3). However, warts are unlikely to be mistaken for ITN lesions unless trauma causes removal of the warts. Due to limited studies on ITN and papillomas being almost ubiquitous it would be unwise to rule out this virus as a potential instigator in early disease investigation. Transmission of the above-mentioned viruses are via direct and indirect contact and therefore can be rapidly spread through the herd.

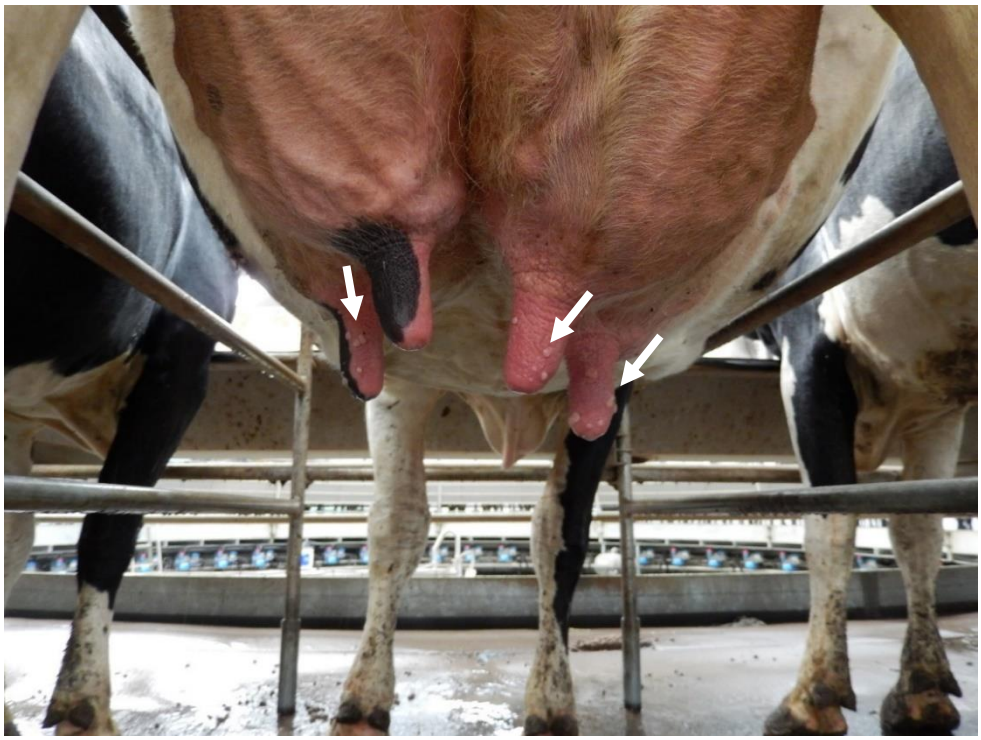


Figure 1.3 Cow's teats with multiple small, raised papillomas (arrows).

1.2.2 Bacterial diseases affecting the teat and udder skin

Other infectious conditions that need to be considered present as lesions that are more targeted towards the teat orifice than the teat base, such as blackspot. This is due to secondary bacterial infection of a milking machine induced lesion, teat end hyperkeratosis, by *Fusobacterium necrophorum* with or without *Staphylococcus aureus* (Blowey and Edmondson, 2010).

A disease that anecdotally farmers feel may be linked to ITN is udder cleft dermatitis (UCD) (Fig 1.4), also known as ulcerative mammary dermatitis. This is a skin disease that typically affects the udder either at the midline cleft between the two halves of the udder or at the junction

of the anterior udder and the abdomen (Olde Riekerink et al., 2014; Bouma et al., 2016; Ekman et al., 2018; Sorge et al., 2019). UCD is considered a multifactorial disease with many risk factors associated with the milking protocols and the environment, but also similar to ITN, has reported associations with Digital dermatitis treponemes (Beattie and Taylor, 2000; Stamm et al., 2009; Evans et al., 2010).

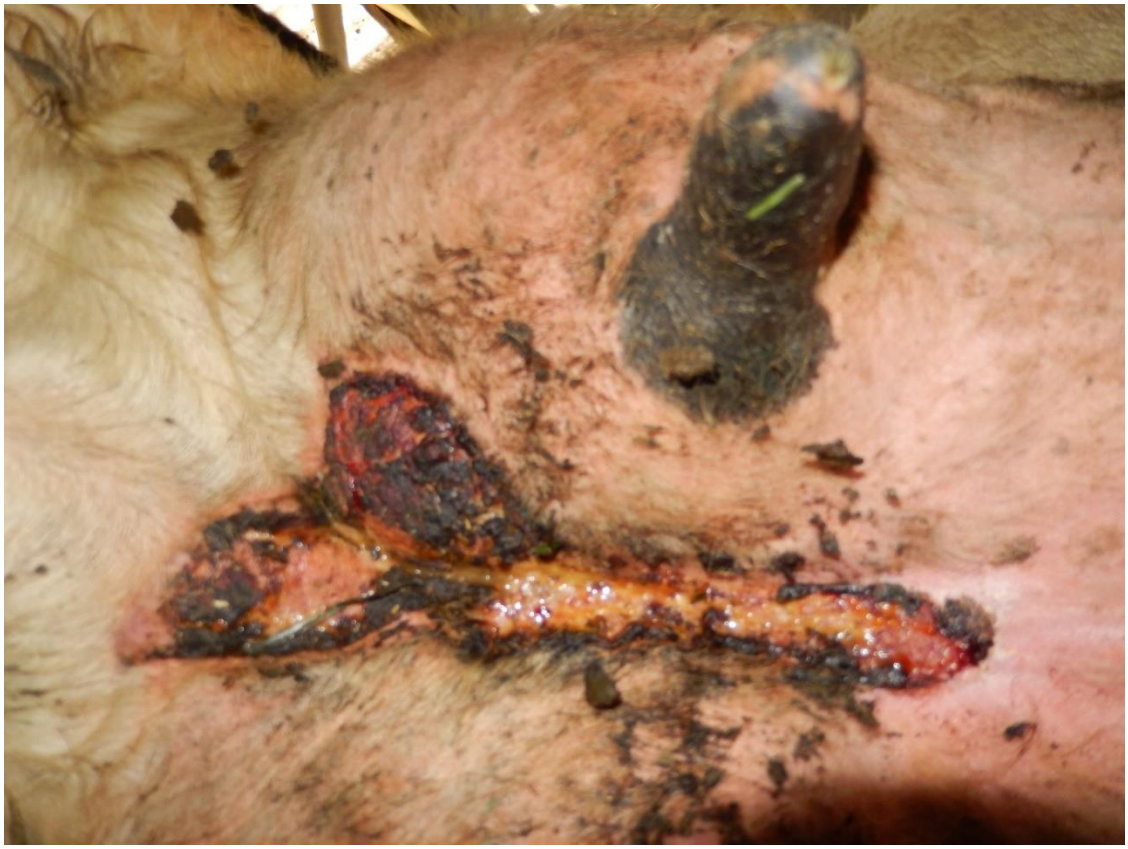


Figure 1.4 The ventral and anterior aspect of the cow's udder. There is a chronic and ulcerative lesion, representative of udder cleft dermatitis between the two halves of the udder and extending cranially to between the anterior portion of the udder and the caudal aspect of the abdomen.

1.2.3 Teat and udder diseases that can be diagnosed with routine procedures

There are other (mostly infectious) lesions that may occur along the length of the teat as well as the teat end and base. These may be excluded based on a clinical exam, but may require additional, readily available tests such as routine microbiology and biochemistry, and include

udder acne, also known as *Staphylococcus* dermatitis, photosensitisation (due to ingesting certain plants or liver disease), and fungal lesions (Blowey and Weaver, 2003). A fungal lesion, such as ringworm, is unlikely to be solely present on the teats. Usually there will be signs on haired skin areas, typically on the neck and thorax.

1.3 Other factors affecting the teat skin in cattle

Infectious diseases are not the only cause of teat lesions with many lesions associated with environmental factors or poor milking systems or techniques. These lesions are considered multifactorial and the result of the interactions of environmental and other factors. Many non-infectious factors that potentially induce teat lesions can be broadly classified into those induced by the mechanics of the milking machine and milking protocols, and ones induced by the farm environment.

1.3.1 Milking machine and milking protocol factors that may induce teat lesions

The milking machine is one of the few pieces of agricultural equipment to harvest a "crop" from living tissue on a daily basis (Jarrett, 1984). As such, a defective or malfunctioning milking machine has the ability to cause harm and lesions to the teats. The milking machine utilises a vacuum and a pulsator in an attempt to mimic the actions of a calf suckling with a massage phase (non-milking phase) to relieve congestion produced by the vacuum (Baines, 1993). The mouth of the milking machine cluster sits close to the base of the teat, exerting force in this region and potentially allows for unintentional trauma or micro-abrasions during the milking process. It has been found that the teat wall can increase by 33% after milking when compared to the pre-milking thickness, as a physiological response to the milking machine (Olechnowicz and Jaśkowski, 2009). The milking machine, and especially the vacuum pressure, is thought to be responsible for lesions located largely at the teat end, such as hyperkeratosis (an overproduction of keratin at the teat orifice) and teat end eversion. Teat end

hyperkeratosis has had multiple grading systems but the simplified 4 point system with a normal smooth teat orifice with no ring as grade 1 and very rough teat orifice with protruding fronds as grade 4 (Mein et al., 2001). However, only severe hyperkeratosis lesions have been associated with increased risk of clinical mastitis (Breen et al., 2009). In addition to teat end hyperkeratosis, if there is insufficient massage phase and excessive milking phase, lesions can arise along the length of the teat, including oedema (blueing of the teat) (Fig 1.5), congestion, haemorrhage, wedging and/or ringing of the teat (Fig 1.6).

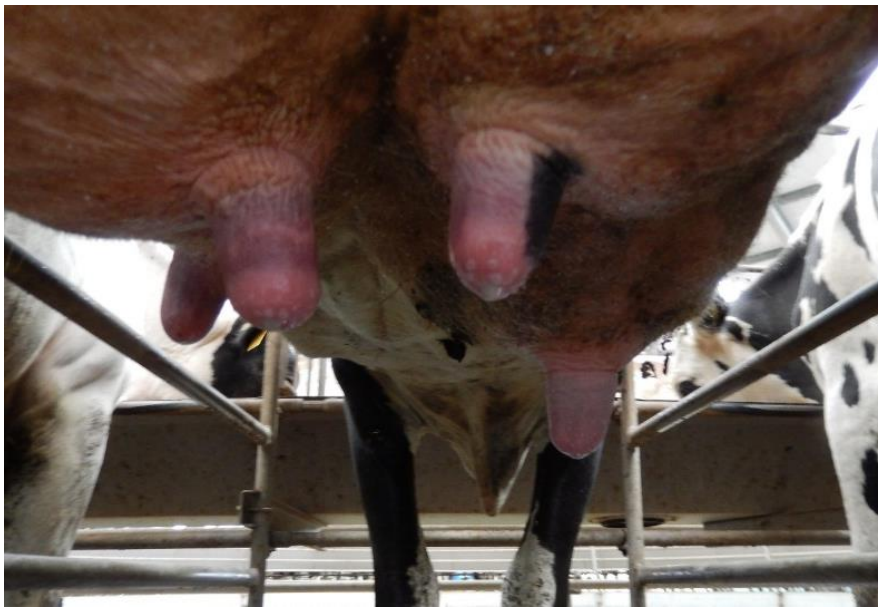


Figure 1.5 Oedema or blueing of the teat after milking due to incorrect set up of the milking machine.

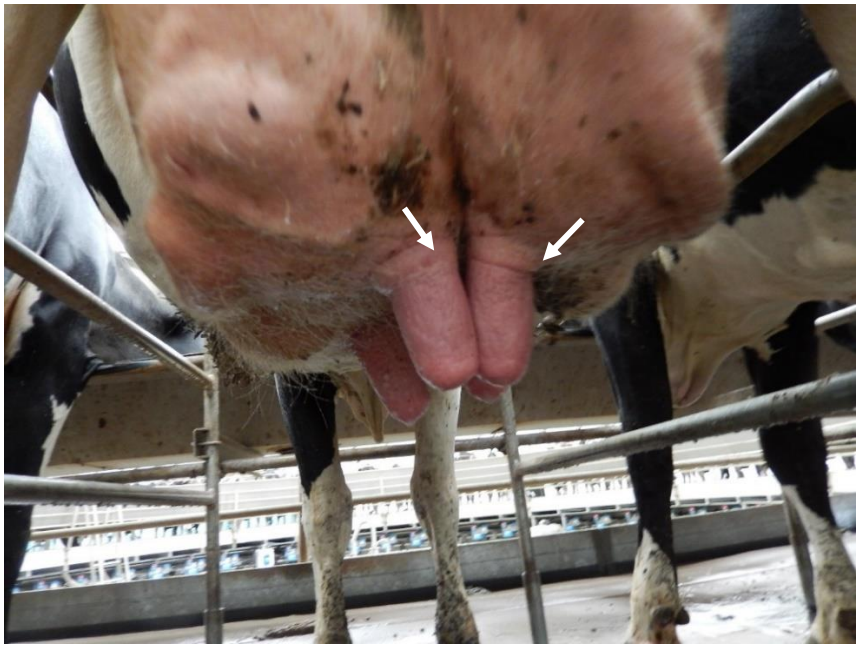


Figure 1.6 Ringing of the teat due to incorrect set up of the milking machine.

These lesions, although considered temporary with the teat returning to normal after milking, can be uncomfortable to the cow (Baines, 1993; Odorčić et al., 2019). Such lesions may be induced in part by the mechanical effect of the pressure on the teats during milking causing blood vessels to temporarily close and cause a fleeting ischaemia, although the blood vessels in cows' teats are thick walled and designed to withstand the pressures from a suckling calf. In addition, if there is already a teat lesion present, the milking machine can exacerbate the lesion (Shearn, 1993) making the milking process uncomfortable and this may lead to behavioural issues associated with milking, such as kicking off the cluster.

Other than the vacuum, the teat liners may be responsible for teat lesions. There are multiple teat liner designs with a combination of advantages and disadvantages for teat health (Gleeson et al., 2004). However, despite teat liner design being geared towards cow comfort, an important consideration of the teat liner is the frequency in which they are replaced (AHDB, 2022c). All common commercial teat liners are composed of rubber or less frequently silicone. These materials perish with time, use and chemical exposure. Damage to the teat liner can lead

to an unstable vacuum and subsequently teat lesions (Mein et al., 2001). Additionally, cracks and crevasses in a perished teat liner can result in inefficient cleaning, allowing the teat liner to act as a potential reservoir/fomite to harbour and spread microbiological agents and the possibility of spreading infectious agents between animals (AHDB, 2022c). As such, the milking machine can be a source of both physical alterations to the teat and transmit infectious agents (Odorčić et al., 2019).

Aside from the milking machine, the milking protocol or routine can also be a source for inducing teat lesions. In part, due to the manipulation of the teats when cleaning and the exposure to chemicals, disposable gloves, towels and sometimes the use of teat brushes as part of pre and post milking routines.

Pre-milking routines are designed to: 1. to clean the teat prior to milking; and 2. to stimulate milk let down (Wagner and Ruegg, 2002). There are manual, semi-automated and fully automated methods. These are mostly based on spraying, dipping or wiping with or without a drying step (Breen, 2019). Both manual and automated methods employing chlorine dioxide to clean the teats prior to milking will reduce bacterial counts on the teat (Baumberger et al., 2016). However, other farm conditions and management practices can have more of an impact on bacterial counts than disinfection of the teat alone (Baumberger et al., 2016). For instance, if general hygiene practices are poor and the teat dipping cups are soiled with faeces (Fig 1.7) then the disinfection will be less effective. If udders and teats are particularly soiled then careful washing, avoiding the introduction of bacteria to the teat canal by contaminated water, may be required prior to milking. Pre-milking cleansing routines are variable which may be as simple as wiping with a clean paper towel, to spraying or dipping the teats in disinfectant and using teat brushes (Fig 1.8). There are different commercial products available; however, the majority contain a few options of active ingredients (Table 1.3.1)..

Table 1.3.1 A selection of options used in the pre-milking routine that can be combined in different ways.

Active ingredient options in pre-milking product	Application options	Drying options	Labour type
Iodine based products	Dip cup	Air dry/no drying	Manual (hand application)
Chlorhexidine	Foam cup	Paper towel	Semiautomated (eg hand held mechanical scrubbing brushes)
Sodium Hypochlorite	Teat sprayer	Washable clothes	Automated (eg. sprayer on entrance to parlour)
Lactic acid Peracetic acid Alcohol Water	Scrubbing brushes	Disposable wipes	

Figure 1.7 An example poor hygiene with teat dipping cups containing little cleaning agent and contaminated by a large volume of faeces. The waste bin is also overflowing and caked in faeces.

Figure 1.8 Teat scrubbing brush used as part of the pre-milking teat cleaning routine.

As with pre-milking, there are a few options in which the post milking routine may be performed. It is ultimately designed to protect the teat end and prevent infectious agents entering the teat canal and the glandular tissue to prevent and control mastitis (Wesen and Schultz, 1970). Important features of a post milking teat dip are: a rapid speed of action, remaining on the skin for a period of time, containing skin conditioners to keep the teat supple, resistance to organic material, and be highly visible allowing the milker to assess if the teat has been coated sufficiently (Breen, 2019). There are multiple commercial products that can be used as part of the pre and post milking routine. However, these tend to have a limited option of active ingredients that contain varying proportions of teat conditioners and emollients. The post milking routine can again be split in to manual or automated systems. Systems are generally either dipping the teat in a dip/foam cup or spraying the product on to the teats after milking (Table 1.3.2).

Table 1.3.2 A selection of options used in the post-milking routine that can be combined in different ways.

Active ingredient options in post milking teat product	Application options	Labour type

Iodine based products	Dip cup	Manual
Chlorhexidine	Teat sprayer	Semiautomated
Sodium hypochlorite		Automated
Dodecyl benzene sulphonic acid		

In the UK, dipping rather than spraying post milking product is considered to provide more coverage although this generally takes longer (Ohnstad et al., 2012). In addition to automated post dipping systems, there are systems that integrate the automatic cleaning of the cluster between cows and are referred to as automatic dipping and flushing (ADF) systems, which have been found to reduce milking time (Ohnstad et al., 2012). It is possible that the products and methods used in the pre and post-milking routine may be potential sources of inducing teat lesions. Although it has not been readily reported in the dairy industry, the use of some chemicals in the milking routine may have the capability to induce a skin reaction or hypersensitivity in some animals. The use of chemicals also has the ability to alter the microbiome of the teat skin and may allow for imbalances in the skin bacteria. In humans, it has been shown that emulsifiers, that are commonly found in teat dips, can favour the growth of potential pathogens such as *Staphylococcus aureus* (Boxberger et al., 2021).

1.3.2 Environmental factors that may induce teat lesions

In the UK, dairy cows are maintained in a number of settings. Many will have a combination of being housed and having access to pasture. Nevertheless, it is becoming increasingly common to find dairy cows that are either housed year-round or at pasture year-round depending on the location of the farm and the type of farming system the farmer maintains (Redman, 2020). Each of these farming systems create different challenges for teat skin health. Cows with pasture access are more at risk to weather changes, from sun burn in the warmer,

drier months, to teat chapping from cold, wet weather and muddy fields. Teat chapping is more frequent in winter and often occurs at the base of the teat which may weep and form a scab and become secondarily infected with bacteria (Sieber and Farnsworth, 1984). Housed cows require bedding material, of which there are numerous options, including sawdust, sawdust and lime, straw and recycled manure products (RMP) to name a few. Cubicle houses frequently utilise rubber mats to increase cow comfort. When compared to concrete lying areas, the general risk of teat lesions was reduced with the use of soft rubber mats or mattresses (Ruud et al., 2010). Although bedding material provides comfort to the cow when lying, the bedding may act a reservoir of infectious agents, particularly bacteria. Nevertheless, the addition of lime to sawdust has been shown to reduce the number of bacterial pathogens on the teat skin (Paduch et al., 2013). However, this was a limited study of two three-week periods. The increasingly common practice of using recycled manure solids (RMS) is of concern as potentially this may be another source of pathogens (Leach et al., 2015). Nevertheless, the risk of subclinical and clinical mastitis has been found to be not significantly different from farms that use RMS to those using straw bedding (Frechette et al., 2022).

1.3.3 Other factors which may induce teat lesions

Cow genetics may pose as another potential risk factor for the development of ITN. Often dairy cow sires are selected on various desirable traits in their daughters. In some instances, the desirable traits are linked to udder and teat confirmation and have been shown to have a large heritability (Poppe et al., 2019). There are many different shaped teats that vary with cylindrical, bottle-shaped and funnel-shaped being described, with genetics also affecting the teat length and placement (Seykora and McDaniel, 1985a, 1985b).

Teats, especially long pendulous teats can be accidentally trodden on by the cow when standing or lying down. Accidental trauma of the teats can also occur via catching the teats on sharp

objects, for example barbed wire fences or broken cubicle dividers; therefore, maintaining a good and safe farm environment is crucial to animal and human safety (Saibaba et al., 2016).

It is possible that the shape of the teat can influence the way the mouthpiece of the machine fits onto the teat. For example, a shorter teat may not go as far into the liner and therefore will have less of a massage phase and longer milking phase, thereby predisposing the teat to oedema and other lesions (Odorčić et al., 2019). These are other potential risk factors that need to be considered for ITN.

1.4 Epidemiological approaches for investigating diseases of veterinary importance

One of the most important aspects of a disease investigation is to gain an understanding of the number and type of animals affected, the different presentations of the disease and the geographical distribution (Hitchcock et al., 2007). These foundations allow for disease monitoring and assessment of changes or detection of disease clusters which may indicate an outbreak. It is also important to understand the demographics of the affected animals and the farms, which may aid in identifying potential risk factors and allow for control measures to be implemented (Hitchcock et al., 2007; Fricker and Rigdon, 2020).

Questionnaires asking the people directly affected by the disease, in this case farmers, are important tools to investigate potential risk factors and have been used many times for hypothesis searching in farm animal diseases (Peeler et al., 2000; Angell et al., 2014; Tunstall et al., 2019). Multiple studies in the dairy industry have utilised the farmer based questionnaire to understand current farm practices and assess how practices have shifted and evolved (Bradley et al., 2007; Relun et al., 2013; Hokkanen et al., 2015). However, notoriously farmer-based questionnaires are frequently subjected to poor response rates. Nevertheless, there are

many ways to increase response rates, such as including a pre-paid return envelope and giving prior warning of an upcoming questionnaire, to gain the essential information the farming community can provide (Edwards et al., 2002).

When a disease is emerging, there is often little awareness across the industry as a whole with only people severely affected by the disease aware of its existence. Therefore, for an epidemiology study into the occurrence of the disease an awareness campaign is essential. There have been many disease awareness campaigns in veterinary medicine aimed to reduce the impact of the disease, engage stakeholders and to combat misinformation. Combating misinformation was highlighted as a major challenge during the Covid-19 pandemic that occurred during the length of this PhD work (Cheng et al., 2021). Veterinary examples of successful awareness campaigns include the World Health Organisation (WHO) campaign to vaccinate dogs against Rabies (TM et al., 2017); the reduction in the use of antimicrobials in production species by the Responsible Use of Medicines in Agriculture alliance (RUMA) and AHDB; and educating sheep farmers regarding the recently emerged disease contagious ovine digital dermatitis (CODD) (Duncan et al., 2022). These campaigns focused their advertising programs both on the disease and on educating stakeholders. As ITN anecdotally appears to be an emerging disease, educating dairy farmers and veterinary surgeons regarding ITN is a vital aspect investigating the any epidemiological aspect of this disease. Gaining an understanding for the basic epidemiological data can provide insight into many areas such as modes of transmission, type of infection, risk factors for disease, management and efficacy of any control protocols, understanding impact on farms and farmers, and on cost of prevention/treatment to name a few.

1.5 The importance of documenting pathology in disease investigations

An essential early aspect of any disease study is to create a case definition and document the different clinical presentations to allow for rapid, accurate and consistent diagnosis around the world (Fricker and Rigdon, 2020). Once this is established and recognised, surveillance measures can be instigated to detect disease cases in new locations and/or in other species (Fricker and Rigdon, 2020). Emerging diseases of unknown aetiologies have caused devastating effects to the UK cattle industry and Worldwide, such as Bovine spongiform encephalopathy (BSE) (Kumagai et al., 2019) and Bovine neonatal pancytopenia, also known as bleeding calf syndrome (Lambton et al., 2012). After successful disease investigation processes the aetiologies were identified as transmissible prions and use of a particular vaccine, respectively. In addition to documenting the clinical and gross presentations, performing a histopathological examination of the affected teats is paramount. The histopathological picture can often provide insights into potential aetiologies and pathogenetic pathways that may not be apparent from the clinical and macroscopic picture.

The previous descriptions of ITN have been restricted to macroscopic or gross findings in a limited number of resources (Blowey, 2004; Andrews et al., 2008; Blowey and Edmondson, 2010; Mauldin and Peters-Kennedy, 2015; Clegg et al., 2016b) . These resulted in other names for ITN, such as summer sores and teat eczema (Blowey and Weaver, 2003), primarily due to confusion on the disease definition. As such, there is urgency to fully characterise and differentiate the disease. A pilot study detected digital dermatitis associated treponemes in 11 of 12 ITN cases via PCR (Clegg et al., 2016b). These bacteria have been detected in a number of skin diseases in farm animals, particularly associated with lameness, including DD in cattle (Evans et al., 2009) and CODD in sheep (Sullivan et al., 2015a). The different presentations of these diseases are well documented including the establishment of grading system (Döpfer et al., 1997; Angell et al., 2015a), which provide convenient ways to follow outcomes and progression of disease. In DD and CODD not only were the DD associated treponemes detected

via PCR but these bacteria were also observed in abundance within the lesions using immunohistochemistry (IHC) (Evans et al., 2009; Angell et al., 2015b) (Fig 1.9). In addition to looking for infectious agents, the same techniques can be developed for inflammatory markers to aid in further understanding pathogenesis of disease (Newbrook et al., 2021).

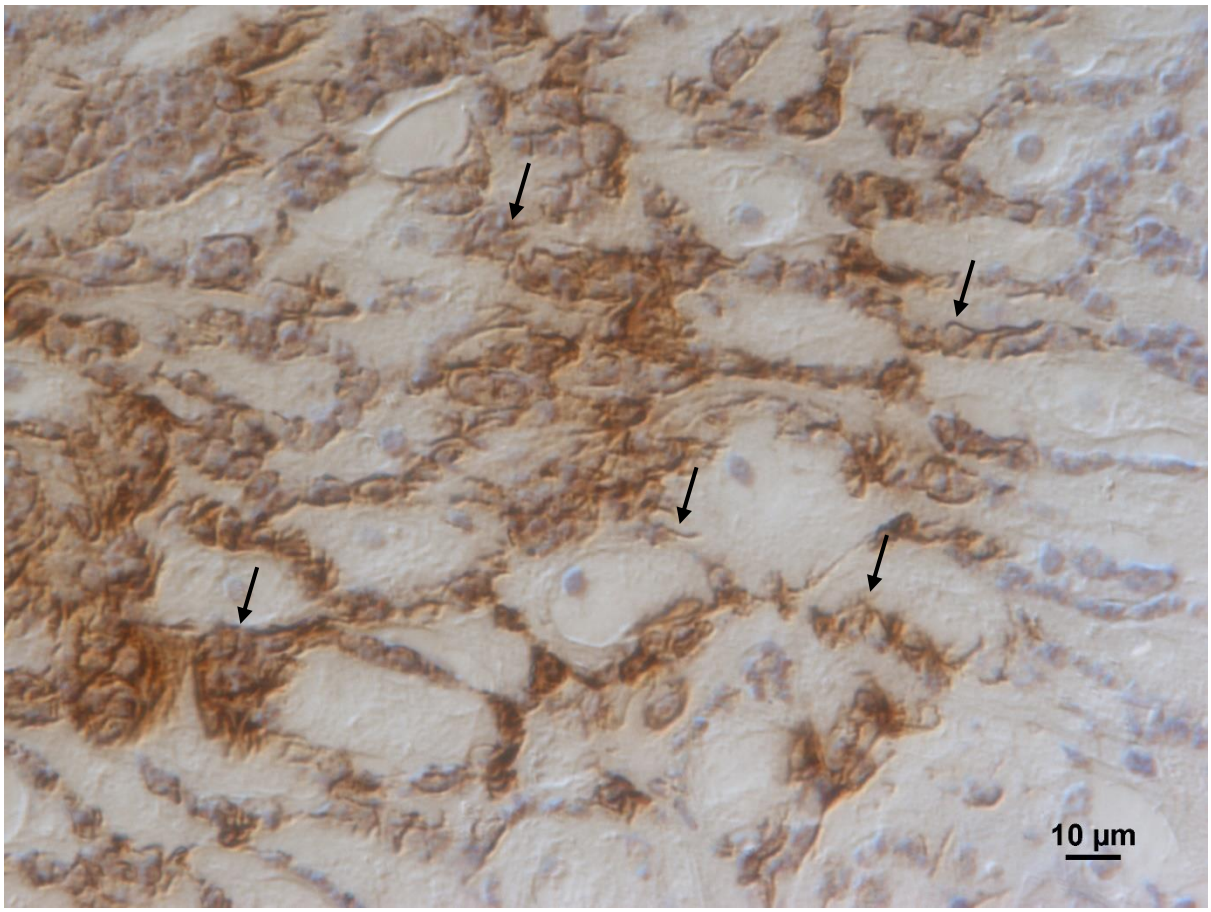


Figure 1.9 Immunohistochemistry from the dorsal horn of a CODD grade two lesion in a sheep using an antibody to target the DD associated treponemes. Brown represents positive labelling with spirochaetal-shaped positivity roughly tracking around keratinocytes (arrow) (400x magnification).

1.6 Infectious skin diseases caused by DD associated treponemes

The potential for involvement of DD associated treponemes in ITN has been discussed above. These bacteria received the collective name due to being associated with an important infectious skin disease that causes lameness in dairy cattle, digital dermatitis (DD). This disease

has been identified as a substantial welfare concern. In addition, DD in dairy cattle was estimated to cost approximately £75.75 per case with the cost likely to have drastically increased since the time of the study (Willshire and Bell, 2009). As yet the aetiology of DD has not been confirmed and is considered polymicrobial. However, several phylogroups of Spirochaetal bacteria, the DD associated treponemes, are considered the most likely aetiology with DD associated bacteria detectable by molecular techniques in all the cases and have successfully been cultured from DD lesions (Wilson-Welder et al., 2015). Three main pathogenic phylogroups of DD associated treponemes are recognised, *Treponema medium*, *T. phagedenis* and *T. pedis* (Evans et al., 2008). These treponemes are separated from non-pathogenic treponemes such as *T. ruminis* that have been isolated from healthy cow rumen (Newbrook et al., 2017). These bacteria are gram-negative, helical, highly motile, highly fastidious and require specific environmental conditions, including an anaerobic environment, to grow and thus historically have been elusive in many diseases (Norris et al., 2015; Brodard et al., 2021). However, molecular techniques, mostly PCR assays, have allowed for molecular detection of DD associated treponemes in an increasing number of diseases, and in an expanding host range, including CODD in sheep (Sullivan et al., 2015a), foot lesions causing lameness in goats (Sullivan et al., 2015b) and elk (Clegg et al., 2015) and skin lesions in pigs (Clegg et al., 2016d). They have also been detected from additional locations of skin lesions in dairy cattle, including hock lesions (Clegg et al., 2016a) and pressure sores (Clegg et al., 2016c) and in a subset of ITN cases (Clegg et al., 2016b). With the increasing number of lesions associated with DD associated treponemes there is the potential for these bacteria to be a main pathogen of ITN and the possibility these bacteria have several reservoirs within the host and the environment. In fact, one study found them in foremilk in UCD animals (another disease where DD associated treponemes have been previously detected) (Sobhy et al., 2020) and

multiple studies have reported them in the environment including the bedding, cattle footprints (Bell, 2017) and hoof knives (Gillespie et al., 2020).

Koch's postulates (Loeffler, 1884) states that for a microorganism to cause disease the following statements must be met:

1. The microorganism must be found in diseased but not healthy animals
2. The microorganism must be cultured from the diseased individual
3. Inoculation of a healthy individual with the cultured microorganism must recapitulate the disease
4. The microorganism must be re-isolated from the inoculated, diseased individual and matched to the original microorganism

Therefore, screening of all samples of ITN cases for DD associated treponemes to assess the potential for Koch's postulated to be fulfilled and the increased sample size will be important for understanding to true impact of these bacteria in ITN cases. For DD, while the pathogenic DD associated treponemes are routinely detected from DD lesions via molecular methods, due to the fastidious nature of treponemes, the final steps to fulfil Koch's postulates for DD are yet to be achieved. The current hypothesis for clinical DD to be apparent there requires multiple infections with different bacteria in waves with DD associated treponemes being a key pathogen but not the single aetiological agent (Krull et al., 2014, 2015). However, it is clear that screening ITN samples for these bacteria is an important step in understanding the aetiopathogenesis of ITN.

1.7 Next generation sequencing for identifying potential disease agents in the microbiome

As a disease may be multifactorial and multi-aetiological, understanding which potential aetiological agents are involved and the importance of such agents in disease development is challenging. In addition, healthy skin harbours large numbers of commensal microorganisms, which collectively are referred to as the microbiome and the composition of the microbiome can vary across anatomical locations (Swaney and Kalan, 2021). Recently, there has been increased interests in studying the microbiome with disease progression (Krull et al., 2014; Boxberger et al., 2021). Advancements to technology have made it possible to rapidly sequence whole genomes allowing for new aetiological agents to be identified that were previously difficult to detect and isolate using traditional time-consuming and narrow microbiological and molecular techniques. Not only have new agents been identified in diseases, but also new relationships between microorganisms with more areas of symbiosis becoming apparent (Swaney and Kalan, 2021). Many diseases that were once considered caused by a single aetiological agent, using traditional techniques have been found to appear more complicated by new deep and broad sequencing techniques. These technological progressions have thus enabled researchers to study the microbiome in health and disease and dissect disease aetiologies (Boxberger et al., 2021; Swaney and Kalan, 2021).

As anecdotally the numbers of ITN cases appeared to be rapidly increasing, an infectious agent has been proposed (Blowey, 2004; Manning, 2016). The pilot study investigating potential pathogens in ITN cases, indicated that BHM and pseudocowpox were unlikely to be primary agents and that DD associated treponemes were a possible aetiology (Clegg et al., 2016b). However, as this is a skin disease that could potentially affect the natural microbiome the pathogenesis is likely to be far more complex than infection with a single group of bacteria,

and there is also the possibility that DD associated treponemes are not a primary pathogen in this disease and maybe detected in some animals as opportunistic and secondary agents.

There are multiple ways to investigate the microbiome, these days mainly involving next generation sequencing (NGS) approaches. One method of NGS is shotgun metagenomics. Briefly, this sequences all the DNA in a sample, including host, and any bacterial, viral, fungal or parasite DNA that may be present (Ilumina, 2022). These technologies also remove the requirement of having a suspected aetiological agent and designing primers to target such agent in PCR assays and therefore are useful for hypothesis searching (Gwinn et al., 2019). A favoured approach employs Illumina technology (Illumina Inc., San Diego, CA, USA) and this has previously been used to investigate potential aetiologies and new associations between microorganisms in diseases that have previously only been associated with DD treponemes such as DD in dairy cattle (Krull et al., 2014), Contagious Ovine Digital Dermatitis (CODD) in sheep (Duncan et al., 2021) and Udder Cleft Dermatitis (UCD) in dairy cattle (Ekman et al., 2020). In addition to looking for potential pathogens, NGS can be used to detect the presence of antimicrobial resistance (AMR) genes, allowing for an understanding not only of the pathogen present but if there is already resistance within the bacterial population that may lead to poor responses to treatment with antimicrobials (de Abreu et al., 2021). Regardless, as the host DNA is also sequenced using this technique, it can overwhelm the microbiome data and reduce the read depth in the area of interest. To compensate for the contamination by host DNA, many studies where a bacterial population is of interest will also include analysis of the 16S rRNA gene amplicon sequencing. This type of sequencing can be performed using multiple platforms including Illumina and more recently using the portable and rapid Oxford Nanopore Technology (ONT, Oxford, UK), which can also be used for whole genome sequencing (WGS). However, ONT is not considered as specific or as sensitive as Illumina but is making improvements in this area (Gwinn et al., 2019; Kerkhof, 2021). As the 16S rRNA

gene is a ubiquitous housekeeping gene present in all bacterial genomes but not present in the mammalian genome, targeting this area can allow for amplification and assessment of the bacteria present without actively depleting the host genome (Heravi et al., 2020). However, while the 16S rRNA gene can identify to the Phylum level it is not always possible for this method to categorise reads to the species level (Ranjan et al., 2016). Therefore, 16S rRNA gene amplicon studies run in parallel to WGS can increase confidence in results especially in tissue samples with a high percentage of host DNA present. These new technologies allow for a greater understanding of the complexity of microbial diseases and aid in studies of transmission dynamics (Gwinn et al., 2019).

As it is currently unknown which aetiological agents are involved in ITN, it is currently not possible to target an gene using PCR methods for a rapid diagnosis. A combination of WGS and 16S rRNA gene amplicon sequence can be used to identify potential pathogens that may be indicated in the development of ITN lesions (Ranjan et al., 2016). Additionally, as the aetiology is unknown it is not possible to provide evidence-based and targeted control measures and treatments.

1.8 Aims and objectives

The hypothesis of DD associated treponemes being the aetiological agent of Bovine ischaemic teat necrosis was proposed and that ITN would be attributable to the presence of pathogenic treponemes within the teat lesions. Furthermore, if DD associated treponemes were detected in all ITN lesions, then farms with a large number of cows with DD foot lesions would likely report ITN cases compared to farms with no active DD foot lesions. Therefore, the main objectives for this multidisciplinary approach to a disease investigation into Bovine ischaemic teat necrosis were:

- Perform an epidemiological study to:

    - Identify the proportion of farmers that have observed ITN on their farm and over what time frame;
    - Assess how widespread ITN is throughout Great Britain;
    - Gain information on when farmers reported the index ITN case on their farm;
    - Identify the reported at-risk animals;
    - Investigate factors potentially associated with ITN at a farm level.
- Apply basic pathological principals to:
    - Record the variations in presentation of the clinical disease;
    - Apply a grading scheme to the different variations in clinical disease;
    - Record the histopathological presentations of the different clinical grades;
    - Apply special stains to investigate potential aetiologies;
    - Use immunohistochemistry techniques to investigate potential aetiologies.
- Investigate the possibility of the involvement of digital dermatitis associated treponemes in ITN via:
    - Screening all ITN samples using a nested PCR assay for DD associated treponemes;
    - Screening parts for the environment for DD associated treponemes;
    - Consider the possibility of milk acting as a reservoir for DD associated treponemes.
- Survey the microbiome for potential ITN aetiological agents by:
    - Performing shotgun metagenomics on diseased ITN teats and compare with non-lesion, healthy teats
    - Comparing 16S rRNA gene amplicons of the diseased ITN teats and compare with non-lesion, healthy teats.

Chapter 2: An observational study investigating potential risk factors and economic impact for bovine ischaemic teat necrosis on dairy farms in Great Britain.

The data presented in this chapter is supported by the paper: An observational study investigating potential risk factors and economic impact for bovine ischaemic teat necrosis on dairy farms in Great Britain. Authors: Hayley Ellen Crosby-Durrani, Al Manning, Roger Blowey, João Sucena Afonso, Stuart D Carter, Nicholas James Evans, Joseph W Angell published in Frontiers in Veterinary Science on 22nd March 2022.

2.1 Introduction

Bovine ischaemic teat necrosis (ITN) is a relatively new disease, first reported in 2004 (Blowey, 2004). The disease affects the teats of dairy cattle (*Bos taurus*) and can lead to sloughing of teat tissues, resulting in pain and discomfort and consequently is a welfare problem (Blowey, 2004). Moreover, ITN is considered to have substantial economic consequences for farmers that have experienced this disease as many animals do not respond to treatment and have to be culled prematurely as a result.

Ischaemic teat necrosis has been associated with the digital dermatitis (DD) *Treponema* bacteria (Clegg et al., 2016b) and thus is considered to potentially be infectious in nature. There are many infectious diseases that can affect the teat of the dairy cow. One of the differential diagnoses for ITN is bovine herpes mammillitis (BHM). Ischaemic teat necrosis and BHM can be differentiated based on their clinical presentations as ITN presents as a focal dry red to black area of necrosis on one or more teats (Blowey and Edmondson, 2010) compared with the exudative lesion produced by BHM that can affect one teat or involve the entire udder (Gibbs, 1984; Shearer et al., 2008). Another different clinical presentation between the diseases is that ITN cases can be highly pruritic in nature (Mauldin and Peters-Kennedy, 2015), which is not a reported sign of BHM.

Infectious diseases are not the only cause of teat lesions with many lesions associated with environmental factors or poor milking systems or techniques. These other diseases of bovine udder skin are considered multifactorial and the result of the interactions of environmental, infectious and other factors. An example of such a disease is udder cleft dermatitis (UCD), lesions of which also reportedly contain DD *Treponema* spp. (Stamm et al., 2009; Evans et al., 2010). UCD typically affects the skin either in-between the two halves of the udder or at the junction of the anterior udder and the abdomen (Olde Riekerink et al., 2014; Bouma et al., 2016; Ekman et al., 2018; Sorge et al., 2019). Clear aetiological, environmental and epidemiological data is lacking for ITN. Moreover, it is unknown how many GB dairy farms have experienced ITN and the associated cost implications of cases, although there are reports that ITN is an increasing problem (Blowey, 2004; Clegg et al., 2016b; Manning, 2016). Hence, it is timely to attempt to identify how widespread this disease has become, its transmission dynamics, associated risk factors and the economic impact of ITN on the GB dairy industry.

Farmer questionnaires have been frequently used to investigate potential areas of interest and risk factors associated with farm animal diseases (Peeler et al., 2000; Angell et al., 2014; Tunstall et al., 2019). They have been used regularly in the dairy industry to gain further understanding of current farm practices and to identify how issues change over time (Bradley et al., 2007; Relun et al., 2013; Hokkanen et al., 2015). However, this method is subjected to poor response rates. There are many studies that have aimed to find ways to increase response rates such as including a pre-paid return envelope and giving prior warning of an upcoming questionnaire (Edwards et al., 2002).

As ITN is a relatively recent teat disease, increasing awareness of this manifestation is important. There have been many disease awareness campaigns in veterinary medicine aimed to reduce the impact of the disease, engage stakeholders and to combat misinformation.

Examples of such awareness campaigns in the veterinary world would be the vaccinating dogs against Rabies (TM et al., 2017); reducing antimicrobial resistance in production species by the responsible use of medicines in agriculture alliance (RUMA) and AHDB; and educating sheep farmers regarding the recently emerged disease contagious ovine digital dermatitis (CODD) (Duncan et al., 2022). These campaigns focused advertising both on the disease and on educating stakeholders. As, such educating dairy farmers and veterinary surgeons regarding ITN should be a key component to any epidemiological survey.

The aims of this study were to: 1) investigate the farmer reported experience of ITN in the GB dairy herd, 2) to identify potential associated risk factors and 3) calculate some of the associated costs involved with ITN by using a farmer-based postal questionnaire, with an online and telephone option.

2.2 Methods

2.2.1 Ethics Statements

The studies involving human participants were reviewed and approved. Ethical approval was granted by University of Liverpool, School of Veterinary Science Ethical Committee (application number: VREC 460). The participants provided their written informed consent to participate in this study. No potentially identifiable human images or data and no animal studies are presented in this study.

2.2.2 Study design

A series of farmer interviews and farm visits to develop an observational study using a twelve-page postal questionnaire, with an additional pictorial guide of diseases affecting the bovine udder, was designed (see Appendix A.1.1).

2.2.3 Sample size calculation

The study population was selected from producers designated as dairy farmers in a database of the AHDB. Formerly known as DairyCo, this board collects a levy from dairy farms across Great Britain (GB). Sample size was calculated using the online tool, OpenEPi (https://www.openepi.com) and farms were randomly selected using simple randomisation to attempt to gain information across all types of dairy farms. There were 10250 dairy farms in the database provided by AHDB Dairy in 2017 composed of 9464 producers in England and Wales, and 786 in Scotland. To be included in the AHDB Dairy database dairy farmers were considered to be a site producing raw material for dairy milk products by the food standards agency (FSA) and aside from dairy cattle included buffalo, goat and sheep milk producers. It was unknown exactly how many non-cattle dairy producers were including in the database and thus they were included in the sample size calculation. For farmers to be eligible to have the completed the questionnaire used in the analysis they had to be within this database, farm in GB and have an active dairy milking cow herd. As the hypothesised percentage frequency for the presence of ITN within the population of dairy farms was unknown, a value of 50% was used with confidence limits set at 5%. The sample size required to detect this value at a 95% confidence level for this population was 371. Potential response rate was estimated to be 20% due to previous AHDB Dairy experiences and relevant publications with questionnaires targeting a similar population (Angell et al., 2014; Cresswell et al., 2014). Therefore, in an

attempt to obtain a sample size of 371, 18.1% of the target population (1855 questionnaires) received a postal questionnaire

2.2.4 Advertising

An essential part of the study was to engage with farmers from the onset to obtain study participants, gather future samples for pathological and microbiological screening and encourage a higher response rate for the questionnaire. Between April and October 2017, a combination of flyers, posters, conference attendance, newsletters, and magazine articles were targeted towards dairy farmers and farm veterinarians along with continuous professional development (CPD) events for veterinary surgeons were conducted, prior to dispatching the postal questionnaire. A webpage on the University of Liverpool website was also created with a link to an email address and telephone number should teat necrosis be searched for in an internet search engine. This was aimed to increase awareness throughout the dairy industry that ITN exists and about this study.

2.2.5 Questionnaire design

The main aims of the questionnaire were to:

1. Identify the proportion of farmers that have observed ITN on their farm and over what timeframe;
2. Gain information on when farmers reported the index ITN case on their farm;
3. Identify the reported at risk animals (animal-level factors);
4. Investigate factors potentially associated with ITN at a farm level.

Due to the constraints on accessing farmer data, only names and addresses of dairy farms were available and therefore a postal questionnaire was chosen as the only suitable mode for obtaining the required data. Many epidemiological resources and previous studies were employed for questionnaire development (Peeler et al., 2000; Bradley et al., 2007; White et al., 2008; Relun et al., 2013; Hokkanen et al., 2015; Bludau et al., 2016; O'Kane et al., 2017). These studies influenced the layout and content of the questions and guide to importance of variables to investigate in the dairy industry. As ITN is a poorly studied disease a pictorial guide was developed as used in Angell et al., (2014) and O'Kane et al., (2017) to aid completion of questions regarding specific diseases. The pictorial guide represented examples of different diseases described in the questionnaire for comparative purposes. This guide also included full written descriptions and was reviewed by farmers and industry experts (Roger Blowey and Al Manning) prior to distribution with the questionnaire to confirm an accurate description of ITN. It was found that farmers were readily able to correctly distinguish between teat skin diseases from this guide. The images and written descriptions were also compared to veterinary textbooks (Blowey and Edmondson, 2010; Mauldin and Peters-Kennedy, 2015; Blowey, 2016). Farmers were asked to refer to the pictorial guide when answering disease specific questions. The survey covered a wide range of topics including: questions related to the farmers' experience with ITN; the health of the udder; general animal health; milking routine and the farm environment. Each question also included a "don't know" and an "other" option. The "other" option had an area for free text to allow farmers to expand on their answers. As part of the questionnaire development, 26 dairy farmers were interviewed extensively during phone calls and farm visits and a pilot questionnaire developed based on these experiences. This pilot postal questionnaire was then distributed to ten different dairy farmers. Five of the ten farmers responded and their feedback, while mostly positive, informed and altered the final questionnaire design.

As with many previous studies, and in a view to increase response rate, one week prior to questionnaire dispatch, a postcard stating that the selected farm address would be receiving a postal survey was sent (Edwards et al., 2002). This questionnaire along with a cover letter, pen and pre-paid return envelope was posted in January 2018. Postal questionnaires included a link to an online version of the survey and a telephone number in case farmers preferred to respond in that way or had questions that required clarification. All questions were optional with any data provided on a voluntary basis. All participants were given the option to withdraw from the study at any time and to self-select in to a prize draw in appreciation of their time completing the survey. The dataset was anonymised.

2.2.6 Geographical location

From the returned questionnaires the geographical location of numbers of farms returning the questionnaire and the number of farms affected were mapped based on reported county level at a midpoint in the county using Google Maps™ mapping service. Google Maps™ mapping service is a trademark of Google LLC (Mountain View, CA, USA) and this study is not endorsed by or affiliated with Google in any way. The farms that returned the questionnaire were mapped separately to those that returned the questionnaire and reported having had a case of ITN on the farm.

2.2.7 Data analysis

A database was constructed with all questionnaire responses manually entered. After this, a series of range and consistency checks were performed to identify any input errors and the returned hard copy of the questionnaire consulted and any errors rectified. Many variables were categorical (Appendix A.2.1.1). Variables that were continuous in nature were transformed into

categorical groups where appropriate. All analyses were carried out using R version 3.5.0 (RStudio, Inc., Boston, USA) using the following packages in alphabetical order: Amelia, base, DescTools, dplyr, lmtest, LogisticDx, Mass, PropCIs, ResourceSelection, sjPlot, and stats.

Exploratory and descriptive statistical investigations were applied to assess the distribution of the data, and any outlying data. A Chi squared test was used to assess differences between groups. Logistic regression analyses were carried out where appropriate. For all analyses, statistical significance was set at p-value ≤0.05 for evidence of a strong association and p-value 0.05-0.2 for evidence of a weak association. The denominator changed per variable to reflect the number of farmers that responded to each question. The primary outcome variable was the presence of ITN on the farm; secondary outcome variables were the presence of UCD and chapped teats.

2.2.7.1 Missing data

Many variables contained some missing data, either where the participant had not answered or was unable to answer, or where they had answered "don't know". The pattern of missingness was assessed as a generalised pattern of missingness (Dohoo, 2015). As attempts at multiple imputation failed, where applicable, multivariable analyses were carried out on constrained datasets whereby observations with missing values were excluded from the model.

2.2.8 Cost of ITN

The costs associated with ITN were calculated using the questionnaire data alongside various industry guides and references. Costs were averaged over all calving systems and data used to calculate the cost per case. Three separate financial calculations were made based on the

following categories: if the animal was an uncomplicated ITN case which recovered; if the cow lost the affected teat or developed mastitis and finally, if that animal was culled early on in the lactation due to ITN complications. For calculation purposes, it was assumed that once an ITN lesion appeared on the teat, milking the affected quarter would be challenging or not possible for the rest of the lactation. The reproductive losses were not calculated for a recovered case or a cull case of ITN but are included for a case with complications. It is assumed that a cull case was culled early in lactation, less than 100 days, due to the severity of the ITN lesion. For calculation purposes, a case was considered to affect only one teat and therefore the milk from one quarter. Therefore, these are likely minimum costs as many reported cases affect more than one teat.

2.2.9 Associations with ITN presence on the farm

Both univariable and multivariable analyses were carried out using logistic regression. Observations were excluded where farmers had not answered a question or had responded with "don't know". All exposure variables with a p-value <0.2 on univariable analysis were included for subsequent investigation within the multivariable regression models.

An initial multivariable model, including all the selected exposure variables, did not generate converging outcomes; consequently, variables were grouped into the following common themes: 1) disease factors: presence or absence of certain diseases on the farm; 2) chemical factors: such as disinfectant usage; 3) farm environment and management factors: including other animals on the farm, vaccination history and calving system.

For each of the three themes, multivariable models were fitted using a step-wise backwards elimination strategy whereby a full model was fitted including all the selected variables for that category. Then, each variable was removed in turn and a likelihood ratio test carried out.

Variables were retained if the resultant p-value was <0.05. Omitted variables were then added back in turn to the final model starting from the lowest p-value. A likelihood ratio test was performed after each addition and the variable retained in the model if p-value <0.05. This process was continued until no further variables could be added to produce the final model.

Following construction, variables retained in each of these models were then combined in an overall model. Stepwise backwards elimination was carried out again as previously described using the explanatory variables from the previous three models to produce the final model. Due to the presence of observations with missing values (for example where a farmer responded with 'don't know') the addition and subtraction of variables was performed on a constrained dataset excluding those with missing data. The final model was presented using all available observations for the variables included, excluding those with missing values.

Post estimation, the final model fit was assessed using the Hosmer-Lemeshow goodness of fit test and estimating the area under the receiver operating characteristic (ROC) curve. The mean predicted probability of the outcome (the presence of ITN on a farm) was then compared to the observed proportion of farms with that outcome to visually assess the reliability of the model.

The final multivariable model included two disease factors which were considered to potentially induce a risk of collider bias. Consequently, fitting a multivariable model was attempted excluding all disease factors as well as those variables where there were large numbers of observations with missing values. However, it was not possible to fit a multivariable model with reliable estimates (with realistic standard errors), and as a consequence the univariable models are presented.

2.2.10 Associations with UCD and chapped teats as secondary outcome variables

From the results using ITN as the primary outcome variable it was clear that UCD and chapped teats were associated with the presence of ITN on the farm. Given that the nature of the questionnaire data gathered was largely transferrable, the analysis was repeated using UCD and chapped teats as secondary outcomes. For UCD, a forward stepwise process was implemented as models did not converge when using a series of backwards approaches. As for ITN, a similar potential risk of collider bias could arise. In the same way, multivariable models were fitted excluding all disease factors and variables with large amounts of missing data.

2.3 Results

2.3.1 Response rate and geographical location

Of the 1855 surveys posted, a total of 263 were returned including 256 paper questionnaires, four online questionnaires and three farmers responding via email or telephone. Postal questionnaires were mostly returned January to March 2018 with a further four returned up to June 2018. Of these, 227 were adequately completed, producing an overall returned response rate of 12.2% (95% CI: 10.8-13.8%). Response rates from each region were similar with 12.3% of 225 (95% CI: 10.6 -14.2%) respondents from England, 13.0% (95% CI: 8.5-18.7%) from Scotland and 13.3% (95% CI: 9.7-17.5%) from Wales. Three respondents did not indicate the country their farm was situated in. When using a 95% CI, there was no statistical difference in response rate per region with farmers from all countries reported having had cases of ITN. England had 86 positive farms from 162 farms (53.1%; 95% CI: 45.4-60.6%); Wales had 15 from 42 (35.7%; 95% CI: 23.0-50.8%); and Scotland had 14 positive farms from 24 respondents (58.3%; 95% CI: 38.8-75.5%) (Fig. 2.1). As not all answers in the questionnaire were completed, or where farmers responded with the "don't know" response, the response

rate per question varied. There were some redundancies within the sampling frame and Table 2.1 shows the reported reasons for not completing the questionnaire.

Figure 2.1. ***Location of reported cases of ITN from the farmer questionnaire****. Google map showing markers at the centre point of the county where farmers reported cases of ITN on farm. Each point represents the county level and therefore one point can represent multiple farms.* (Google, 2022)

Table 2.1 Reported response reasons for not completing the questionnaire.

Responded via post	**Number**
No longer in dairy farming	17
Not a dairy farm	2
No reason	2
Not the right address	1
Responded via phone or email	
Not a dairy farm	2
No longer in dairy farming	1
Total	25

2.3.2 Descriptive statistics

One hundred and sixteen of 227 (51.1%; 95% CI: 44.4-57.8%) farmers reported that they had observed a case of ITN at some point between 1985 and 2018. Of those that provided a date when they first observed the disease on their farm (n=108), fifty farmers (46.3%; 95% CI: 36.7-56.2%) reported seeing the first case of ITN in the three years up to 2018 (Fig. 2.2). There was a reported increase in farmers witnessing cases for the first time within the last decade.

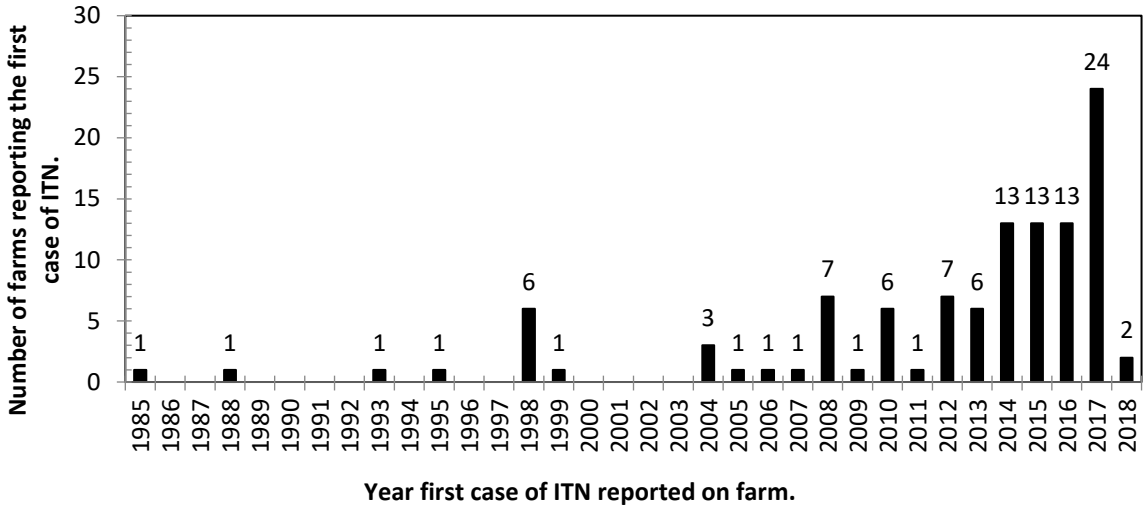


Figure 2.2 ***Frequency of the year farmers reported seeing the first case of ischaemic teat necrosis (ITN) on their farm.*** *The number of farmers reporting the first case of ITN observed on the farm is persistently higher from 2012 than earlier years. Note there are only two farms reporting the first case in 2018 as the questionnaire was submitted in January 2018.*

Participants also reported that they had previously called ITN by other names including: teat sores, udder sores, cracked teats, dermatitis, 'dermo', sores, wart teats, black teat, teat scabs, manure burn, teat rot, cow pox, teat necrosis, orf, herpes mammillitis, 'digi of the udder', and licking teat.

The age group of animals affected was allocated based on the production age depending on which lactation the affected cows were in or if they were pre-lactation heifers. To the question asking in which lactations the farmers had seen cases of ITN, 116 farmers responded, with 25 seeing ITN in more than one age group, therefore giving a total of 146 responses (Fig. 2.3). The reported production age of animals indicated that first lactation cows were significantly more likely to develop ITN lesions with 47.3% (95% CI: 38.7-55.9%) of cases reported in first lactation cows (p-value <0.001) and less than 15% (95% CI: 0.8-29.2%) in any other lactation and only 3% (95% CI: -11.7-17.7%) pre-lactation.

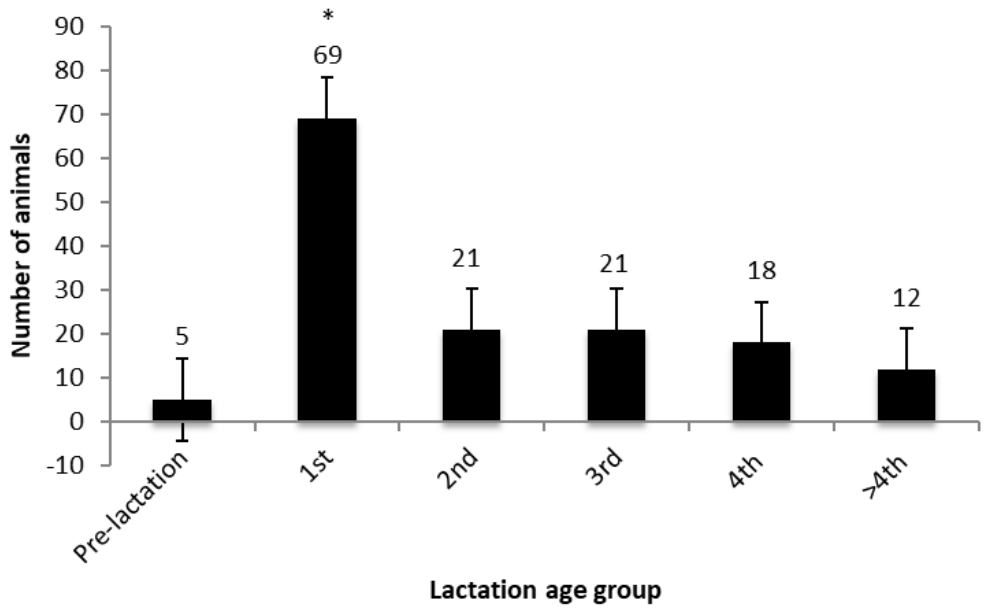


Figure 2.3 ***The production age of animals depending on the lactation the cow presented with an ischaemic teat necrosis (ITN) lesion on the teat****. First lactation heifers are significantly over-reported as developing ITN lesions on their teats. * Represents a significant difference (p-value <0.001).*

Farmers also reported that there were significantly more animals affected by ITN lesions within the first 90 days in milk (DIM) (78.9%; 95% CI: 75.2-82.6%) compared to animals over 201 DIM and animals in the dry period (9.4%; 95% CI -6.4-25.2%; p-value <0.001) (Fig. 2.4). Seventeen farmers (14.8%; 95% CI: -0.9 – 30.5%) of 115 that responded reported the lesions appearing in more than one DIM category.

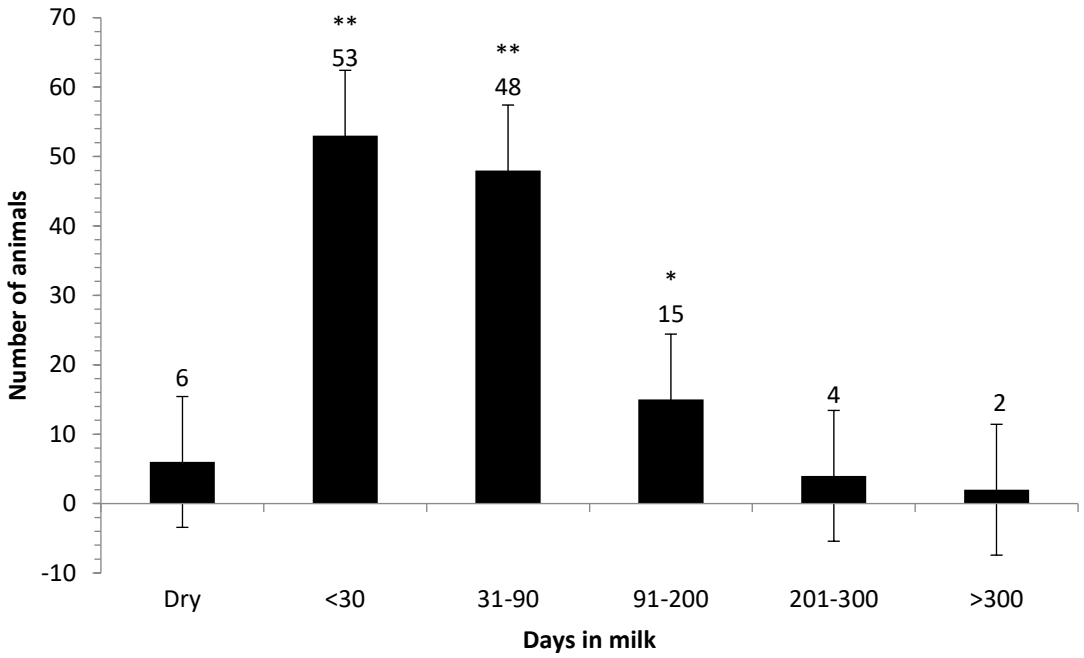


Figure 2.4 ***Days in milk that the affected cows are first observed with an ischemic teat necrosis (ITN) lesion.*** *The time period that cows are reported to first be observed with an ITN lesion on their teats are the categories of less than 30 days and 31-90 days in milk. Later in the lactation and during the dry period cows are reportedly less likely to present with an ITN lesion. ** Very strong evidence of a difference (p-value <0.001), * strong evidence of a difference (p-value <0.02).*

When questioned on the time of year that farmers observed ITN lesions, 116 farmers answered with 46 (39.7%, 95% CI: 28.7-50.7%) seeing the disease in more than one season (*n*=225). Farmers reported fewer cases during springtime compared with other seasons. There were 26 ITN cases (11.6%, 95% CI: 0-23.2%) reported in spring, 82 (36.4%, 95% CI: 28.1-44.7%) in summer, 66 (29.3%, 95% CI: 20.1-38.6%) in autumn and 51 (22.7%, 95% CI: 12.6-32.8%) in winter. However, once cofounding factors, such as lactation number and calving pattern were considered, it was not possible to fit a model giving reliable estimates.

Farms varied in size from 5 to 1923 milking cows and were grouped into five categories: small, 5-100 milking cows (*n*=45; 20.2%; 95% CI: 9.8-30.8%); small to medium, 101-140 milking cows (*n*=45; 20.2%; 95% CI: 9.8-30.8%); medium, 141-200 milking cows (*n*=51; 22.9%; 95%

CI: 12.8-33.1%); medium to large, 201-300 milking cows (*n*=52; 23.3%; 95% CI: 13.2-33.4%); and large, more than 300 milking cows (*n*=30; 13.5%, 95% CI: 2.1-24.9%). These categories were devised so there were approximately similar numbers of farms in each category. All variable coding is provided in Appendix A.2.1.1. Of the 223 farmers that responded to the specific question, 171 (76.7%; 95% CI: 70.6-82.1%) farms had year round calving; 47 (21.1%; 95% CI: 15.9-27.0%) had seasonal calving systems and five (2.2%; 95% CI: 0.7-5.2%) had a combination of year round or seasonal patterns. When asked about housing, 28 of 226 respondents (12.4%; 95% CI: 8.4-17.4%) had lactating cows that were housed inside all year, 23 (10.2%, 95% CI: 6.6-14.9%) had cows at pasture all year and 175 (77.4%; 95% CI: 71.4-82.7%) had cows with pasture access and housing.

To investigate the representation and similarity between the sampled study population and the GB dairy population, comparisons were made between the distributions of various characteristics in this study population and published figures for the GB dairy industry. Variables considered included: mean herd size, average milk yield, rates of clinical mastitis, somatic cell count, and proportion of farmers using seasonal and year round calving systems, with comparisons made with similar published national data for GB. In this study, 77% of farmers stated that their farm had an all year around calving system while 21% were seasonal and 2% had a combination of the 2 systems with one group of cows following a seasonal pattern and the remaining cows following year round systems. The estimate from this dataset were found to be broadly similar to the published GB data (Appendix A.2.1.2).

2.3.3 Univariable associations with the presence of ITN on the farm (primary outcome variable).

Variables significantly associated with the presence of ITN are shown in Table 2.2.1, 2.2.1 and 2.2.3. Other factors investigated are included as supplementary data (Appendix A.2.1.3).

Table 2.2.1. ***Univariable "disease" associations with ischaemic teat necrosis (ITN) as the outcome variable****. The table shows the number of farms reporting each variable along with the proportion of farms in each ITN status (positive if have cases of ITN, negative if do not report cases of ITN), the odd's ratio and p-value of the association of variable to ITN status. The number of farmers responding to each question varied with n= the number of farmers that answered. The numbers within the parenthesis next to each variable indicates the code used within the statistical models. The number of farms with/or without the variable in question was recorded alongside the ITN status (+/-) with the percentage indicated in parenthesis. Odds ratio is indicated along with the Wald method of calculating the lower confidence interval (lci) and the upper confidence interval (uci). Variables with p-value >0.05 are included as Appendix A.2.1.3.*

Variable with (coding)	ITN + farms	ITN - farms	Odds ratio (lci-uci)	p-value
Teat licking present on farm *n*=224				
No teat licking (0)	28 (12.5%)	100 (44.6%)	*	
Teat licking (1)	88 (39.3%)	8(3.57%)	39.29 (17.02-90.67)	<0.01
Presence of Bovine papilloma virus/warts *n*=217				
No cases of bovine warts (0)	49 (22.6%)	66 (30.4%)	*	
Cases of bovine warts (1)	61 (28.1%)	41 (18.9%)	2.00 (1.17-3.44)	0.01
Presence of udder cleft dermatitis *n*=217				
No cases of UCD (0)	59 (27.2%)	81 (37.3%)	*	
Cases of UCD (1)	51 (23.5%)	26 (12.0%)	2.69 (1.51-4.81)	<0.01
Presence of chapped teats *n*=217				
No cases of chapped teats (0)	90 (41.5%)	103 (47.5%)	*	
cases of chapped teats (1)	20 (9.2%)	4 (1.8%)	5.72 (1.89-17.37)	<0.01
Presence of DD in the summer *n*= 212				
Farms never had DD in summer (0)	50 (23.6%)	64(30.2%)	*	
Farms with DD in summer (1)	59 (27.8%)	39 (18.4%)	1.94 (1.12-3.35)	0.02
Presence of DD in the autumn *n*=212				
Farms never had DD in autumn (0)	21 (9.9%)	34 (16.0%)	*	
Farms with DD in autumn (1)	88 (41.5%)	69 (32.5%)	2.06 (1.10-3.87)	0.02
Type of mastitis present on the farm *n*=152. 7 (4.6%) not interpretable				
No testing for mastitis (0)	22 (14.5%)	38 (25.0%)	*	
Environmental mastitis (1)	26 (17.1%)	25 (16.4%)	1.66 (0.78-3.55)	0.19
Contagious mastitis (2)	4 (2.6%)	6 (3.9%)	2.59 (0.66-10.19)	0.17
Mixed environmental & contagious (3)	9 (5.9%)	11 (7.2%)	2.11 (0.76-5.89)	0.15
Test but don't specify (5)	1 (0.66%)	3 (2.0%)	5.18 (0.51-52.90)	0.17

** indicates the reference group used for each variable.*

Table 2.2.2 ***Univariable "chemical" factors associations with ischaemic teat necrosis (ITN) as the outcome variable.*** *The table shows the number of farms reporting each variable along with the proportion of farms in each ITN status (positive if have cases of ITN, negative if do not report cases of ITN), the odd's ratio and p-value of the association of variable to ITN status. The number of farmers responding to each question varied with n= the number of farmers that answered. The numbers within the parenthesis next to each variable indicates the code used within the statistical models. The number of farms with/or without the variable in question was recorded alongside the ITN status (+/-) with the percentage indicated in parenthesis. Odds ratio is indicated along with the Wald method of calculating the lower confidence interval (lci) and the upper confidence interval (uci). Variables with p-value >0.05 are included as Appendix A.2.1.3.*

Variable with (coding)	ITN + farms	ITN - farms	Odds ratio (lci-uci)	p-value
Use of an automated dipping and flushing (ADF) system *n*=213				
Don't use ADF (0)	74 (34.7%)	82 (38.5%)	*	
Do use ADF (1)	37 (17.4%)	20 (9.4%)	2.05 (1.09-3.84)	0.03
Disinfection of clustered between cows *n*=208				
Don't disinfect clusters (0)	25 (12.0%)	47 (22.6%)	*	
Disinfect clusters between every cow (1)	38 (18.3%)	29 (13.9%)	2.46 (1.24-4.89)	0.01
Disinfect cluster if mastitis/high SCC (2)	41 (19.7%)	28 (13.5%)	2.75 (1.39-5.45)	<0.01

** indicates the reference group used for each variable.*

Table 2.2.3 ***Univariable management and milking machine factors associations with ischemic teat necrosis (ITN) as the outcome variable.*** *The table shows the number of farms reporting each variable along with the proportion of farms in each ITN status (positive if have cases of ITN, negative if do not report cases of ITN), the odds ratio and p-value of the association of variable to ITN status. The number of farmers responding to each question varied with n= the number of farmers that answered. The numbers within the parenthesis next to each variable indicates the code used within the statistical models. The number of farms with/or without the variable in question was recorded alongside the ITN status (+/-) with the percentage indicated in parenthesis. Odds ratio is indicated along with the Wald method of calculating the lower confidence interval (lci) and the upper confidence interval (uci). Variables with p-value >0.05 are included in Appendix A.2.1.3.*

Variable with (coding)	ITN + farms	ITN - farms	Odds ratio (lci-uci)	p-value
Presence of teat ringing after milking *n*=169				
No teat ringing (0)	53 (31.4%)	65 (38.5%)	*	
Cases of teat ringing (1)	32 (18.9%)	19 (11.2%)	2.07 (1.05-4.05)	0.03
Presence of teat end keratosis *n*=169				
No teat end keratosis (0)	36 (21.3%)	56 (33.1%)	*	
Cases of teat end keratosis (1)	49 (29.0%)	28 (16.6%)	2.72 (1.46-5.09)	<0.01
Foremilk cows before milking *n*=224				
Don't foremilk (0)	9 (4.0%)	22 (9.8%)	*	
Yes, always foremilk (1)	42 (18.8%)	29 (12.9%)	3.54 (1.42-8.78)	0.01
Foremilk most of the time (2)	12 (5.4%)	14 (6.3%)	2.10 (0.70-6.25)	0.19
Foremilk occasionally (3)	14 (6.3%)	17 (7.6%)	2.01 (0.70-5.75)	0.19
Foremilk if suspect mastitis (4)	38 (17.0%)	27 (12.1%)	3.44 (1.37-8.63)	0.01
Site of heifer rearing for the farm *n*=220. 7 (3.2%) don't rear heifers				
Heifers are reared on the same site as milking herd (1)	82 (37.3%)	62 (28.2%)	*	
Heifers reared on the same farm but different site from the milking herd (2)	21 (9.5%)	31 (14.1%)	0.51 (0.27-0.98)	0.04
Reared on different farm (3)	7 (3.2%)	10 (4.5%)	0.53 (0.19-1.47)	0.22
Freshly calved cow management *n*=216				
Fresh cows housed year round (1)	25 (11.6%)	12 (5.6%)	*	

Fresh cows at pasture housed at night (2)	17 (7.9%)	12 (5.6%)	0.68 (0.25-1.87)	0.45
Fresh cows at pasture housed in winter (3)	52 (24.1%)	59 (27.3%)	0.42 (0.19-0.93)	0.03
Fresh cows at pasture housed at night and in winter (4)	10 (4.6%)	9 (4.2%)	0.53 (0.17-1.66)	0.28
Fresh cows at pasture (5)	9 (4.2%)	11 (5.1%)	0.39 (0.13-1.20)	0.10
Freshly calved cow housing *n*=216				
Fresh cows in cubicle housing (1)	44 (20.4%)	58 (26.9%)	*	
Fresh cows in loose housing (2)	50 (23.1%)	30 (13.9%)	2.20 (1.21-4.00)	0.01
Fresh cows cubicles and loose housing (3)	16 (7.4%)	13 (6.0%)	1.62 (0.71-3.72)	0.25
Fresh cows no housing (4)	2 (0.93%)	3 (1.4%)	0.88 (0.14-5.49)	0.89
Freshly calved cows bedded on straw *n*=210				
Fresh cows not on straw (0)	34 (16.2%)	47 (22.4%)	*	
Fresh cows on straw (1)	75 (35.7%)	54 (25.7%)	1.92 (1.09-3.37)	0.02
Heifer housing *n*=207. 2 (0.96%) have no heifers				
Heifers in cubicles (1)	49 (23.7%)	35 (16.9%)	*	
Heifers in loose housing (2)	25 (12.1%)	39 (18.8%)	0.46 (0.24-0.89)	0.02
Heifers in cubicles and loose (3)	27 (13.0%)	20 (9.7%)	0.96 (0.47-1.99)	0.92
No housing (4)	7 (3.4%)	3 (1.4%)	1.67 (0.40-6.90)	0.48
Time calves with dams *n*=221				
0-1 hours (1)	3 (1.4%)	11 (5.0%)	*	
1<12 hours (2)	47 (21.3%)	29 (13.1%)	5.94 (1.53-23.10)	0.01
12<24 hours (3)	27 (12.2%)	26 (11.8%)	3.81 (0.95-15.22)	0.06
24<48 hours (4)	20 (9.0%)	18 (8.1%)	4.07 (0.98-16.97)	0.05
>48 hours (5)	19 (8.6%)	21 (9.5%)	3.32 (0.80-13.72)	0.10
Average number of dry cows at one time for year round calving systems *n*=219, with 47 (21.5%) having seasonal calving systems excluded from this variable.				
1-20 dry cows (1)	47 (21.5%)	48 (21.9%)	*	
21-40 dry cows (2)	32 (14.6%)	28 (12.8%)	1.17 (0.61-223)	0.64
41-65 dry cows (3)	10 (4.6%)	3 (1.4%)	3.40 (0.88-13.15)	0.08
65+ dry cows (4)	3 (1.4%)	1 (0.46%)	3.06 (0.31-30.52)	0.34
Number of cows in milk for year round calving systems *n*=220, with 47 (21.4%) having seasonal calving systems (coded as 0) and excluded from this variable.				
1-50 cows in milk (1)	4 (1.8%)	8 (3.6%)	*	
51-100 cows in milk (2)	23 (10.5%)	25 (11.4%)	1.84 (0.49-6.94)	0.37
101-150 cows in milk (3)	17 (7.7%)	20 (9.1%)	1.7 (0.43-6.65)	0.45
151-200 cows in milk (4)	23 (10.5%)	12 (5.5%)	3.83 (0.96-15.37)	0.06
201-250 cows in milk (5)	8 (3.6%)	10 (4.5%)	1.6 (0.35-7.30)	0.54
251-300 cows in milk (6)	9 (4.1%)	4 (1.8%)	4.5 (0.84-24.18)	0.08
301+ cows in milk (7)	9 (4.1%)	1 (0.45%)	18 (1.65-196.28)	0.02
Average milk yield per cow per year on farm *n*=216				
<6000 litres	17 (7.9%)	22 (10.2%)	*	
6001-8000 litres	28 (13.0%)	44 (20.4%)	0.82 (0.37-1.82)	0.63
8001-10000 litres	53 (24.5%)	30 (13.9%)	2.29 (1.05-4.96)	0.04
>10001 litres	14 (6.5%)	8 (3.7%)	2.26 (0.77-6.63)	0.14
Milking herd size *n*=223				
Small milking herd (1)	15 (6.7%)	30 (13.5%)	*	
Small to medium milking herd (2)	22 (9.9%)	23 (10.3%)	1.91 (0.82-4.49)	0.14
Medium milking herd (3)	29 (13.0%)	22 (9.9%)	2.64 (1.15-6.05)	0.02
Medium to large milking herd (4)	28 (12.6%)	24 (10.8%)	2.33 (1.02-5.33)	0.04
Large milking herd (5)	21 (9.4%)	9 (4.0%)	4.67 (1.72-12.65)	<0.01
Total herd size *n*=223				
Small total herd (1)	12 (5.4%)	29 (13.0%)	*	
Small to medium total herd (2)	25 (11.2%)	21 (9.4%)	2.88 (1.18-6.99)	0.02
Medium total herd (3)	24 (10.8%)	20 (9.0%)	2.9 (1.18-7.11)	0.02
Medium to large total herd (4)	21 (9.4%)	18 (8.1%)	2.82 (1.12-7.08)	0.03
Large total herd (5)	33 (14.8%)	20 (9.0%)	3.99 (1.67-9.54)	<0.01

* indicates the reference group used for each variable.

Of 117 possible variables, 23 were strongly associated with the presence of ITN on a farm (p-value <0.05) and a further 30 variables were weakly associated (p-value <0.2). These variables, included: other diseases (Table 2.2.1); chemical factors (Table 2.2.2); management and milking machine factors (Table 2.2.3).

2.3.4 Multivariable analysis

The final multivariable model included the presence of UCD (OR: 2.80; 95% CI: 1.54-5.07; p-value <0.01) and chapped teats (OR: 6.07; 95% CI: 1.96-18.76; p-value <0.01) on the farm (Table 2.3).

Table 2.3 ***The final multivariable model with the reported presence of ischaemic teat necrosis (ITN) on the farm as the outcome variable.*** *Indicates strong ITN associations with udder cleft dermatitis (UCD) and chapped teats (n=217 farms). The Wald's method was used to calculate the lower confidence interval (lci) and upper confidence interval (uci) and is indicated in parenthesis next to the value for the odds ratio. UCD- udder cleft dermatitis on the farm. * indicates the reference group used for each variable.*

Variable	Odds ratio (lci-uci)	p-value
Intercept	0.61 * (no UCD or chapped teats)	
UCD	2.80 (1.54-5.07)	<0.01
Chapped teats	6.07 (1.96-18.76)	<0.01

For this model, the Hosmer-Lemeshow goodness of fit test was 0.96 and the area under the receiver operating characteristic (ROC) curve was 0.67 (0.60-0.73) and indicated evidence of a good fit. Where possible, visual comparisons of the mean predicted and observed percentages of farms with ITN, with each combination of the explanatory variables from the model were similar, and examination of the 95% CIs around each percentage revealed no significant differences (Appendix A.2.2).

It was not possible to fit a multivariable model with reliable estimates (standard errors were too large to be deemed realistic) including disease variables and those variables with large

amounts of missing data. As such, the univariable associations are presented (Tables 2.2.1, 2.2.2 and 2.2.3).

2.3.5 The cost of ITN

One hundred and eight farmers reported the clinical outcomes of 250 ITN cases. Fifty-two cases recovered (20.8%; 95% CI: 15.9-26.4%) and 57 were culled (22.8%; 95% CI: 17.8-28.5%). The remaining cases (n=141) (56.4%; 95% CI: 50.0-62.6%) either lost the teat and were milked on reduced numbers of teats and/or the cow subsequently developed mastitis. Costs were calculated based on these three clinical outcomes. Performance averages were obtained from across all calving patterns in the dataset and compared with industry standards and literature in similar fields (Tables 2.4.1, 2.4.2, 2.4.3 and 2.4.4).

*Table 2.4.1. **The estimated cost of a case of ITN**. Breakdown of the components and assumptions used for the calculations. The source or reference used to devise these calculations are also indicated in the table. These key figures were used to calculate the costs in Tables 2.4.2, 2.4.3, 2.4.4.*

Component	Breakdown	Cost per component	Source
Milk yield/lactation	8000/l	-	Dataset (Redman, 2020)
Milk yield /quarter/ day	6.15 l	-	Dataset
Price per litre of milk	£0.28		(AHDB, 2020b) (Redman, 2020)
Length of lactation	325 days	-	Dataset
ITN lesion onset	25 DIM	-	Dataset

ITN- ischaemic teat necrosis; DIM- days in milk; l-litres, £-pounds Sterling.

Table 2.4.2 ***The estimated cost for an uncomplicated case of ITN that recovers.*** *The calculations utilise the assumptions displayed in Table 2.4.1. The source or reference used to devise these calculations are also indicated in the table.*

Component	Breakdown	Cost per component	Source
Milk loss from ¼ for 300 days	£0.28/l at 6.15l/quarter/day	£516.60	Dataset (AHDB, 2020b)
Vet visit & medicines	Vet visit ~£80, medicines ~£45	£125	
Milk loss for 7 day withdrawal period	£0.28/l, 24.6l/day	£48.22	Dataset (AHDB, 2020b)
Extra labour costs for a case of ITN	Extra 30 minutes/day, for 7 days at £8.72/h	£30.52	(Redman, 2020) (Beattie, 2019)
Total costs for an uncomplicated ITN case that recovered		**£720.34**	

ITN- ischaemic teat necrosis, l- litres, h- hour, ~- approximately.

Table 2.4.3 ***The estimated cost for a complicated case of ITN.*** *The calculations utilise the assumptions displayed in Table 2.4.1. The source or reference used to devise these calculations are also indicated in the table. One reference the currency was in US Dollars and thus the exchange rate used to calculate the cost in pounds Sterling is shown.*

Component	Breakdown	Cost per component	Source
Average costs for a case of mastitis	$453.17, exchange rate at $:£ 0.76 equals£344.41	£344.41	(Rollin et al., 2015; Down et al., 2017; Doehring and Sundrum, 2019)
Costs included in the average mastitis calculations that need to be excluded here	Vet fees & medicines £125; Milk loss for withdrawal period £48.22; Extra labour costs £30.52	-£203.74	
Total cost for a complicated case of ITN	£720 + £342.45 - £203.74	**£860.67**	

ITN- ischaemic teat necrosis, l- litres, ~- approximately; $- US dollar, £- pounds Sterling, $:£- US dollar to pounds Sterling exchange rate.

Table 2.4.4 ***The estimated cost for a case of ITN that required culling before the end of lactation****. The calculations utilise the assumptions displayed in Table 2.4.1. The source or reference used to devise these calculations are also indicated in the table.*

Component	Breakdown	Cost per component	Source
Replacement animal	First lactation animal (year round calving pattern)	£1500	(AHDB, 2020a)
Average value back from the cull cow	Assuming is acceptable for slaughter and meat production	-£400	(AHDB, 2020a; Beattie, 2019; Redman, 2020)
Extra loss of milk if culled before 100 DIM	200 DIM, at £0.28/l for ¾ of 24.6l/d	£1033.20	
Total cost for a cull case		**£2133.20 (not including any cost for treatments)**	

ITN- ischaemic teat necrosis; DIM- days in milk; l-litres, £-pounds Sterling, d-day.

For cows experiencing ITN, 20.8% recovered, 22.8% were culled and 56.4% had complications; therefore, the cost per case varied, depending on the outcome, between £720.34 and £2133.02. To calculate the average cost per farm per year the probability of each clinical outcome was multiplied by the cost of the outcome and combined to give an average cost per case per farm per year £1,121.62. This was a minimum figure as it was assumed that each farm would experience only a single case of ITN each year.

2.3.6 Associations with the presence of UCD on the farm

Univariable analysis with UCD as the outcome variable revealed strong associations with 93 variables (p-value ≤0.05) and weak associations with a further 12 variables (p-value 0.05-0.2) (Appendix A.3.1). As with ITN, the associated variables were from all three categories (disease, chemical and farm management factors). The final multivariable model included three parameters, namely the presence of ITN on the farm, having lactating cows bedded on sawdust and cases of teat end eversion after milking all of which were associated with an increased likelihood of reporting cases of UCD on the farm (Table 2.5).

Table 2.5 ***The reported associations with presence of udder cleft dermatitis (UCD) on the farm:*** *final multivariable model with UCD as the outcome variable. (n =158). Wald method was used for calculating the lower confidence interval (lci) and upper confidence intervals (uci) and is indicated in parenthesis next to the value for the odds ratio.*

Variable	Odds ratio (lci-uci)	p-value
Intercept	0.66 * (no ITN, no sawdust and calves removed from dams <1 hour)	
ITN	3.14 (1.42-6.97)	0.01
Lactating cows bedded on sawdust	2.94 (1.37-6.29)	0.01
Teat end eversion	3.05 (1.06-8.77)	0.04
Calves with dams:		
1-12 hours	0.12 (0.027-0.54)	0.01
12-24 hours	0.41 (0.095-1.75)	0.23
24-48 hours	0.33 (0.074-1.47)	0.15
>48 hours	0.089 (0.017-0.46)	<0.01

*UCD- udder cleft dermatitis. ITN- ischaemic teat necrosis on the farm. OR- odds ratio. * indicates the reference group used for each variable.*

For this model, the Hosmer-Lemeshow goodness of fit test was 0.80 and the area under the ROC curve was 0.76 (0.68-0.83) implying that the model was a good fit of the data. Due to the added number of variables in this model and the complexities of the variables, the predicted percentage probabilities are not presented for these data.

A multivariable model excluding disease variables and variables with large amounts of missing data was fitted (Appendix A.3.2). This multivariable model was based on data from 196 farms and included the variables: type of housing used for lactating cows, if lactating cows were bedded on sawdust, the average milk yield per cow per year and if there was no isolation period on the farm when introducing new animals. The Hosmer-Lemeshow goodness of fit test was 0.69 and the area under the ROC curve was 0.78 (0.71-0.84) indicating that the model was a fair fit of the data.

2.3.7 Association with presence of chapped teats on the farm

Univariable analysis with chapped teats as the outcome variable revealed strong associations with 97 variables and weak associations with two variables (Appendix A.4.1). The final multivariable model contained two variables (Table 2.6).

Table 2.6 ***The reported associations with chapped teats as the outcome variable, (n=101 farms).*** *Wald method was used for calculating the lower confidence interval (lci) and upper confidence intervals (uci) and is indicated in parenthesis next to the value for the odds ratio.*

Variable	Odds ratio (lci-uci)	p value
Intercept	0.04 * (no peracetic acid in pre dip and no ADF system)	
Peracetic acid in pre dip	8.91 (2.06-38.59)	<0.01
Use an ADF system	4.04 (1.04-15.69)	0.04

*ADF- automated dipping and flushing system is used during milking. * indicates the reference group used for each variable.*

The Hosmer-Lemeshow goodness of fit test was 0.71 and area under the ROC curve was 0.73 (0.58-0.90) indicating that the model was a fair fit of the data. The probability of reporting a

case of chapped teats on the farm was predicted from the final model and compared to the observed probability of having chapped teats on the farm; these were very similar (Appendix A.4.2).

It was not possible to fit a multivariable model with reliable estimates excluding disease variables and those variables with large amounts of missing data. As such, the univariable associations are presented (Appendix A.4.1).

2.4 Discussion

2.4.1 Descriptive statistics

Ischaemic teat necrosis is a disease which poses a significant and increasing challenge for the dairy industry but has not been well studied (Clegg et al., 2016b). This is the first national study that investigated farmer experiences of ITN within GB. This study has revealed some key foundations and hypotheses for further investigation. In particular ITN was reported on over half (51%; 95% CI: 44.4-57.8%) of GB dairy farms that responded to the survey conducted in Chapter 2 in 2018, which asked for farmer reports of ITN between 1985 and 2018. Furthermore, farms from most parts of GB reported cases and there were no differences in reporting between geographical areas. This high proportion, as well as reports from across GB, is concerning particularly as this study identified that the number of farms experiencing the disease for the first time appears to have increased in recent years. Hence, based on these data, ITN could be considered already endemic in GB, although given the continued yearly increases reported in this study it could also be designated as emerging.

To investigate the generalisability of these data to the rest of the GB dairy population, various analyses were carried out. This study farming systems were similar to the reported demographic approximation whereby 85% of the GB dairy farmers report as having all year

round calving systems (Redman, 2020). The apparent difference may be due to the increasing popularity of seasonal farms in GB in attempts to improve efficiency (AHDB, 2020a). Nevertheless, all year round calving systems predominate and this gives further confidence that this study aligns with and is representative of the GB dairy population. Additional comparisons were made using other variables, demonstrating the similarities of the study dataset with available published data for the GB dairy population. These comparisons revealed broad similarities between the study population and the GB dairy population as a whole (Appendix A.2.1.2).

Considering the question of whether the farmers knew the ITN lesions by another name, it was clear that there were misunderstandings around identification of the individual diseases that affect the bovine udder and for this reason the pictorial guide accompanying the survey was essential to raise awareness of different lesions and their associated names, as well as to ensure accuracy when answering questions in relation to a specific lesion. Farmers were requested to answer questions relating to specific diseases using the supplied descriptions and pictorial guide. This guide proved an important educational aid for farmers for future reference. From interviews with farmers prior to submission of the questionnaire, the authors identified that farmers could readily distinguish between teat skin diseases using this guide and they were encouraged to make contact to discuss questions if they were unsure how to answer. Inevitably, this is not an ideal format to obtain such information as it can introduce observational and misclassification bias. However, the use of pictorial guides to aid farmer surveys is a well-established methodology to ensure collection of reliable data (Kaler and Green, 2008; Angell et al., 2014). In addition, due to the limitations on accessing farmer data (only farmer names and addresses were accessible) the study was restricted to a postal questionnaire. It was debated if the questionnaire should be submitted to veterinary surgeons but it was deemed that farmers

would know their animals and farms the best and provide the most accurate results on the milking, chemical, management and environmental factors.

From the data presented, there were several key findings that are worth pursuing as potential intervention strategies. For example, this study found that first lactation animals in the first 90 days in milk appear to be most at risk of ITN development. This requires follow up longitudinal studies as this information could be utilised to encourage regular careful inspection of early lactation animals at every milking to identify the disease early on in its clinical presentation. There are many studies that encourage the monitoring of early lactation animals for clinical mastitis (potentially affecting profitability), which indicate infections acquired in the dry period (Bradley et al., 2007; Barkema et al., 2009; Rollin et al., 2015; Down et al., 2017). The same measures could potentially aid in the rapid detection of ITN and thus, its control.

2.4.2 Study limitations and weaknesses

As with all questionnaires, there is the potential for reporting bias as farmers that have seen the disease may be more likely to respond and there is also the issue of recall bias when asked to think of an event in the past (Choi and Pak, 2005). There is a suggestion of recall bias in the data where there are apparent peaks in cases in 1998 and 2008 (20 and 10 years before the questionnaire). These results may also be biased depending on how long the farmer had been actively farming. For example, if the majority of farmers were younger or had recently started farming, then data could be more bias towards recent emergence of the disease compared to older farmers. Unfortunately, the length of time the farmer had been in farming was not captured using the questionnaire and is a weakness of the study.

The overall response rate in this study was lower than anticipated which was partly due to redundancies within the sampling frame with more respondents than anticipated not eligible to

complete the questionnaire. The questionnaire was lengthy and this may have discouraged some potential participants. In addition, a follow up telephone interview, to discuss their answers was planned to attempt to increase the response rate, but due to unforeseen circumstances and budget restraints this did not occur. However, there were still a substantial proportion, almost half, of farmers (49%, 95% CI: 42.2-55.6%) that responded who had not seen the disease. It is also possible that farmers were motivated to gain further knowledge, or understood the potential devastating effects ITN could pose.

The potential for collider bias was explored within this dataset. Collider bias happens when the outcome of the variables can affect the likelihood of being sampled (Griffith et al., 2020). In this study both ITN and UCD are skin diseases of the udder and this may cause farmers who have experienced one or the other to self-select to complete the questionnaire. Unfortunately, this cannot be mitigated for entirely with voluntary farmer-based observational studies. However, to explore the possibility of the presence of collider bias a comparison of key variables within the dataset was made with those of existing published studies (Appendix A.2.1.2). These analyses demonstrated that whilst this study represents a small sample of the GB dairy farmer population, the sample farms were broadly similar in terms of milking herd size, average milk yield, rates of clinical mastitis and average yearly somatic cell count. As such, whilst the possibility of collider bias cannot be totally eliminated it is not readily apparent within this study. Additionally, attempts were made to construct multivariable models without disease factors to reduce the risk of collider bias in the analysis. However, it was not possible to fit multivariable models with reliable estimates and realistic standard errors for ITN as an outcome or chapped teats and so the data within univariable analyses are suggested for future investigations.

2.4.3 Economic implications of ITN

In this study, farmers reported 22.8% (95% CI: 17.8-28.5%) of cows with ITN were culled and only 20.8% (95% CI: 15.9-26.4%) recovered. The remaining cases (56.4%) had complications such as teat loss and/or mastitis. This set of outcomes is hugely important not only for animal welfare but also has an economic impact. From the data, a recovered case of ITN is estimated to cost £720, a complicated case to cost around £859 and a culled case to cost at least £2992. Therefore, the average cost per farm, taking in to consideration the expected proportions of each clinical presentation and assuming one case per farm per year, was estimated to be £1121. Similar to the study by Down *et al.*(2013), whereby the costs associated with clinical mastitis were investigated, the costs of both ITN and mastitis increase substantially when a cow is culled. Given that 22.8% of ITN cases require culling, many of them first lactation heifers, this is likely to be a significant loss for farmers not only in monetary terms but also in genetic potential. Due to the reported increasing numbers of cases observed over the last few years and due to increasing costs of treatment, the number appears likely to increase with each year. This is the first estimate of the economic impact of ITN.

2.4.4 Potential risk factors for ITN

Regression analysis of questionnaire data has been utilised frequently to identify potential risk factors for diseases (Peeler et al., 2000; Angell et al., 2014; O'Kane et al., 2017). In this study, if the farm had cases of UCD (OR: 2.8, 95% CI: 1.54-5.07; p-value <0.01) or cases of chapped teats (OR: 6.07; 95% CI: 1.96-18.76, p-value <0.01), then farmers were more likely to have reported a case of ITN. The predicted probabilities from the multivariable models demonstrated

the likelihood of reporting ITN when either UCD or chapped teats are present individually or in combination. Multiple methods were applied to denote confidence in these models showing that UCD and chapped teats were important factors associated with ITN that warrant further investigation. These associations may have a causal or reverse causal link, or may reflect some third factor not detected in this study.

2.4.5 Potential risk factors for UCD and chapped teats

The same dataset was used to investigate potential farmer reported risk factors for reporting cases of UCD and chapped teats. Although the original questionnaire was not designed for such investigation, due to the nature of the questions asked, it was deemed a logical approach to analyse the data to investigate these significant diseases and investigate potential farm level risk factors for both and consequently identify additional potential areas for intervention. Interestingly, UCD and ITN were strongly related as both appeared as potential farm level risk factors for each other. However, chapped teats were more associated with chemical factors, specifically the use of peracetic acid in a pre-milking formulation (OR:8.91; 95% CI: 2.06-38.59; p-value <0.01) and the use of some form of automated dipping and/or flushing system (OR: 4.04; 95% CI: 1.04-15.69; p-value 0.04). Compared to the model for ITN, the number of observations were reduced for these models as a result of missing values. As such, validation tables were used to assess if there was a significant amount of missing data for the farms with and without the disease (Appendix A.5.1 and A.5.2). There were no significant differences identified due to missing data and the pattern of missingness was mostly a generalised pattern of missingness (Dohoo, 2015). Therefore, the models were unlikely to have been biased in this manner.

The findings of ITN and UCD as potential farm level risk factors for each other were biologically plausible and may indicate a common underlying aetiopathogenesis. It is also common amongst the medical and veterinary fields to find an infectious or non-infectious disease process which will predispose to another disease, for example many bacterial pneumonias will be preceded by a viral respiratory infection (Hament et al., 1999; Hodgson et al., 2005; Griffin et al., 2010). Whilst submission bias could skew associations this reported risk factor warrants further investigation.

2.4.6 ITN and other diseases

In this study, there was no association of ITN with DD. The reported hypothesis that ITN is associated with DD treponemal bacteria may not hold true and further work is needed to clarify this area (Clegg et al., 2016b). From the model investigating UCD as the outcome variable, it was hypothesised that lactating cows that were bedded on sawdust and the presence of teat end eversion in lactating animals within the milking herd also increased the likelihood of developing UCD and thus potentially ITN. Studies in the Netherlands and Sweden have identified risk factors for UCD such as conformational traits at an individual level, the use of a foot bath, high producing herds, breed and housing factors at a farm level (Olde Riekerink et al., 2014; Persson Waller et al., 2014; Ekman et al., 2018). This study has highlighted potential differences in risk factors for UCD between the GB and other countries.

As there was also the potential for collider bias with this model using UCD as the outcome variable, a multivariable model excluding disease factors and variables with large numbers of missing observation was fitted with similar reliability to the model including these excluded variables. The variables included in this model included the type of housing that lactating cows are in, with farmers that have lactating cows without housing more likely to report cases of

UCD. Cows bedded on sawdust and higher yielding herds with no isolation periods are also more likely to report cases of UCD, which is consistent with the findings in the Netherlands and Sweden (Olde Riekerink et al., 2014; Persson Waller et al., 2014; Ekman et al., 2018). These findings require further investigation as they may lead to farmers being able to reduce cases of UCD on their farms.

The final model investigating factors associated with the presence of chapped teats was much simpler than the model investigating potential causes of UCD. Only two explanatory variables remained in the model: peracetic acid in the pre-milking teat preparation; and use of an automated dipping and flushing system. Peracetic acid is a common disinfectant used in the dairy industry and has not been linked to any major hypersensitivities or dermatitis in animals or humans unless used at high concentrations for prolonged periods (Müller et al., 1988; Laven and Hunt, 2002; Bore and Langsrud, 2005; Pechacek et al., 2015; Megahed et al., 2019). This is potentially useful information in that farmers can be made aware of the risk of teats becoming chapped in such situations and thereby potentially increasing the risk of developing a case of ITN. In fact, a recent study found that using a flushing system with water alone, without the addition of peracetic acid was effective in reducing bacterial numbers on the teat skin (Skarbye et al., 2020) and may be a way to decrease the risk of ITN . Other potential interventions a farmer could take to reduce the incidence of chapped teats would be to use a post milking teat dip with a high emollient and perform a dynamic milking machine test, especially in the proposed high-risk group of first lactation heifers. While chapped teats in themselves may appear relatively minor problems, the potential subsequent increased risk of ITN should not be over looked. As, there was a high number of missing observations in this model, multivariable models were attempted excluding variables with large amounts of missing data and disease factors. However, once these variables were excluded it was not possible to fit a multivariable

model with reliable estimates and so the univariable association are presented (Appendix A.4.1).

Although research into ITN is in its infancy, this study demonstrated several possible areas of intervention that farmers and veterinary surgeons could investigate should a case of ITN occur on farm. Further studies are required to understand the potential for causality of these associated risk factors further, especially at an individual animal level. Furthermore, determination of disease aetiology and studies into the prevention and treatment of ITN is greatly needed. Whilst this study is only focused on GB farms, it highlights a disease that should be monitored in the rest of the world's dairy cow populations, especially given its severity and potential economic impact.

2.5 Conclusions

Ischaemic teat necrosis has been reported more frequently in recent years and may cause significant losses on dairy farms. Over half of the farmers that responded to this study had experienced a first case of ITN between 1985 and 2018. First lactation cows up to 90 days in milk are reported to be the greatest risk of developing ITN. Potential farmer reported farm level risk factors for having cases of ITN on a farm included having cases of udder cleft dermatitis and or chapped teats. These udder and teat presentations were found to have specific associated farm level risk factors, which could be mitigated to improve teat health on farms.

Chapter 3: The clinical and pathological features of bovine ischaemic teat necrosis

The data presented in this chapter is supported by the paper: Clinical and pathological features of Bovine Ischaemic Teat Necrosis. Authors: H.E. Crosby-Durrani, S.D. Carter, R.J. Blundell, A. Manning, R. Blowey, N.J. Evans accepted by the Journal of Comparative Pathology 28th July 2022 and available online on 30th August 2022.

3.1 Introduction

Bovine ischaemic teat necrosis (ITN) is a newly emerging skin disease causing severe lesions on udder tissues of UK dairy cattle. ITN affects the base of the teat and may extend distally along the teat towards the teat end and/or proximally on to the adjacent skin of the udder (Blowey, 2004). The lesion severity can cause the affected teats to slough, and in other cases the teats can become firm and difficult to milk. Some animals may find ITN lesions highly irritating or pruritic and lick their own teats until the tissue is further traumatised and eventually removed, as such prognosis is guarded.

ITN is a differential diagnosis overlapping with several other diseases that affect the skin of the teat which mostly have a better prognosis. Such diseases include: bovine herpes mammillitis (BHM), the main differential diagnosis, caused by bovine herpesviruses 2 and 4; the bovine parapox viruses, cowpox virus and bovine warts caused by multiple bovine papilloma viruses (Gibbs, 1984; Wellenberg et al., 2002; Ogawa et al., 2004; Anon, 2007; Kemp et al., 2008; Cargnelutti et al., 2017). Foot and mouth disease virus (FMDV) may also affect the skin of the teats (Wellenberg et al., 2002); however, other systemic signs of FMDV in the same animal and herd would lead to rapid diagnosis. Another disease often considered, anecdotally by farmers as part of ITN is udder cleft dermatitis (UCD), also referred to as bovine ulcerative dermatitis, and ulcerative mammary dermatitis (Olde Riekerink et al., 2014). UCD affects the skin of the udder where there are folds, usually at the cranial aspect between the udder and abdominal wall but also can occur between the two halves of the udder. UCD is not considered to affect the teats and older cows with more pendulous udders are more susceptible

(Persson Waller et al., 2014; Bouma et al., 2016). In contrast to UCD, ITN mostly affects first lactation heifers with smaller udders. Consequently, the clinical presentations strongly suggest that ITN and UCD are different diseases.

There are few previous descriptions and reports of ITN (Blowey, 2004; Andrews et al., 2008; Blowey and Edmondson, 2010; Mauldin and Peters-Kennedy, 2015; Clegg et al., 2016b). In some literature, ITN has been referred to as summer sores and teat eczema (Blowey and Weaver, 2003). In all previous reports, the macroscopic and histological descriptions of ITN are either inadequate or completely lacking. For a consistent diagnosis and a better understanding of the aetiopathogenesis of ITN, a more robust characterisation of the disease is required. In a pilot study, ITN was associated with the detection of digital dermatitis (DD) treponemal bacteria (Clegg et al., 2016a). These bacteria have been detected in abundance in other lesions affecting the skin and extremities of production animals such as within DD lesions in cattle (Evans et al., 2009), in contagious ovine digital dermatitis (CODD) in sheep (Sullivan et al., 2015a), in foot lesions in goats (Sullivan et al., 2015b) and elk (Clegg et al., 2015) and in pig skin lesions (Clegg et al., 2016d). For the more established of these diseases, DD and CODD, a clinical grading system has been developed (Döpfer et al., 1997; Angell et al., 2015a). Grading systems have been utilised many times to improve awareness and to categorise lesions to better follow up of outcomes and progression. In several of these diseases, the DD treponemes were visualised within tissue sections by immunohistochemistry (IHC) (Evans et al., 2009; Angell et al., 2015b; Crosby-Durrani et al., 2016) further implicating these bacteria in the aetiopathogenesis of these diseases. These studies also queried the presence of round bodies within these tissues and the interpretation of intensely labelled granular material observed on IHC. Round bodies are thought to be a product of a spirochaetal bacteria, such as *Treponema* spp., responding to unfavourable conditions for growth by becoming cystic and

may be able to return to the spirochaetal form (Margulis et al., 2009). Thereby, providing an opportunity for transmission that requires further investigation.

In this project, the aims were to formally and systematically detail the clinical presentation of ITN. To: 1) develop an appropriate ITN grading system; 2) describe associated histopathological changes and 3) investigate the role of potential pathogens. This would then allow for a more rapid and consistent diagnosis of potential ITN cases.

3.2 Materials and methods

3.2.1 Ethics Statements

The studies involving human participants were reviewed and approved. Ethical approval was granted by University of Liverpool, School of Veterinary Science Ethical Committee (application number: VREC 460). The participants provided their written informed consent to participate in this study.

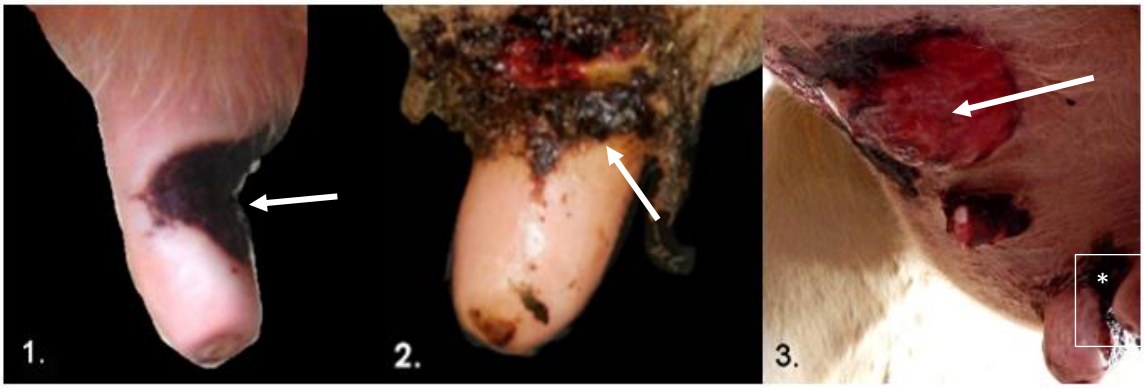
3.2.2 Macroscopic examination

Veterinary surgeons (VS) visited farms with suspected ITN cases and submitted photographs of the lesions along with the clinical history to a board-certified veterinary pathologist. The pathologist (HCD) reviewed each individual case to eliminate those lesions bearing the clinical or macroscopic hallmarks of other well recognized skin diseases of the bovine teat and udder. Images that were consistent with diseases such as BHM, and those caused by parapox viruses were excluded from the study. Cases were also excluded if the photographs were deemed of inadequate quality to readily describe the lesion or to confidently disqualify other well-characterized teat and udder diseases. Next, cases were assessed to see if the lesion was

consistent with the current working definition of ITN compiled from written descriptions (Blowey, 2004; Blowey and Edmondson, 2010; Clegg et al., 2016b) and author experience as starting as a focal, well demarcated dry, red to black area of cutaneous necrosis commonly at the teat udder junction. Lesions identified as ITN were described macroscopically and from these descriptions, were categorized based on a set of morphological criteria (Table 3.1). Forty-seven cows presenting a total of 73 affected teats (from 188 at-risk teats) from 28 different farms were used to develop a grading system for the lesions. Categories were developed from the following presentations: presence or absence of the teat; where the teat was still present then each was categorized on the length of the lesion, presence or absence of scab formation (crusting), udder skin involvement and concurrent UCD lesions. Notes were also made on the number of teats affected per animal, if the lesions were actively haemorrhaging and on which aspects of the teat the lesions were found. Chi-square tests were used to determine statistical differences in different aspects of clinical presentation, such as the number of teats in each category, the extent of the ITN lesion and the number of teats affected per cow. Statistical significance level was set to p-value <0.05.

Table 3.1. Categories of bovine ischemic teat necrosis by macroscopic clinical appearance (n =73) and the number of teats available for histological examination (n =17).

	Type 1 (Fig. 3.1)	**Type 2 (Fig. 3.2)**	**Type 3 (Fig. 3.3)**
Description	Dry Red to black Well demarcated	Type 1 plus proliferative epidermal lesion with crusting	Teat sloughed or partially sloughed
Macroscopic teats	26	22	25
Histology	7	5	5



Figures 3.1 - 3.3 ***Bovine ischemic teat necrosis, teat and udder****, cow. Figure 3.1* ***Type 1 lesion****. There is a focal well-demarcated red to black area of necrosis on the medial aspect of the left rear teat (arrow). Figure 3.2* ***Type 2 lesion****. The lesion has a proliferative epidermal lesion with crusting at the edge of the lesion (arrow). Figure 3.3* ***Type 3 lesion****. The right front quarter has granulation tissue in place of the sloughed teat (arrow). The lesion also involved the skin of the udder. N.B. there is also a Type 2 lesion on the right rear teat at the edge of the figure (*).*

3.2.3 Histological examination

A set of histological teat samples (*n*=8) were obtained by the VS surgically removing severely affected teats on clinical grounds from live animals; for example, from cows with multiple affected teats after one teat had sloughed. This was an attempt to contain the disease or to prevent the animal causing further trauma when the lesions were pruritic in nature.

Other histological teat samples (*n*=9 with ITN and *n*=1 control) were obtained from animals that presented as cull cows at meat inspection in abattoirs and in fallen stock centres. The teats were removed from the carcass using sterile scalpel blades; a normal teat from a healthy animal was taken as a negative control for immunohistochemistry studies. The teats were then halved longitudinally through the lesion. One half of the teat was placed into 10% neutral buffered formalin and the other half stored on ice prior to freezing at -20 °C for future microbiological studies.

Histological samples were retained in 10% neutral buffered formalin for at least 48 hours to allow for adequate fixation. Samples were sectioned horizontally into approximately 4 mm slices (to include lesion and non-lesioned tissue) and embedded in paraffin wax. Tissue blocks were cut into 4 µm sections and placed on glass slides, processed through a series of xylene washes and stained using standard haematoxylin and eosin (HE) protocols. Additional sections were also stained using Gram-stain, periodic acid Schiff (PAS) and Warthin-Starry sliver stain using standard laboratory procedures.

3.2.4 Immunohistochemistry

Immunohistochemistry (IHC) for detection of treponeme bacterial and orthopox viral antigens was utilised. Briefly, the DD associated treponeme IHC was performed using a rabbit polyclonal antibody to the three phylogroups *T. medium*, *T. phagedenis* and *T. pedis* (Evans et al., 2009) with an antigen retrieval step and stained using an DAKO Autostainer Link 48 (Dako, Agilent Technologies, Carpinteria, California, USA) (Crosby-Durrani *et al.* 2016) using a CODD grade 2 lesion as a positive control. In a similar manner, the orthopox IHC utilised the DAKO Autostainer Link 48 with a protease (P8038, Sigma) pre-treatment for antigen retrieval and using a rabbit polyclonal IgG anti-vaccinia virus antibody (Abcam ab35219) at a 1:1000 dilution as optimised by Pereira da Costa (2021) using a feline cowpox lesion and a ovine orf lesion as positive controls.

3.2.4.1 *Investigation of treponeme round bodies with immunohistochemistry*

Previous studies had found intense granular labelling for the DD associated treponeme on IHC and queried the interpretation of this material as to whether it represented background labelling or specific staining indicative of round body formation. To investigate this, briefly, the two phylogroups of DD associated treponeme bacteria (groups 2, T320A and 3, T3552B) were cultured in liquid media (detailed method included in section 4.2.2) and left to grow in

anaerobic conditions until large numbers of round bodies were identified by phase contrast microscopy. On detection of round bodies approximately 700µl of sample was removed and placed into an Eppendorf. This was subsequently spun down to form a pellet and the supernatant removed. Ten percent normal buffered formalin was added to the pellet and the bacterial pellet processed in the same manner as tissue for histochemical and immunohistochemical examinations.

3.2.5 Transmission electron microscopy (TEM) examination

Areas that contained sites of interest on light microscopy had 20 µm sections cut from the formalin fixed, paraffin embedded tissue. These were de-waxed, re-hydrated in 0.1 M cacodylic acid and fixed in cacodylic acid buffered 2.5% glutaraldehyde. Sections were then further fixed in 1% osmium tetroxide, stained with 2% uranyl acetate in 0.69% maleic acid. These were then dehydrated and embedded in araldite resin. Semi-thin (0.5 µm) sections were cut and stained with 1% toluidine blue to select areas for the 90 nm ultrathin sections which were contrasted with 3% lead citrate and 2% uranyl acetate (Cheville and Stasko, 2014) and examined under a Phillips EM208S transmission electron microscope (FEI UK Limited, Cambridge, UK) at 80kV.

3.3 Results

3.3.1 Macroscopic description

From forty-seven animals, 188 teats (not including supernumerary teats) were examined, with 73 teats with what were considered to be typical ITN lesions consistent with the working definition previously described. After reviewing the archive of submitted ITN photographs, the lesions were divided into three main categories based on the most readily distinguishable

and common features between lesions (Table 3.1). Categories were named Type 1, Type 2 and Type 3 and there was no statistical difference between the numbers in each category (p-value = 0.90). In addition to the three main categories, extra observations on the number of teats and which teats were involved per animal (Table 3.2) with any extension of the lesion were also noted. Most cows with the disease had only one or two teats affected at the time of observation.

Table 3.2. Ischaemic teat necrosis (ITN) location and whether teats sloughed or present.

Teat	Sloughed teats	Not sloughed	Total
Right front	5	10	15 (20.5%)
Right back	6	12	18 (24.7%)
Left front	5	11	16 (21.9%)
Left back	5	9	14 (19.2%)
Unknown location	4	6	10 (13.7%)
Total	25 (34.2%)	48 (65.8%)	73

There was a difference in how many teats the animals had affected: 28 of 47 (59.6%) cows had one teat affected, 12/47 (25.5%) cows had two teats affected and 7/47 (14.9%) cows had three teats affected. No cow had all four teats affected.

Collectively, there was no apparent predilection for a teat of a specific anatomical location to be more affected than another (p-value=0.72). Nor was there a site where teats were more likely to be sloughed (p-value = 0.99) or non-sloughed (p-value = 0.73) (Table 3.2).

Fourteen of 73 (19.2%) teats were haemorrhagic at the time of photographic documentation.

The location of the ITN lesion on the individual affected teat was variable. The medial aspect of the teat was the most common site reported for ITN lesions (39.6%, p-value <0.01). For other sites, the cranial aspect of the teat was affected in 10.4%, the caudal aspect in 8.3%, the lateral aspect in 4.2% of cases. Many cases had lesions on more than one aspect (29.2%), and in 8.3% only one unknown aspect was affected.

The length of the lesion compared to the teat length was assessed and scored as one of the following: proximal half only; over half the length of the affected teat; the whole length of the teat; also described was, if the lesion had extended to the skin of the udder. Proximal only lesions made up 35.4% of affected teats, 22.9% of lesions affected over half the length of the teat, and 41.6% affected the whole teat. Thirty-seven (50.7%) of affected teats had evidence of the lesion extending to the haired skin of the udder.

Three animals of the 47 (6.4%) with ITN also had concurrent lesions consistent with UCD, that is a separate UCD lesion on the udder and ITN on a teat, with no visible signs of lesions coalescing or being part of the same pathological process.

3.3.2 Microscopic examination

Haematoxylin and eosin stained sections of teats were viewed by light microscope. A transection through normal microanatomy of the bovine teat, in a superficial to deep direction, consists of a thick keratinised stratified squamous epidermis and dermis comprising the teat skin; and deep to this the lamina propria of the teat sinus and a bistratified cuboidal to columnar teat sinus epithelium. In this location the dermis lacks hair follicles and associated adnexa and the transition to teat sinus epithelium is demarcated by bundles of smooth muscle. The lamina propria of the teat sinus is highly vascular with many large muscular vessels often surrounded by several smaller vessels. The number of teats examined for each lesion type are shown in Table 3. Sections from four ITN teats were fragmented and difficult to interpret although they all contained superficial epidermis with fragments of laminated keratin (presumed hyperkeratosis), large colonies of 1-2 µm coccoid bacteria, degenerate neutrophils, extravasated erythrocytes (haemorrhage), eosinophilic fibrillary material (fibrin) and occasional eosinophils. In the remaining sections, the teat anatomical structures were retained

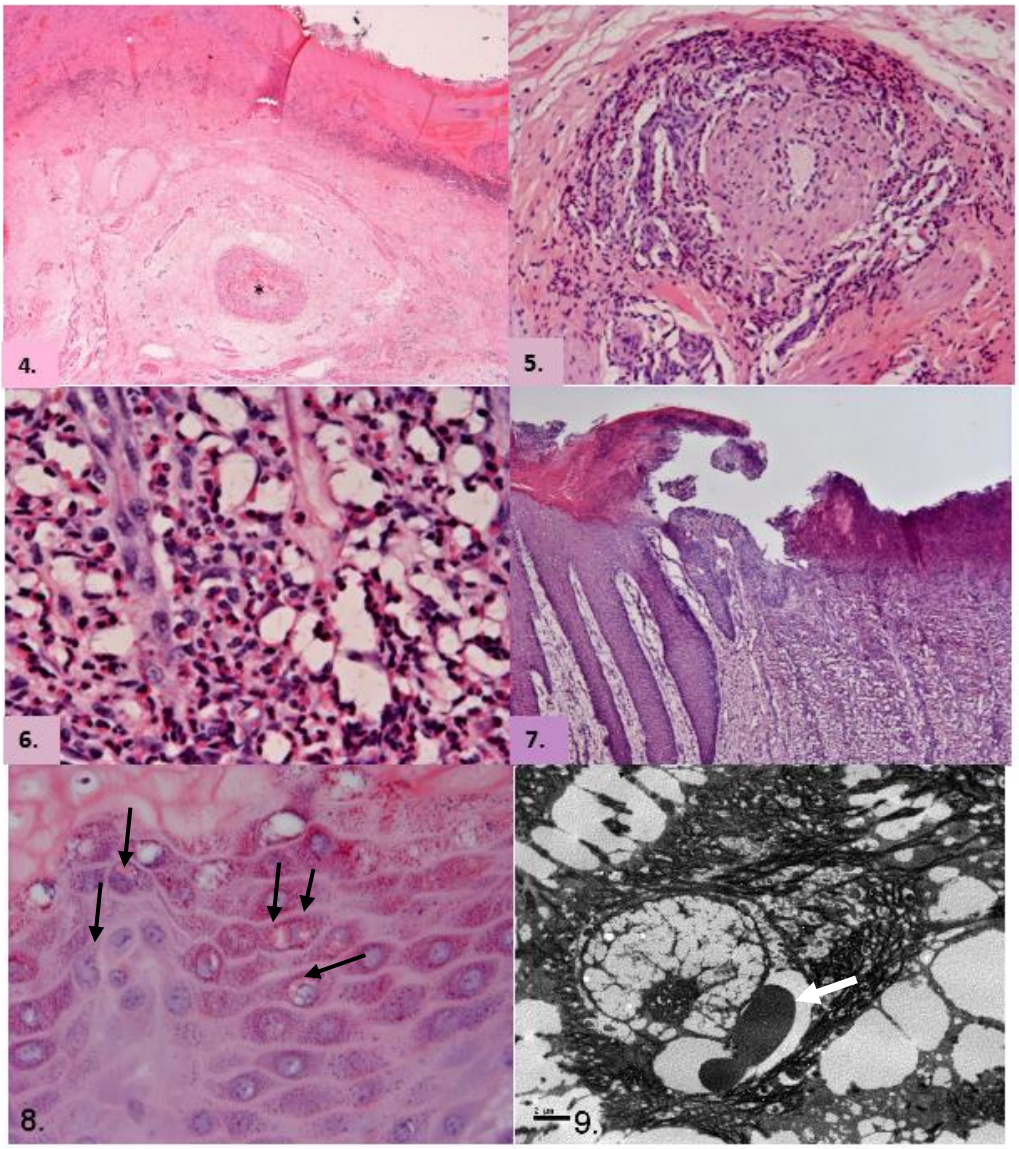
and composed of epidermis, dermis, lamina propria and sometimes teat sinus epithelium (as a transverse section through the teat). The main commonalities of ITN affected teats with the histopathologic presentation compared to the three main categories are presented in Table 3.3.

Table 3.3 Summary of ITN histopathological findings in relation to the macroscopic grading (n= 17)

Lesion	**Macroscopic clinical type 1 (least severe) (*n*=7)**	**Macroscopic clinical type 2 (*n*=5)**	**Macroscopic clinical type 3 (most severe) (*n*=5)**
Epidermis			
Epidermal necrosis	4 (57.1%)	0 (0.0%)	0 (0.0%)
Ulceration	5 (71.4%)	1 (20.0%)	4 (80.0%)
Hyperplasia	2 (28.6%)	3 (60.0%)	2 (40.0%)
Intracorneal pustules	1 (14.3%)	3 (60.0%)	2 (40.0%)
Intracytoplasmic inclusions	1 (14.3%)	2 (40.0%)	1 (20.0%)
Serocellular crust	2 (28.6%)	3 (60.0%)	2 (40.0%)
Ballooning degeneration	1 (14.3%)	0 (0.0%)	0 (0.0%)
Dermis/lamina propria changes			
Suppurative infiltrate	7 (100%)	5 (100%)	5 (100%)
Haemorrhage	4 (57.1%)	3 (60.0%)	3 (60.0%)
Granulation tissue	2 (28.6%)	1 (20.0%)	3 (60.0%)
Eosinophilic infiltrate	2 (28.6%)	1 (20.0%)	4 (80.0%)
Vasculitis	4 (57.1%)	1 (20.0%)	2 (40.0%)
Thrombosis	2 (28.6%)	0 (0.0%)	1 (20.0%)
Coagulative necrosis	2 (28.6%)	0 (0.0%)	0 (0.0%)

All of the samples from all lesion types had a suppurative infiltrate. There were a few lesions that were more common in each clinical macroscopic type presented. Type 1 lesions were the only type histologically to have the presence of epidermal necrosis, coagulative necrosis of the dermis and/or the lamina propria of the teat sinus and had observed thrombosis (Fig. 3.4). Thrombosis was observed in thick walled medium sized vessels containing values and erythrocytes (veins). However, thrombosis was also observed in a Type 3 lesion and vasculitis was observed in both other types (2 and 3) in smaller proportions (Fig. 3.5). Vasculitis varied in nature from a non-specific lymphoplasmacytic to eosinophilic around medium size blood vessels and leukocytoclastic of smaller vessels (mostly small veins). Eosinophils (Fig. 3.6)

were observed in the highest proportion in Type 3 lesions. Serocellular crusting was observed most frequently in Type 2 lesions. Ulceration (Fig. 3.7) was observed more frequently in Type 1 and Type 3 compared to Type 2 disease. Hyperplasia with rete peg formation and intracorneal pustules were less frequent within Type 1 lesions. Large brightly eosinophilic intracytoplasmic inclusions were observed sporadically in the epidermis of all macroscopic types, which prompted IHC analysis for orthopox virus (Fig. 3.8). The teat sinus epithelium, when present, was within normal limits.



Figures 3.4 – 3.9 ***Bovine ischemic teat necrosis****, teat, cow.* ***Figure 3.4*** *The lamina propria of the teat sinus with a thrombosed vessel (*) surrounded by coagulative necrosis extending to the associated dermis. (HE, 20x magnification).* ***Figure 3.5*** *The lamina propria of the teat cistern with eosinophilic and lymphoplasmacytic vasculitis. (HE, 100x magnification).* ***Figure 3.6*** *The dermis subjacent to the ulceration showing marked infiltration of eosinophils. (HE, 400x magnification).* ***Figure 3.7*** *There is a focally extensive, well-demarcated area of epidermal ulceration with a superficial serocellular crust adjacent to a hyperplastic epidermis. There is a large infiltrate of degenerate leukocytes in the superficial dermis. Haematoxylin and eosin (HE, 40x magnification).* ***Figure 3.8*** *The thickened stratum granulosum of the epidermis with variable intracytoplasmic globules of eosinophilic keratin (arrows). (HE, 400x magnification).* ***Figure 3.9*** *Transmission electron micrograph of a keratinocyte with an intracytoplasmic keratin in a vacuole (arrow).*

3.3.2.1 Additional staining

A selection of special staining techniques was utilised to investigate possible aetiological agents including Gram stain, Warthin-Starry stain and PAS. Across all sections there were multifocal either Gram-negative, Gram-positive or a combination of both Gram-negative and Gram-positive variably sized (from 1- 3 µm) coccobacilli bacteria in small to large colonies mostly in the serocellular crust or within the necrotic areas in the ulcerated dermis. The Warthin-Starry stain correlated with the gram stain in showing large numbers of coccobacilli, and failed to detect any spirochaetal bacteria. PAS did not detect any fungal or yeast elements.

3.3.3 Immunohistochemistry

All samples for histopathology were also processed for immunohistochemistry for DD associated treponemes and for vaccinia (orthopox) virus using specific antibodies. Six of 17 samples had some small areas of intense granular labelling using the anti-DD-treponeme antibody; however, no spirochete morphology was detected throughout the sections. This was interpreted as non-specific labelling and therefore negative for *Treponema spp*. All samples were negative for orthopox.

3.3.3.1 *Investigation of treponeme round bodies with immunohistochemistry*

The immunohistochemistry for both group 2 (T320A) and group 3 (T3552B) phylogroups exhibited strong and specific labelling of the treponeme bacteria and the round body form, including observation of a transition phase of spirochete morphology to the round body form (Fig. 3.10).

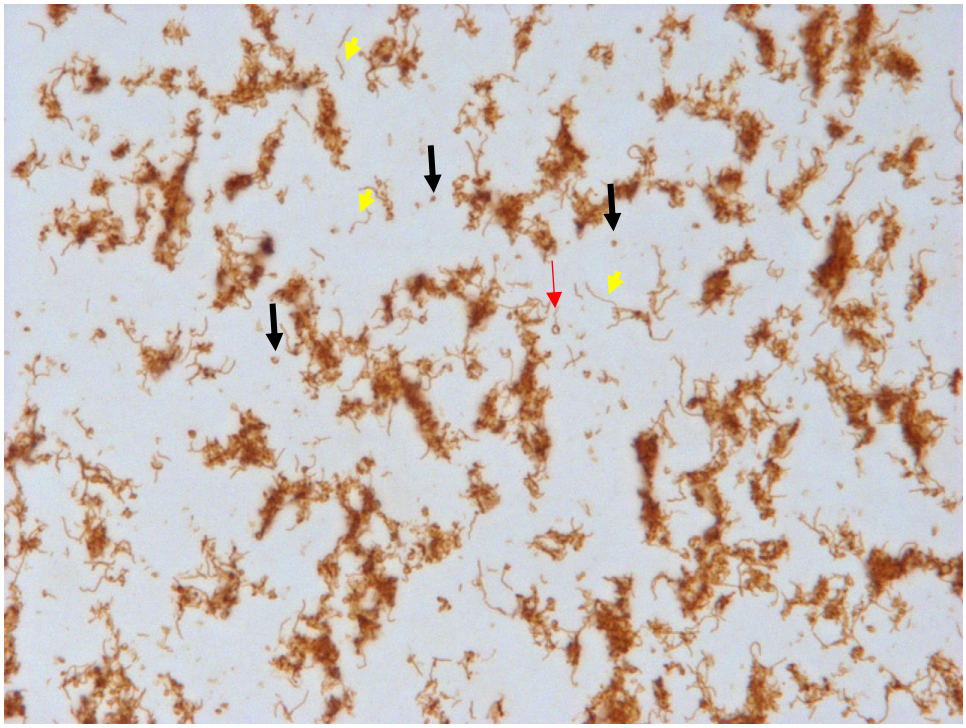


Figure 3.10 *Photomicrograph of Treponeme immunohistochemistry of treponeme cultures phylogroup 2 (T320A). Small yellow arrows are mature treponemal bacteria with spirochete morphology. Black arrows show intense labelling of the treponeme round bodies. Red arrow demonstrates a mature treponeme undergoing transformation to the round body form (400x magnification).*

3.3.4 Ultrastructure examination

Areas where there were intracytoplasmic inclusions as determined by light microscopy (Fig. 3.8) were subsequently examined using TEM. There were multiple keratinocytes, which contained variably sized cytoplasmic vacuoles close to the nucleus. Within the vacuoles there were single to multiple, variably sized, round to oval, homogenous electron dense material consistent with keratin (Fig. 3.9) and/or abnormally large keratohyalin granules.

3.4 Discussion

ITN is a severe disease leading to economic and animal welfare concerns that can result in early culling of animal. One study reported a culling rate of 38% rising to 71% with more than one teat affected (Manning, 2016). Until now, stages of disease were not well defined and corresponding macroscopic and microscopic descriptions were lacking (Blowey and Weaver, 2003; Blowey, 2004; Mauldin and Peters-Kennedy, 2007; Andrews et al., 2008; Blowey and Edmondson, 2010; Clegg et al., 2016b). From this study, ITN lesions were placed into three broad macroscopic categories: presence of teat with the lesion (Type 1), teat lesion with crust formation (Type 2) and absence of teat due to disease (Type 3). Other notes that can expand the overall description of the lesions are observations such as the length of the lesion; spread to other teats and the udder skin; and the presence of active haemorrhage. From clinical histories provided with cases, it is apparent that the time scale from lesions appearing to the absence of the teat is variable. However, it is clear that Type 3 lesions are end stage lesions. Animals with highly irritable or pruritic lesions may lose the teat between consecutive milking and others may have a delay prior to the teat sloughing, or it may not slough at all and enter an apparent recovery stage. The time interval of lesion development and direction of progression between grades is still unclear and further longitudinal studies are required. However, of note, no cow had all four teats affected. This is likely due to the fact many cows were rapidly culled on economic and welfare grounds before the disease could affect all teats. It is plausible and likely that all teats could be affected if the natural course of the disease were allowed to progress.

Another study constraint is the inability to know which farm/individual cow will be affected to monitor in observational and longitudinal studies. This poses a problem in that, it is assumed teats are sloughed due to the necrotising disease process unless there is a report of the cow observed actively licking or biting their teats in a self-destructive manor. Currently, it is not

known why some lesions are pruritic while others are not. An interesting point highlighted by the histopathological examination is that Type 3 lesions had a higher percentage of samples with eosinophils present, mostly perivascular, within both the dermis and lamina propria. Eosinophils are part of the immune system that are not present within healthy skin. The presence of eosinophils is often linked to hypersensitivity reactions as they release a range of chemokines and can degranulate to release inflammatory proteins which is thought to be a mechanism of inducing pruritis (Radonjic-Hoesli et al., 2021). Therefore, it may be that the presence of eosinophils in the tissue stimulates the cow to lick the teats more, inflicting trauma on the necrotic friable teat tissue and allowing the teat to slough more readily. Almost a fifth of affected teats were haemorrhagic at the time the photographs were taken. Twenty-five teats were either lacerated or partially/completely missing. It is unclear if all of these outcomes were due to necrosis or as a result of self-trauma or a combination of the two.

There is no anatomical bias to which teat (eg. front-left or back right) was affected. Despite this, there is a bias to the involvement of the medial aspect of the teat, as speculated by Blowey and Edmondson (2010) and Manning (2016). A predisposing factor may be compression of the medial aspect of the teat by the udder when the cow is lying down, causing a moist localised environment in that area, weakening the natural skin barrier defences and allowing the skin to become more easily abraded.

Potentially, it may be unjustified to rule out BHM from the macroscopic appearance alone. However, there are a few key differences that would suggest an ITN diagnosis over BHM, which can then be confirmed by diagnostic tests to rule out BHM if required (Cargnelutti et al., 2017). Such key differences are: ITN tends to be initially a dry lesion and BHM are moist lesions with ruptured vesicles (Shearer et al., 2008); ITN can lead to sloughing of the teat whilst BHM does not cause sloughing of the whole teat, although it can cause the epidermis to ulcerate and slough. Although, BHM can be very severe, lesions usually heal readily with regular and

persistent washing, drying and moisturizing the skin (Syring et al., 2010), whereas ITN takes a long time to heal if it does at all. In addition, BHM has not been reported to be pruritic in nature.

As DD-associated treponemes had previously been detected via PCR methods in ITN cases (Clegg et al., 2016b) it was important to attempt to visualise spirochetes microscopically via special stains such as Warthin-Starry (WS) and using more specific IHC methods (Angell et al., 2015b; Crosby-Durrani et al., 2016). In this study neither WS or anti-DD treponeme IHC detected spirochaetal bacteria within ITN lesions. This is in contrast to previous ruminant treponeme-associated diseases where treponemes detected by PCR were subsequently visualised in abundance within lesions such as with DD and CODD and a similar disease in goats (Evans et al., 2009; Angell et al., 2015b; Crosby-Durrani et al., 2016). What was noted was the abundance of mixed bacterial flora on Gram-stain. Further microbiological investigations are required to investigate the diversity of these bacteria. The IHC of treponeme cultures suggested that there is a presence of round bodies that may give an intense granular appearance on IHC. It may be that the more aerobic environment on the skin causes stresses within the treponeme leading to more cystic forms to be observed and reversal to the helical forms with a more favourable anaerobic environment, such as necrosis, may develop in some skin lesions. This process has been reported to occur in spirochetes including *Treponema* sp. (Margulis et al., 2009). Therefore, the previous IHC studies for detecting treponemes in DD and CODD lesions may need to be reviewed in light of the finding of the appearance of round bodies on IHC sometimes having a granular appearance, and demonstrates the importance of concurrent molecular data.

When considering the histological appearance, the epidermis and the lamina propria of the teat have the most interesting features in terms of understanding the pathogenesis. In the epidermis the bacterial colonies observed were mostly cocci embedded into serocellular crusts. These

were likely to be commensal skin bacteria or environmental contaminants that have opportunistically invaded the lesioned tissue as secondary agents. The intracytoplasmic inclusions were observed in multiple sections from animals from different farms. From the TEM, these inclusions most likely represent an abnormal premature formation of intracellular keratin (dyskeratosis). They were always in the epidermis and mostly in the hyperplastic area of the stratum granulosum. The observation of intracytoplasmic inclusions and ballooning degeneration led to the suspicion of an orthopox virus. However, immunohistochemistry designed to detect orthopox virus was unable to demonstrate this virus within the tissues examined. The keratohyalin granules were prominent, enlarged, coarse and often clumped in these regions, as sometimes occurs with papilloma viruses (Gulbahar et al., 2003). There was no evidence of viral infection on the TEM unlike findings in teat papilloma lesions (Maeda *et al.*, 2007).

The blood vessels in the lamina propria also appeared to be involved in the pathogenesis. Thrombi were observed in 17.6% of cases examined histologically and may have been present in other cases if the thrombosis was transient and re-cannulation of a thrombosed blood vessel had occurred. However, there was well-demarcated necrosis present in each section suggestive of ischemia, meaning it was likely that a thrombosed vessel had been present and not detected histologically possibly due to the variable nature of sampling these lesions. Ulceration was a prominent feature and likely a consequence of necrosis. These types of vascular lesions are usually indicative of one of two common potential pathogeneses. The first is that the vessels are affected by an immune mediated inflammation, eg. hypersensitivities, with the possibility of immune complex deposition in the vessel wall or secondly, that the vessels were damaged by direct invasion of an infectious agent (Kumar et al., 2015). There was some evidence of the potential for immune complex deposition and thus a hypersensitivity reaction by the presents of leukocytoclastic vasculitis in some small veins in the dermis/lamina propria.

Although, from this study it is not possible to determine the duration of the Type 1 and Type 3 lesions from the macroscopic appearance, it was clear that Type 2 lesions that were chronically active were associated with a proliferative epidermal response with crust formation. It is possible that the Type 2 lesions previously were necrotic and ulcerated and the crust represents an aberrant healing process. From the histopathological examinations, some lesions were more chronic than the others with the presence of granulation tissue and both orthokeratotic and parakeratotic hyperkeratosis in some samples. There were a few small histopathological differences between the clinical macroscopic grades with only Type 1 lesions found to histologically exhibit epidermal necrosis, coagulative necrosis of the dermis and/or lamina propria with observable thrombosis. Serocellular crusting was more common in the Type 2 lesions. It seems Type 1 lesions can have a degree of chronicity histologically (presence of granulation tissue) that could not be appreciated macroscopically.

To summarise, there are three macroscopic categories for ITN (Type 1, 2 and 3); although these do not correspond completely with the histological categories, there are some key differences observed between grades histologically that are useful for future diagnostic and research into pathogenesis. The main histological findings are: focal extensive severe purulent to eosinophilic necrotising and ulcerative dermatitis; serocellular crust formation; epidermal hyperplasia with dyskeratosis; small veins with leukocytoclastic vasculitis; medium-sized veins with lymphoplasmacytic to eosinophilic vasculitis; thrombosis with ischaemic necrosis. The histopathology indicates that there may be a hypersensitivity response in Type 3 lesions. The immunohistochemistry studies were not supportive of DD-associated treponeme or orthopox involvement. There was a large number of mixed bacterial colonies present and further studies are required to investigate the microbiome and potential aetiopathogenesis to better understand this disease of substantial animal welfare and economic concern.

Chapter 4: PCR screening of ITN samples for putative pathogens and investigations into infection reservoirs of digital dermatitis treponemes.

4.1 Introduction

Bovine ischaemic teat necrosis (ITN) has previously been suggested to be associated with DD associated treponemes (Clegg et al., 2016b). This previous study was based on 12 animals with ITN lesions, 11 of which were positive for the presence of DD associated treponemes by PCR. DD associated treponeme phylogroups have also been detected in another skin lesion of the udder, UCD in approximately 10 % of cases (Stamm et al., 2009; Evans et al., 2010); the foremilk from cows with UCD lesions (Sobhy et al., 2020) and in skin lesions of the hocks of dairy cattle (Clegg et al., 2016c). DD associated treponemes have been frequently associated with DD infectious lameness in dairy cattle as part of a polymicrobial disease (Wilson-Welder et al., 2015; Moreira et al., 2018; Staton et al., 2020; Caddey and De Buck, 2021). One hypothesis is that when an animal with DD lesion on the hindfeet lie down the feet can come into contact with the udder and allowing microbes to be directly transferred from the feet to udder. In addition, while lying, parts of the udder may become compressed, such as between the two halves of the udder and between the anterior aspect of the udder and the abdomen, the site of UCD lesions. Of note, lying could cause the teats to fold at the junction between the teat and udder, leading to compression near the teat base especially on the medial aspect, resulting in two parts of the skin to be in-contact, which could lead to a moist and anaerobic environment that would be a niche environment for the growth of DD treponemes. Moreover, it has been hypothesised that DD associated treponemes may migrate along the skin to areas of poor skin integrity (Clegg et al., 2016c), such as an area of necrotic skin. As this microenvironment could potentially be supportive of treponeme growth, and with the publication from Clegg et al. (2016b), it was considered timely to further investigate the hypothesis of DD associated

treponeme presence in ITN lesions using a larger number of animals across a wider geographical location.

DD associated treponemes have also been detected in different host tissues including gingiva, rumen and recto-anal junction (Evans et al., 2012). Subsequently, studies found DD associated treponeme bacteria in multiple sites in the dairy environment, such as in slurry and cow faeces (Klitgaard et al., 2014), hoof knifes (Gillespie et al., 2020) and foot trimming equipment, and cow hoof footprints, gloves worn by foot trimmers (Bell, 2017). Bell (2017a) also found that DD associated treponemes could survive in different types of bedding used in the dairy farm using *in vitro* experiments that added cultured DD associated treponemes to clean bedding samples. This demonstrates a wide range of areas were DD associated treponemes have been detected. However, little assessment of the milking environment has been documented to look for environmental reservoirs of DD associated treponemes that regularly come into contact with the teat and udder.

There are many infectious agents that can cause lesions on the teats in dairy cattle and with the findings presented in Chapter 3 of sporadic identification of inclusion bodies in small numbers of keratinocytes, the possibility of an initial viral infection was queried. As the inclusion bodies on H & E sections appeared as large, intracytoplasmic and eosinophilic within keratinocytes (reminiscent of Bollinger bodies), pox virus was considered a potential aetiology (Maxie, 2007). There are numerous pox viruses that affect the skin of multiple species. Notable viruses include Parapox viruses such as bovine popular stomatitis and Pseudocowpox, which are almost ubiquitous in the dairy industry (Underwood et al., 2015), Cowpox virus, mostly identified in rodents and cats (Bennett et al., 2008) and Orf (contagious erythema) virus in sheep (Shapiro, 2017) . All these viruses can potentially cause minor teat lesions but may allow for entry of a secondary infectious agent.

As described previously, DD associated treponemes have been detected in the foremilk of cows with UCD lesions (Sobhy et al., 2020). As such, it was important to investigate if DD associated treponemes were able to survive for any length of time in milk at body temperature (37°C) and bulk milk tank temperature (4°C). It was also important to see if a cow presenting with DD lesions on the hindfeet were also able to have DD associated treponemes present in the foremilk as with UCD positive animals. If this was the case then there would be further implications around the control of DD and another infection reservoir to consider.

The main aims of this chapter were to screen all ITN lesions for DD associated treponemes and some key areas of the environment that the teat regularly comes into contact which such as teat liners of the milking machine and bedding. It was also, important to attempt to investigate the potential for any pox viral involvement and the possibility of the foremilk as a potential reservoir for DD associated treponemes.

4.2 Materials and Methods

4.2.1 Ethics statement

Ethical approval for this study was granted by the University of Liverpool, School of Veterinary Science Ethical Committee (application number: VREC 460). The participants provided their written informed consent to participate in this study and allowed for samples to be retained and used for research purposes.

4.2.2 Culture of digital dermatitis associated treponemes

Digital dermatitis associated treponeme bacteria were used to investigate treponeme survival in milk. For ease of use the *T. phagedenis* phylogroup strain T320A and *T. pedis* phylogroup strain T3552B, stains previously isolated by this laboratory, were utilised. T320A and T3552B were previously stored in 10% (v/v) glycerol at -80^{o}C and were thawed and transferred to an

anaerobic cabinet under the following conditions: 85% N_2, 10% H_2 and 5% CO_2 at 37ºC. Once thawed, approximately 300 µl (10 drops from a sterile glass Pasteur pipette) was inoculated into oral treponeme enrichment broth (OTEB, Anaerobe systems, CA, USA) supplemented with 10% (v/v) foetal calf serum (FCS). Inocula growth was assessed by a 5 point grading scheme (Bell, 2017) under phase contrast microscopy on days 4 and 7 after inoculation. If growth was adequate, the cultures were subsequently subcultured taking approximately 90 µl (3 drops) and passaged into new OTEB 10% (v/v) FCS every 7 days.

4.2.3 Sample collection

4.2.3.1 Tissue samples

A set of tissue samples for microbiological studies were obtained by an VS surgically removing teats from live animals in an attempt to contain the disease (*n*=8). A further 16 samples were obtained during debridement for treatment purposes from the VS and debrided tissue placed into a sterile container and stored frozen awaiting delivery or collection. Other teat tissue samples (*n*=9 with ITN) were obtained from animals that presented as cull cows at meat inspection in abattoirs and in fallen stock centres. Cull cow teats were removed from the carcass using sterile scalpel blades; 20 normal teats from healthy animals were taken as negative controls for microbiome studies (see Chapter 5). The teats were then halved longitudinally through the lesion. One half was stored on ice prior to freezing at -20 °C for microbiological studies and the other half placed in 10% neutral buffered formalin to be used for histological examination as previously described (section 3.2.1).

4.2.3.2 Swab samples

Where tissue samples were not obtainable, veterinary surgeons obtained plain swabs from the teat lesions. For swabbing the lesions, swabs were rubbed against the relevant necrotic areas.

For swabbing healthy teats, the swab was first wetted with sterile saline. Sixty-two swabs of ITN lesions from 32 animals were obtained along with 18 swabs from non-affected teats of the affected animals. When possible, affected animals were matched with cows from the same farm at a similar age and stage of production and their healthy teats were swabbed at the same site where ITN lesions develop (*n*= 10 cows). Swabs were stored frozen at -20 °C and transported on ice to the laboratory.

4.2.3.3 Environmental samples

When possible, environmental samples were taken. This included used bedding from animals that were currently housed (*n*=14), swabs of milk liners after milking an ITN positive teat (*n*= 20) and a disposable milker's gloves after milking (*n*=4).

4.2.3.4 Collection of milk for assessment of treponeme survival in milk

Whole milk and whole ultra-high temperature (UHT) milk were obtained from the supermarket for investigations into the ability for DD treponemes to survive in milk.

For investigating the presence of treponemes in foremilk, foremilk was taken immediately prior to milking the cow and a simplified DD status (acute, chronic, no DD) of the cow recorded by use of an inspection mirror in the parlour. The university dairy farm was utilised for this sample collection due to the close proximity to the laboratory. Samples were immediately processed for DNA extractions and inoculated into OTEB 10% (v/v) FCS within 2 hours after milking.

4.2.4 DNA extraction

4.2.4.1 Extraction from swabs and tissue of ITN lesions

Swabs and tissue samples obtained from ITN lesions were chopped using a sterile scalpel blade and placed into an eppendorf. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) as per manufacturer instructions. Briefly, the kit has a simplified

method that utilises enzymatic breakdown of samples with Proteinase K and silica-based DNA extraction to produce high yields of DNA from a variety of samples. This kit has been used a number of times to detect DD-associated treponeme bacteria (Evans et al., 2009; Clegg et al., 2015, 2016a, 2016c; Sullivan et al., 2015b). The same kit was also used for the environmental samples with a small amount, approximately up to 0.5 ml in a 1.5ml eppendorf, of bedding or the forefinger from a milker's disposable glove added to the first incubation step.

4.2.4.2 Extraction from liquid culture

Samples inoculated into OTEB liquid culture and treponeme culture stocks for positive controls required extraction using the Chelex extraction method as described previously (de Lamballerie et al., 1992; Gillespie et al., 2020). Briefly, a 5% Chelex resin solution was formulated by dissolving 0.5 g Bio-Rad BT Chelex® 100 Resin (Bio-Rad, Watford, UK) in 10 ml of distilled water. Approximately 1.5 ml of liquid culture was removed from the OTEB tubes and placed into a clip-lock eppendorf and the sample centrifuged at 13,000 RPM for 5 mins and approximately 800ml of the supernatant removed. The pellet was then re-suspended in the remaining supernatant and 250µl of the 5% Chelex resin added. The sample and Chelex solution were suspended in a boiling water bath for 10 mins before centrifuging at 13, 000 RPM for 10 mins. The supernatant was placed in a new Eppendorf and frozen at - 20 ° C until required.

4.2.4.3 Extraction from milk

Extraction of DNA from milk was performed using the Milk Bacterial DNA Isolation kit (Norgen Biotech Corp., Thorold, ON, Canada) according to manufacturer's instructions, which utilises enzymatic and chemical lysis of bacteria.

4.2.4.4 Assessing quality of extracted DNA

After extraction, the quality and quantity of DNA was assessed using a NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™, Waltham, MA, USA).

4.2.5 Polymerase chain reaction

4.2.5.1 Nested PCR for detection of digital dermatitis associated treponemes

A nested PCR approach was utilised for the detection of DD associated treponemes and was performed on all samples in triplicate with DNA extracted from an appropriate DD associated treponeme phylogroup liquid culture as a positive control, and nuclease free water and the appropriate non-targeted DD associated phylogroups as negative controls for each assay. The assays were a two-step process with the initial PCR step a universal bacterial 16S rRNA gene amplification and then a second DD associated treponeme phylogroup specific amplification step. The PCR primers used in each step are listed in Table 4.2.1. Both steps utilised the PCR reaction mix: 13.8µl water, 0.6µl forward primer (100 pmol/µl), 0.6µl reverse primer (100 pmol/µl), 4µl 5 x FIREpol® Ready to Load Master Mix (7.5 mM $MgCl_2$) (Solis BioDyne, Tartu, Estonia), 1µl template DNA. The template DNA for the second step was the product produced from the first universal bacterial 16S rRNA gene amplification.

Table 4.2.1 PCR assay primers for DD associated treponeme screening

Primer	Primer sequence (5'-3') (forward and reverse)	16S rRNA gene position	Band size (bp)	Reference
Universal 16S rRNA gene	AGAGTTTGATCCTGG TACCTTGTTACGACTT	7-26 1491-1506	1526	(Rurangirwa et al., 1999)
T. medium phylogroup	GAATGCTCATCTGATGACGGTAATCGACG CCGGCCTTATCTAAGACCTTCTACTAG	472-500 1001-1029	475	(Evans et al., 2008)
T. phagedenis phylogroup	GAAATACTCAAGCTTAACTTGAGAACTTGC CTACGCTACCATATCTCTATAATATTGC	612-640 1006-1029	400	(Evans et al., 2008)
T. pedis phylogroup	GGAGATGAGGGAATGCGTCTTCGATG CAAGAGTCGTATTGCTACGCTGATATATC	459-484 1017-1045	475	(Evans et al., 2008)
Treponema genus	AARCATGCAAGTCGARCGGCAAG TCCATTGCGGAATATTCTTA	49-71 365-384	335	(Moore et al., 2005)

For each PCR assay different PCR cycling conditions were required. PCR cycling conditions were specified for 16S rRNA gene (Table 4.2.2), DD associated *T. medium* phylogroup (Table 4.2.3), *T. phagedenis* phylogroup (Table 4.2.4) and *T. pedis* phylogroup (Table 4.2.5).

Table 4.2.2 PCR cycling conditions for universal bacterial 16S rRNA gene.

Operation	Temperature	Time	Cycles
Initial denaturation	95ºC	5 mins	1
Denaturation	94ºC	1 min	24
Annealing	55ºC	3 mins	
Elongation	72ºC	3 mins	
Final elongation	72ºC	7 mins	

Table 4.2.3 PCR cycling conditions for Treponema medium phylogroup

Operation	Temperature	Time	Cycles
Initial denaturation	95ºC	5 mins	1
Denaturation	95ºC	1 min	39
Annealing	68ºC	1 min	
Elongation	72ºC	2 mins	
Final elongation	72ºC	10 mins	

Table 4.2.4 PCR cycling conditions for Treponema phagedenis phylogroup

Operation	Temperature	Time	Cycles
Initial denaturation	95ºC	5 mins	1
Denaturation	95ºC	1 min	39
Annealing	64ºC	1 min	
Elongation	72ºC	2 mins	
Final elongation	72ºC	10 mins	

Table 4.2.5 PCR cycling conditions for Treponema pedis phylogroup

Operation	Temperature	Time	Cycles
Initial denaturation	95ºC	5 mins	1
Denaturation	95ºC	1 min	39
Annealing	68ºC	30 s	
Elongation	72ºC	2 mins	
Final elongation	72ºC	10 mins	

4.2.5.2 PCR for detection of Treponema genus specific 16S rRNA gene

In addition to the nested PCR approach, all samples were also screened targeting the *Treponema* genus specific 16S rRNA gene (table 4.2.1). The PCR reaction mix for the *Treponeme* genus assay was: 13.8µl water, 0.6µl forward primer (100 pmol/µl), 0.6µl reverse primer (100 pmol/µl), 4µl 5 x FIREpol® Ready to Load Master Mix (12.5 mM $MgCl_2$) (Solis BioDyne, Tartu, Estonia), 1µl template DNA. The PCR reaction cycling conditions differed from the nested approach and are presented in Table 4.2.6.

Table 4.2.6 PCR cycling conditions for Treponema genus specific 16S rRNA gene

Operation	Temperature	Time	Cycles
Denaturation	95ºC	30 s	40
Annealing	64ºC	1 min	
Elongation	72ºC	1 min	
Final elongation	72ºC	10 mins	

4.2.5.3 PCR for detection of pan-pox virus

To investigate the presence of an unknown pox virus a pan-pox screening assay as described by Li *et al*. (2010) was used. Briefly, the study described a way of identifying pox viruses based

on the GC content with the primer sequence and universal Pox viral target genes (Table 4.2.7). Using a sample from a confirmed case of cowpox, an Orthopoxvirus, in a Cheetah (*Acinonyx jubatus*) and Orf virus infection, (a Parapoxvirus in a lamb (*Ovis aries*) cases submitted for diagnostic post mortem examination at the University of Liverpool), were used as positive controls for the low GC and high GC content respectively. A temperature gradient was used to optimise the PCR assays prior to screening samples and the final PCR cycle conditions for the low and high GC content in Tables 4.2.8 and 4.2.9 respectively. Ten ITN samples, including the four samples where potential inclusion bodies were observed on H & E, were screened using the Pan-pox PCR assays. Nuclease free water was used as a negative control. Positive samples were purified and submitted for Sanger sequencing.

Table 4.2.7 PCR assay primers for Pan-pox virus screening

Primer	**Primer sequence (5'-3') (forward and reverse)**	**Target gene position**	**Band size (bp)**	**Reference**
Pan-pox Low-GC content	ACACCAAAAACTCATATAACTTCT	Insulin metalloproteinase-like protein gene, G1L ortholog	220	(Li et al., 2010)
	CCTATTTTACTCCTTAGTAAATGAT	Intracellular mature virion membrane protein gene, G3L ortholog		
Pan-pox High-GC content	CATCCCCAAGGAGACCAACGAG	RNA polymerase subunit gene, VAC-COP J6R ortholog	630	(Li et al., 2010)
	TCCTCGTCGCCGTCGAAGTC	RNA polymerase subunit gene, VAC-COP J6R ortholog		

Table 4.2.8 PCR cycling conditions for Pan-pox low GC content

Operation	**Temperature**	**Time**	**Cycles**
Initial denaturation	92ºC	2 mins	1
Denaturation	92ºC	10 s	40
Annealing	52ºC	30 s	
Elongation	72ºC	1 min 40 s	
Final elongation	72ºC	5 mins	

Table 4.2.9 PCR cycling conditions for Pan-pox high GC content

Operation	Temperature	Time	Cycles
Initial denaturation	92ºC	2 mins	1
Denaturation	92ºC	10 s	30
Annealing	62ºC	30 s	
Elongation	72ºC	1 min 40 s	
Final elongation	72ºC	5 mins	

4.2.6 Assessment of treponeme survival in milk

Two processes were used to investigate the ability for DD associated treponemes to survive in milk. The first was to see if treponemes could be detected by PCR after incubation over different time points. The second was to use phase contrast to look for treponemes at various timepoints after an initial passage.

4.2.6.1 Treponemes survival in milk

It is not always possible to detect treponemes with different DNA extraction techniques. Therefore, an *in vitro* experiment was first devised to investigate the methodology in priming experiments, prior to using the technique to assess if treponemes were present in the foremilk of cows with and without DD lesions in their hindfeet.

Whole milk and whole UHT milk were obtained from the supermarket for investigations into the ability for DD treponemes to survive in milk. DD associated *T. phagedenis* phylogroup strain T320A and DD associated *T. pedis* phylogroup strain T3552B were cultured as shown in Chapter 4.2.2. and 6 drops of a known concentration (0.43 optical density on the spectrometer set at 540nm wavelength) were spiked into 1 ml of milk and incubated under different conditions.

Initially serial 1 in 10 dilutions of the bacteria were used to assess if the DNA extraction kit could detect low numbers of treponemes present in the sample.

Then treponemes were added to the milk types to see for how long DNA could be successfully extracted and detected by PCR methods after incubation in an anaerobic environment at 37 °C. Samples were taken for DNA extraction using Milk Bacterial DNA Isolation kit (Norgen Biotech Corp., Thorold, ON, Canada) according to manufacturer instructions, at the following time points: 10 mins, 30mins, 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours post treponeme inoculation.

After the incubation, the effect of temperature on treponeme DNA integrity was also assessed. Treponemes were spiked into milk and incubated either at 4 °C (to represent storage in a refrigerated bulk milk tank) or at 37 °C (to represent body temperature) for 24 hours, DNA was extracted as stated before and also 0.5ml of the milk/treponeme emulsion inoculated into OTEB 10% (v/v) FCS. A sample (1 drop) of the liquid culture was taken using a glass pipette at 24 hours, 1 week, 2 week, 3 weeks, 4 weeks, 5 weeks and 6 weeks (Gillespie et al., 2020) and assessed under phase contrast microscopy to look for treponeme morphology and growth. After 6 weeks incubation in an anaerobic cabinet a Chelex extraction was performed on a sample of the liquid culture and the extracted DNA used in the DD associated treponeme PCR assays. This process was repeated with two strains of bacteria (T320A and T3552B) and 3 technical replicates.

The above process was also repeated but the milk/treponeme emulsion passaged into OTEB+ 10% (v/v) FCS supplemented with 35 µl rifampicin (5mg/ml stock), 3.5µl enrofloxacin (10mg/ml stock in 1M KOH) and 3.5 µl 1M hydrochloric acid (to balance the pH of the enrofloxacin) to assess the effects on treponeme growth of an antibiotic mixture to suppress growth of other contaminating bacteria.

4.2.6.2 Treponeme detection from fresh foremilk from DD symptomatic and asymptomatic cows

For investigating the *in vivo* presence of treponemes in milk, foremilk was taken immediately prior to milking the cow and a simplified DD status (acute, chronic, no DD) of the cow recorded by use of an inspection mirror in the parlour. The university dairy farm was used for this sample collection due to the close proximity to the laboratory. Samples were immediately processed and all DNA extractions initiated and all sampled inoculated into OTEB 10% (v/v) FCS within 2 hours after milking.

One ml of foremilk from DD symptomatic and asymptomatic cows was subjected to DNA extraction using the Milk Bacterial DNA Isolation kit (Norgen Biotech Corp., Thorold, ON, Canada) by manufacturer instructions. Extracted DNA was then screened for DD associated treponemes using the PCR assays described above.

4.2.6.3 Survival of treponemes in fresh foremilk from DD symptomatic and asymptomatic cows

Here, 0.5 ml of foremilk was added to 7 ml OTEB 10% (v/v) FCS and incubated at 37 °C in an anaerobic cabinet. At the following timepoints a sample (1 drop) was removed from the liquid culture using a glass pipette and examined under the phase contrast microscope looking for treponeme morphology and growth: 24 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks and 6 weeks. After 6 weeks, the a Chelex extraction (section 4.2.4.2) was performed on the liquid culture and the extracted DNA screened using the DD associated treponeme PCR assays (section 4.2.5).

4.3 Results

4.3.1 Screening ITN samples for DD associated treponemes

Sixty-two swabs, and 33 tissue samples from ITN lesions were screened using the nested DD associated PCR assays. In addition, 18 swabs from healthy teats from the ITN positive cows and when possible swabs from the teats of a healthy cow from the same farm of a similar age and production stage (*n*=10) were also screened for DD associated treponemes. All screening samples from healthy live animals were swabs. Of the 95 ITN lesions, 34 (35.8%) were positive for at least one DD associated treponemes and only 1 of 18 (5.6%) teats from a non-affected teat from a cow with a ITN lesion were positive (Table 4.3.1). No swabs from the age and production stage matched animals were positive for DD associated treponemes using the PCR assays.

Table 4.3.1 Summary of PCR screening of ITN samples for DD associated treponeme bacteria phylogroups.

Sample (ITN +/- teat and +/- cow)	**Sample type (swab/tissue)**	**Number**	***Treponema* genus**	**Group 1**	**Group 2**	**Group 3**
ITN positive teat	Swab	62	21/62 (33.8%)	12/62 (19.4%)	10/62 (16.1%)	17/62 (27.4%)
ITN positive teat	Tissue	33	13/33 (39.4%)	4/33 (12.1%)	6/33 (18.2%)	10/33 (30.3%)
Total ITN positive teat samples (both swabs and tissue)	Swab and Tissue	95	34 (35.8%)	16 (16.8%)	16 (16.8%)	27 (28.4%)
ITN negative teat but positive cow	Swab	18	1/18 (5.6%)	0	0	1/18 (5.6%)
Matched ITN negative cow	Swab	10	0	0	0	0

Group 1- DD associated Treponema medium phylogroup, Group 2 – DD associated Treponema phagedenis phylogroup, Group 3 – DD associated Treponema pedis phylogroup

When using the Chi squared test there was a statistical difference between the number of ITN positive teats (tissue and swab samples) on *Treponema* genus PCR assay and the numbers positive from the non-affected teat on the same animal (X^2, (1, N =113) = 6.47, p =.01). There were 113 samples from ITN positive cows including 18 swabs of teats without ITN lesions. Twenty-one (18.6%) of samples were positive for one DD-associated treponeme phylogroup; 4 (3.5%) were positive for two DD associated treponeme phylogroups and 10 (8.8%) were positive for the three recognised DD associated treponeme phylogroups. Thirty-four (35.8%) were positive for at least one DD associated treponeme phylogroup.

4.3.2 Screening environmental samples for DD associated treponemes

Ten percent (2 of 20) swabs from teat liners after milking two animals with an ITN affected teat positive for a DD associated *Treponeme* on PCR and 3 (15%) of animals were positive on *Treponema* genus PCR. Only two cows with ITN lesions were recorded as having DD lesions on the hindfeet. The DD lesions were swabbed and were positive for DD associated treponemes on PCR assay but the ITN lesions in the same cows were negative. The bedding (including sand, sawdust and recycled manure products) and milker's gloves were all negative on the DD associated treponemes PCR assays.

Table 4.3.2 Screening of environmental samples indicating the numbers positive for Treponema genus and DD associated trepomene bacteria on PCR assays

Sample	**Number**	***Treponema* genus**	**Group 1**	**Group 2**	**Group 3**
Swabs from teat liners after milking ITN teat	*N*=20	3 (15.0%)	0	1 (5.0%)	1 (5.0%)
Bedding samples	N=14	0	0	0	0
Milker's disposable gloves	N=4	0	0	0	0

Group 1- DD associated Treponema medium phylogroup, Group 2 – DD associated Treponema phagedenis phylogroup, Group 3 – DD associated Treponema pedis phylogroup

4.3.3 Screening ITN samples for pox virus

Four of 10 (40%) ITN samples had faintly positive broad bands for poxvirus (all type 1 ITN lesions, including one with ballooning degeneration and eosinophilic inclusions on histopathology) using the high GC content pox virus PCR assay. All other samples were negative for this assay and all samples were negative using the low GC content pox virus PCR assay. The samples with faint broad bands were purified and submitted for Sanger sequencing. However, sequencing failed multiple times and given the bands were faint and an incorrect size these bands were considered non-specific.

4.3.4 Survival of DD associated treponemes in milk

Optimisation of the protocol found that DD associated treponemes spiked in whole and UHT milk, obtained from the supermarket, could be detected routinely within two hours of incubation at different temperatures and there was no difference in the treponeme growth if the milk/treponeme emulsion was passaged into OTEB 10% (v/v) FCS with or without antibiotics. As such the protocol used to investigate DD associated treponeme presence in foremilk obtained from cows with and without DD lesions excluded antibiotics and was processed within 2 hours of milking (Table 4.2.3).

Table 4.3.3 Summary of the length of time treponemes can be detected via the nested PCR assays for DD associated Treponema phagedenis phylogroup and Treponema genus.

	Time							
PCR Assay	**10 min**	**30 mins**	**1 h**	**2 h**	**4 h**	**8 h**	**24 h**	**negative control**
T. Phagedenis	+	+	+	+	-	-	-	-
Treponema genus	+	+	+	+	+	+	-	-

+ indicates a positive result on PCR and – indicates a negative result on PCR.

DD associated treponemes could not be observed using phase contrast microscopy at any time points from 24 hours to 6 weeks after inoculation into OTEB 10% (v/v) FCS and were not

detectable using the DD associated treponeme PCR assays. Cultures were allowed to grow for six weeks, as treponemes are fastidious and it can take several weeks for growth to be detected and is the time used to assess for DD associated treponeme survival in a previous study (Gillespie et al., 2020).

4.3.4.1 Detection and survival of DD associated treponemes in foremilk from DD symptomatic and asymptomatic cows

Foremilk was collected from 50 cows prior to milking. Nineteen cows had lesions on the hind feet consistent with chronic DD, 31 cows had healthy hind feet (Table 4.3.4).

Table 4.3.4 DD associated treponeme PCR positive foremilk samples (DNA was extracted within 2 hours of milking).

Sample	**Number**	***Treponema* genus**	**Group 1**	**Group 2**	**Group 3**
Cows with healthy hindfeet	31	6 (19.4%)	0	0	0
Cows with DD lesions on hind feet	19	1 (5.3%)	0	0	0

Group 1- DD associated Treponema medium phylogroup, Group 2 – DD associated Treponema phagedenis phylogroup, Group 3 – DD associated Treponema pedis phylogroup

There was no statistical difference using the Chi squared test (X^2 (1, N = 50) = 1.94, p = .16) between the number of positive foremilk samples identified using the *Treponema* genus PCR assay from cows with healthy feet and cows with DD lesions on the hind feet. Nested DD associated treponeme PCR assays were all negative on the foremilk samples.

After inoculation of milk into OTEB 10% (v/v) FCS, all samples examined using phase contrast microscopy did not detect spirochaetal morphology after 24 hours incubation or at any time point in the weekly checks up to and including 6 weeks post inoculation. All samples were negative when assayed using the treponemes PCR assays on the 6 weeks post Chelex extraction.

4.4 Discussion

4.4.1 PCR screening of teat samples for DD associated treponemes

An original hypothesis by Clegg et al., (2016b) suggested that ITN may be another disease that is associated with the presence of *Treponema* bacteria, due to the detection of DD associated treponemes by PCR in 11 of 12 (91.7%) cows with ITN lesions. The screening of the samples collected in this study, 113 samples from cows with ITN lesions (18 samples from teats without lesions), using the same nested DD associated treponeme PCR assays found that 31.0%, less than a third of samples screened were positive for any of the DD associated bacteria. There was predominantly a single DD associated treponeme phylogroup detected in these samples compared to 12.4% of samples with 2 or more different DD associated treponeme phylogroups. This is in stark contrast to Clegg et al., (2016b) where 10 contained two or more DD treponeme phylogroups. These findings, however, are consistent with epidemiological findings of Chapter 2 and the clinical history obtained during sampling, that farms with ITN do not report having issues with DD lameness in their dairy herd. Two animals with DD lesions and concurrent ITN lesions had both anatomical locations screened for DD associated treponemes via PCR assays. In both of these animals the DD lesions on the hindfeet were positive for DD associated treponemes but the ITN lesions were negative. While the number of animals with concurrent lesions are low, these findings along with the screening of larger numbers of ITN lesions and the epidemiological data are beginning to suggest that the DD treponemes may not be a primary pathogen for ITN as once thought. It is possible that the detection of DD associated treponemes may indicate a higher burden of DD treponemes in the environment and contaminating damaged tissue of the teat as a secondary agent (Clegg et al., 2016c). Alternatively, this difference may be as with non-healing horn lesions of white line disease and sole ulcer which can become infected with DD treponemes but these bacteria are not considered to be the aetiology (Evans et al., 2011; Staton et al., 2020). Potentially the initial earlier observed high association with these taxa may result from complicated ITN lesions that do contain DD

treponemes, whereas this study the reduction in association may result from the inclusion of more non-complicated ITN samples.

A similar disease of the udder with a relatively low numbers (10%) of samples detected as containing DD associated treponemes phylogroups via PCR is UCD (Evans et al., 2010b). The authors of that study indicated that it was likely that UCD was a polymicrobial disease rather than involvement of a single aetiological agent. Given there are similarities between ITN and UCD, in that both affect the skin of the udder it seems appropriate to investigate the potential polymicrobial nature of ITN. In addition, more recent studies investigating the microbiome of cows with and without DD lesions also suggests that DD may be more of a polymicrobial disease (Wilson-Welder et al., 2015; Moreira et al., 2018; Caddey and De Buck, 2021) indicating diseases initially considered likely to be due to a single aetiological agent with recent multi-omics studies now appear as more likely a polymicrobial pathogenesis.

4.4.2 Screening the environment for DD associated treponemes

The dairy environment has been shown to have multiple sites where DD associated treponemes can be detected. These include cow faeces and slurry, cow footprints, hoof trimming equipment, and especially hoof knives and gloves used during the foot trimming process (Bell, 2017; Gillespie et al., 2020). In addition, different dairy farm bedding material has been shown to enable survival of DD associated treponemes in laboratory settings (Bell, 2017). However, little investigations into the milking environment has been performed. A key area that comes into contact directly with the teat every day during lactation is the teat liners. This study found that 10% of teat liners had DD associated treponemes detectable via PCR after milking an ITN affected teat. However, the bedding samples and milker's disposable gloves were all negative for DD associated treponemes by PCR. Although the sample size was low, these findings

suggests that there is unlikely to be a high DD associated treponeme burden in the environment on the farms where the samples were collected. Nevertheless, 10% of teat liners were positive for DD associated treponemes via PCR assays after milking a ITN teat. This is interesting and requires further investigation. Although these are relatively low numbers, if there are no disinfection protocols of the milk clusters between cows, the treponemes could potentially be transferred between animals quickly and could be a reservoir site for DD associated treponemes. In fact, direct contact is considered an important transmission route in other treponeme diseases that infect humans such as yaws and syphilis (Lukehart and Giacani, 2014). As such, direct contact of a teat liner from an infected cow to a non-infected cow could transmit these bacteria with further research required in this area to clarify this potential transmission route.

4.4.3 Screening of samples for Pox virus

As demonstrated in Chapter 3, there were a small number of samples with eosinophilic inclusion bodies present in the epidermis of four samples on histopathological examination. Eosinophilic inclusion bodies are frequently observed in acute infections with multiple different pox viruses (Maxie, 2007). Pox viruses are often associated with a proliferative lesion (Maxie, 2007) and therefore it was considered a potential ITN aetiology, especially for the type 2 lesions presented in Chapter 3. In addition to the 4 samples where potential inclusions were visualised on H and E, another 6 samples were screened with the pan-pox PCR assays.

Despite multiple attempts to confirm the presence of pox viruses in a selection of samples, there were only faint broad, non-specific bands with the high GC pox virus PCR assay and the products were unable to be sequenced using Sanger sequencing these samples were interpreted as negative.

Many of the animals affected by ITN lick the affected teats. This could allow for agents that are commensals in the mouth and pharynx to infect the teats. In addition to Bovine Papular Stomatitis virus, DD associated treponemes have been detected in gingival tissue (Evans et al., 2012) which may have been a host reservoir for the DD associated treponemes detected in ITN cases. However, further studies including swabbing ITN affected cows' mouths and comparing with the ITN lesion results are required to confirm or exclude this. Cross suckling can be a common problem in dairy herds, particularly in calves and heifers. This is where an animal will suck another animal's body parts or teats and self-suckling is an animal suckling its own teats. This can be a sign of poor welfare and has been implicated with health problems, including mastitis and teat deformities in cows (Mahmoud et al., 2016). While cross or self-suckling may cause teat deformities alone, the mouths of suckling animals could harbour and potentially act as a reservoir for aetiological agents for ITN.

4.4.4 The assessment of DD associated treponemes in milk

Another potential reservoir for aetiological agents and more specifically DD associated treponemes is milk. Given that detection of DD associated treponeme phylogroups via PCR assays are present in a proportion of ITN and UCD cases it was timely to see if the foremilk and milk could act as a potential reservoir. In fact, DD associated treponemes have been detected in foremilk from cows with UCD lesions (Sobhy et al., 2020). As the teat canal seals after milking, the teat canal could potentially lead to an anaerobic site suitable for fastidious bacteria such as treponemes to proliferate. However, for treponemes to be able to proliferate in milk they must be able to survive in the substance. The findings presented in this chapter demonstrate that the treponemes spiked in milk and incubated at different temperatures (one to represent body temperature at 37°C and another to represent refrigeration at 4°C) could not

survive to be passaged and cultured for 6 weeks. As treponeme bacteria are slow growing, cultures were left for six weeks to provide ample opportunity to detect growth. Another interesting finding of this chapter was when investigating for treponeme growth in milk, there was no difference in treponeme growth when adding a previously documented antimicrobial cocktail to the liquid culture broth (Clegg et al., 2016b) and there was no difference in the high numbers of contaminating bacteria in both cultures (with and without antimicrobials), thus the survival experiments were devised without the antibiotic cocktail for the assessment of treponemes in foremilk. Despite not being able to detect live spirochaetal bacteria via phase contrast microscopy at weekly intervals during the milk survival experiments, the treponeme bacteria can be readily detected via PCR assays up to 2 hours after inoculating the bacteria into milk. Whilst this demonstrates the presence of non-degraded treponemal DNA rather than live bacteria it could, while speculative, potentially in theory allow for infection to a calf or another human. However, further studies are required to determine this. In reality, milk will be stored in the bulk milk tank and transported and processed more than two hours before there is any potential for the public ingesting milk and the treponeme bacteria are likely to have degraded enough to not cause a risk for human ingestion. There may however be a risk to the dairy farmers who obtain their milk direct from the milk tank if less than 2 hours of milking. This chapter's data also suggests that the possibility of DD associated treponeme phylogroups being detected in foremilk from cows with DD is unlikely and therefore from these data, milk and foremilk do not seem to pose as a further important viable infection reservoir for DD associated treponeme bacteria.

4.4.5 Conclusions

From the data presented in this chapter it seems unlikely that DD associated treponemes are the primary infectious agent involved in the development of ITN lesions. They are probably contaminants or secondary invaders and therefore less important for disease control. It was not possible to detected pox virus associated with ITN lesions ruling this potential infectious agent out. DD associated treponemes do not seem to be able to survive for longer than 2 hours in milk and foremilk samples when incubated at 37°C or 4°C. Whilst it was not possible to detect DD treponemes within cattle milk, we did detect them on 10% of teat liners implicating a potentially novel DD treponeme fomite that is quickly moved between animals and warrants future studies for DD transmission implications.

Chapter 5: Next generation sequencing approaches to investigate the aetiology of ITN

The shotgun metagenomic bioinformatic analysis presented in this chapter was performed by Dr. Matthew Gemmell, Centre for Genomic Research, University of Liverpool using a pipeline developed at CGR by Dr. Richard Gregory.

5.1 Introduction

Bovine ischaemic teat necrosis (ITN) is currently of unknown aetiology. An initial hypothesis was that ITN was a disease associated with Digital Dermatitis (DD) associated treponemes, as has been reported for several other ruminant skin diseases (Clegg et al., 2016a, 2016c). This initial suspicion was due to a pilot study with 11 out of 12 samples positive to DD treponemes on PCR (Clegg et al., 2016b). When a larger panel of diseased tissues where investigated the findings from Chapter 4 suggest that while in some samples it is possible to detect these bacteria, for the majority this is not the case. Therefore, it is unlikely that treponemes are the key aetiology and may be a secondary or opportunistic pathogen in a small proportion of animals. As such, further investigations into other potential aetiologies are required. Recently, increasing numbers of studies are utilising next generation sequencing (NGS), often using Illumina technology (Illumina Inc., San Diego, CA, USA), to implicate new or potential infective aetiologies. Such studies include investigations into DD in dairy cattle (Krull et al., 2014), Contagious Ovine Digital Dermatitis (CODD) in sheep (Duncan et al., 2021) and Udder Cleft Dermatitis (UCD) in dairy cattle (Ekman et al., 2020), which have been previously detected DD associated treponemes in tissue lesions for these diseases. Illumina technologies (Illumina Inc., San Diego, CA, USA) utilises sequencing by synthesis allowing for base by base sequencing of short reads to be used for many applications such as shotgun metagenomics, which sequences all DNA present in the sample including host, bacteria, fungi and viruses, (Ilumina, 2022). These new technologies are allowing for a greater understanding of the complexity of microbial diseases (Gwinn et al., 2019). Many diseases that were once considered caused by a single aetiological agent, when investigated using traditional culture-

based techniques and the narrow, time-consuming process of multiple PCR techniques, are being found to appear more complicated with new deep and broad sequencing techniques. These technologies also remove the requirement of identifying a suspected aetiological agent and designing primers to target this agent by PCR assays (Gwinn et al., 2019). NGS allows for samples to be sequenced without the bias imposed by culture techniques and PCR assays and has been used to investigate the microbiome of the skin (Ekman et al., 2018). One of the major constraints of NGS with Ilumina technologies is the cost and the prolonged, complex method for analysing the sequencing data. However, these time constraints are often outweighed by the high specificity and sensitivity outputs. As the aetiology of ITN is currently unknown and the results from Chapter 3 suggested there may have been an initial viral involvement, a shotgun metagenomic approach was considered appropriate. This methodology has the added advantage of the ability to identify the taxa present, provide an estimation of organism abundance and also look into the presences of pathogenetic pathways and potential antimicrobial resistance (AMR) genes.

As well as Illumina, there are now more devices for NGS that are increasingly available, portable and allow for more rapid analysis. In recent times, a new technology that allows for such portable sequencing and rapid analysis has become more available to the research community and vastly decreased in cost, is the Oxford Nanopore Technology (ONT). Briefly, ONT utilises a nanopore, and sequences with base-calling as the DNA strand passes through the pore that detects a variation in electrical current (Oxford Nanopore Technologies, 2008). This allows for rapid alignment and can even allow for enrichment or depletion of a targeted genome in real time. The disadvantage of this technology is that it currently is not as specific and sensitive as the Illumina sequencing and initially in its development was not as suitable for high throughput (Gwinn et al., 2019). However, in the last few years the ONT has made improvement in these areas and is now considered as a useful tool for most laboratories

(Kerkhof, 2021). Additionally, studies are now being published that demonstrate the versatility and the usefulness of this technology in investigating the skin microbiome in veterinary species (Cuscó et al., 2017, 2019).

As it is currently unknown which aetiological agents are involved in ITN it is not possible to give a targeted and evidence-based disease treatment or control methods. Treatments currently are non-specific and often ineffective. In addition, understanding the potential aetiologies can allow farmers to implement potential biosecurity protocols to aid in preventing ITN and stop the spread to other animals and/or to other teats on the same animal. For instance, this may include fly control if it is thought to be transmitted by vectors or isolation of an effected animal if deemed infectious. Therefore, the aim of this study was to explore potential infective aetiologies that could be targets for further investigations.

5.2 Materials and Methods

5.2.1 Ethics statement

Ethical approval for this study was granted by the University of Liverpool, School of Veterinary Science Ethical Committee (application number: VREC 460). The participants provided their written informed consent to participate in this study and allowed for samples to be retained and used for research purposes.

5.2.2 Sample collection

Tissue samples were obtained as described in chapter *4.2.3.1 Tissue samples*. For the Illumina shotgun metagenomic study 10 ITN teats and 10 healthy teats were used. One healthy teat was a non-diseased teat from an ITN positive cow to see if there was a difference in microbiome

between the ITN teats and non-ITN teats on an affected animal. As there was a time delay between the two experiments and to avoid the risk of potential degradation from prolonged freezing of samples, 10 additional ITN and 10 additional healthy teats whose microbiomes were investigated, were used for the experiments using the MinION (Oxford Nanopore, Oxford, UK).

5.2.3 Shotgun metagenomic study using Illumina sequencing

5.2.3.1 DNA extraction

For the Illumina sequencing all tissue samples were processed as described in section 4.2.4.1 "Extraction from swabs and tissue of ITN lesions". This included the use of the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) as per manufacturer instructions with an additional step included after tissue lysis to remove any RNA that may interfere with the shotgun metagenomic sequencing and analysis. Twenty µl of RNase A (100mg/ml) was added to the lysed sample and incubated for 3 minutes prior to addition of the AL buffer step. The DNA extraction was then continued as normal.

5.2.3.2 Assessment of DNA concentration post extraction

All samples to be submitted for Illumina sequencing had the concentration and quality of the DNA assessed in two ways. The first used a NanoDrop™ (Thermo Scientific™, Waltham, MA, USA). The second used a Qubit fluorometer (Thermo Scientific™, Waltham, MA, USA) as per manufacturer guidelines.

5.2.3.3 Illumina sequencing

Samples were submitted on ice to the Centre for Genomic Research (CGR) at the University of Liverpool for Illumina sequencing on a NovaSeq^{TM} 6000 (Illumina Inc., San Diego, CA, USA). Bioformatic analysis was performed by Dr. Matthew Gemmell, CGR.

5.2.3.4 Initial processing and quality assessment of the sequenced data

Quality assessment and initial processing of the sequence data was performed using a pipeline developed at CGR by Dr. Richard Gregory. Briefly, base-calling and de-multiplexing of indexed reads was performed by CASAVA version 1.8.2 (Illumina) to produce 10 samples sequence files in FASTQ format. The raw FASTQ files were trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011). The option "-O 3" was set, so the 3' end of any reads which matched the adapter sequence over at least 3 bp was trimmed off. The reads were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 20 bp were removed. If both reads from a pair passed this filter, each was included in the R1 (forward reads) or R2 (reverse reads). If only one of a read pair passed this filter, it was included in the R0 (unpaired reads).

5.2.3.5 Host removal from reads

Trimmed reads were processed to remove any host reads. Trimmed paired reads were aligned to the *Bos Taurus* representative genome, ARS-UCD1.2, with bowtie2 (Langmead and Salzberg, 2012). Read pairs where one or both reads aligned to the human reference were removed from further analysis.

5.2.3.6 Taxonomic classification of trimmed reads and abundance estimation of species

Prior to taxonomic classification a Kraken 2 custom database was created (Wood et al. 2019). The bacterial and viral Kraken libraries were added to the custom database. Fusobacterium genomes from the National Center for Biotechnology Information (NCBI) were added; the assembly accessions of these are GCA_004006635.1, GCA_003812825.1, GCA_003732525.1, GCA_003019715.1, and GCA_003732505.1. All available Treponeme complete genomes from NCBI Reference Sequence Database (RefSeq)(at 21st November 2019) were added; the RefSeq assembly accessions of these are GCF_000008185.1, GCF_000008605.1, GCF_000195275.1, GCF_000212415.1, GCF_000214355.1, GCF_000214375.1, GCF_000217655.1, GCF_000219725.1, GCF_000447675.1, GCF_000604125.1, GCF_000755145.1, GCF_000775995.1, GCF_005885795.1, GCF_008152505.1, GCF_008152825.1, GCF_008153055.1, GCF_008153205.1 and GCF_008153345.1. Six Treponema genomes which were previously compiled from our laboratory were added and named MEDIUM, PEDIS, REITER, RU1, T19 and T320A. Complete viral genomes for Pseudocowpox, Bovine popular stomatitis virus, Cowpox, Vaccinia virus, Orf virus, Buffalopox virus, Parapox virus, Bovine Herpesvirus 4, and Bovine Herpesvirus 2 from NCBI were added (21st November 2019); the assembly accessions of these were NC_013804, NC_005337, NC_003663, NC_006998, NC_005336, MG599038, NC_025963, AF318573, and AY357736.

Paired reads were classified with Kraken 2 using the custom Kraken 2 database (Wood and Salzberg, 2014). Kraken 2 carries out taxonomic classification of short DNA reads by examining k-mers within a read and querying a database with those k-mers. Interactive summary plots of the taxa found via Kraken 2 were created through krona (Ondov et al., 2011).

5.2.3.7 Functional profiling of microbial communities

The presence/absence and abundance of microbial pathways was profiled for each sample using HUMAnN2 (Franzosa et al., 2018). HUMAnN2 does not take account for the paired relationship of paired reads. Therefore, the R1 and R2 sequences were combined into the same FASTQ file with HUMAnN2 treating the R1 and R2 from the same sample as two distinct reads.

5.2.3.8 Biomarker detection

Biomarker detection of the taxa abundances created by Bracken and the microbial pathway abundances created by HUMAnN2 was carried out with LefSe (Segata et al., 2011). Prior to LefSe per-sample normalisation of the sum of the values to 1 million was carried out on Bracken and HUMAnN2 results.

5.2.3.9 Resistance gene identification

Prior to resistance gene identification, host removed paired reads were assembled into contigs with MEGAHIT (Li et al., 2015). This was carried out with the k-mer sizes 29, 49, 69, 89, 109, 129, 149, 169, and 189. Contiguity of assemblies was assessed with QUality ASsement Tool (QUAST) (Gurevich et al., 2013).

Resistance gene identification of the MEGAHIT produced contigs was carried out with the Resistance Gene Identifier (RGI) using the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2019). Perfect, Strict, and Loose hits were included in the output. A heatmap of the strict and perfect hits to CARD wad produced with the command “rgi heatmap”.

RGI utilises three algorithms which produces three different types of hits: the Perfect algorithm, the Strict algorithm, and the Loose algorithm. The Perfect algorithm detects perfect matches to the reference sequences and mutations listed in CARD and is often used for clinical surveillance. The Strict algorithm allows for detection of unknown variants of known AMR genes and the Loose algorithm allows for detection of new and emerging threats to AMR and works out side of the model cut-off areas. As such the Loose algorithm will also detect sequences that may not lead to AMR.

5.2.4 Microbiome 16S amplicons investigations using Oxford Nanopore Technology (ONT) MinION

5.2.4.1 DNA Extraction for MinION

Ten more recent cases of ITN and ten new non-diseased teats were used for the investigations of the microbiomes with ONT MinION. For this study, both the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) (see section 5.2.2.1) and the QIAamp DNA Microbiome kit (Qiagen, Manchester, UK) were used following manufacturer guidelines. Briefly, the microbiome kit utilises both enzymatic and beating steps to reduce large proportions of host DNA and to allow for maximum bacterial lysis for exposure of the microbiome DNA.

5.2.4.2 Assessing quality of the extracted DNA

DNA quantity and quality were assessed the same as in section 5.2.3.2.

5.2.4.3 Library preparation of rapid sequencing of amplicons

To attempt to reduce the issues around small yields of bacterial DNA and relatively large yields of host DNA from tissue samples, a targeted approach to amplify the 16S rRNA gene amplicon area of the genome was implemented here. This was carried out twice: 1) DNA extractions obtained from both the DNeasy Blood and Tissue Kit and 2) for the DNA extractions obtained from the QIAamp DNA Microbiome kit. Briefly, the ONT Rapid Sequencing of amplicons 16S barcoding kit (SQK-16S024, ONT, Oxford, UK) was used as per manufacturer instructions to prepare the library for sequencing on a MinION Mk1C (ONT, Oxford, UK). The library preparation included a PCR step to amplify the 16S rRNA region using the primers included in the kit. Samples were multiplexed with the barcodes included in the kit to reduce the costs per sample. A purification step using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) and a magnet was included. Molecular grade water was used as a negative control and ZymoBIOTICS Mock community standards (Zymo Research, Irvine, CA, USA) were used as a positive control.

The prepared library was loaded on to the primed flow cell (model R9.4.1, FLO-MIN106, ONT, Oxford, UK) and 24-hour sequencing including fast basecalling, minimum score 8, and alignment active initiated using the MinKNOW^{TM} software (ONT, Oxford, UK) built into the MinION Mk1c (ONT, Oxford, UK).

5.2.4.4 Bioinformatic analysis of rapid sequencing of amplicons

After sequencing, the output files were transferred from the MinION mk1c and uploaded in to EPI2ME (a cloud-based platform, ONT, Oxford, UK) for simple, rapid and real-time analysis of the FASTQ files. The standard pipeline for Fastq 16S analysis for the Rapid sequencing of amplicons 16S barcoding kit (SQK-16S024, ONT, Oxford, UK) was used.

5.3 Results

5.3.1 Results of the Shotgun Metagenomics from the Illumina sequencing

5.3.1.1 Quality control for Illumina sequencing

Samples M1-M10 were from teats with ITN lesions and samples M11-M20 were teats without ITN lesions and were otherwise healthy in macroscopic appearance (Table 5.3.1.1). Prior to submitting to CGR for Illumina sequencing, all extracted DNA from samples M1-M20 were loaded on to the NanoDrop™ (Thermo Scientific™, Waltham, MA, USA) for assessment of concentration and quality (Table 5.3.1.2).

Table 5.3.1.1 Sample description table with the clinical grade of ITN present on the teat and the cow it came from selected for Illumnia shotgun metagenomic sequencing.

Sample	Animal	Clinical grade
M1	Cow 1	Grade 3
M2	Cow 2	Grade 3
M3	Cow 2	Grade 3
M4	Cow 3	Grade 1
M5	Cow 3	Grade 1
M6	Cow 4	Grade 1
M7	Cow 5	Grade 2
M8	Cow 6	Grade 1
M9	Cow 7	Grade 2
M10	Cow 8	Grade 2
M11	Cow 2	Control
M12	Cow 9	Control
M13	Cow 10	Control
M14	Cow 11	Control
M15	Cow 12	Control
M16	Cow 13	Control
M17	Cow 14	Control
M18	Cow 15	Control
M19	Cow 16	Control
M20	Cow 17	Control

Table 5.3.1.2 NanoDrop™ results for DNA extractions before Illumina sequencing

Sample	Concentration ng/µl	A260	A280	260/280	260/230
M1	32.9	0.659	0.408	1.62	0.49
M2	50.8	1.017	0.659	1.54	0.71
M3	616.8	12.336	13.190	0.94	0.26
M4	45.4	0.905	0.632	1.44	0.37
M5	54.9	1.098	0.615	1.79	0.67
M6	50.4	1.009	0.537	1.88	1.18
M7	180.8	3.615	3.587	1.01	0.24
M8	43.0	0.860	0.476	1.81	1.33
M9	33.9	0.678	0.628	1.08	0.17
M10	30.4	0.607	0.376	1.62	0.47
M11	37.9	0.759	0.403	1.88	1.74
M12	18.7	0.374	0.202	1.86	0.60
M13	12.6	0.252	0.157	1.61	0.40
M14	17.3	0.345	0.219	1.57	0.26
M15	32.1	0.645	0.341	1.88	0.87
M16	26.6	0.531	0.293	1.81	0.87
M17	25.3	0.507	0.329	1.54	0.32
M18	79.3	1.586	0.844	1.88	1.72
M19	55.9	1.118	0.608	1.84	1.23
M20	50.3	1.005	0.555	1.81	0.83

The sequencing data was uploaded on to the Laboratory Information Management System (LIMS) under project number LIMS120325. The total number of reads and the numbers of paired and single reads were assessed prior to downstream analysis and the quality of the sequencing data summarised in Figures 5.3.1.1 and 5.3.1.2 and Table 5.3.1.3.

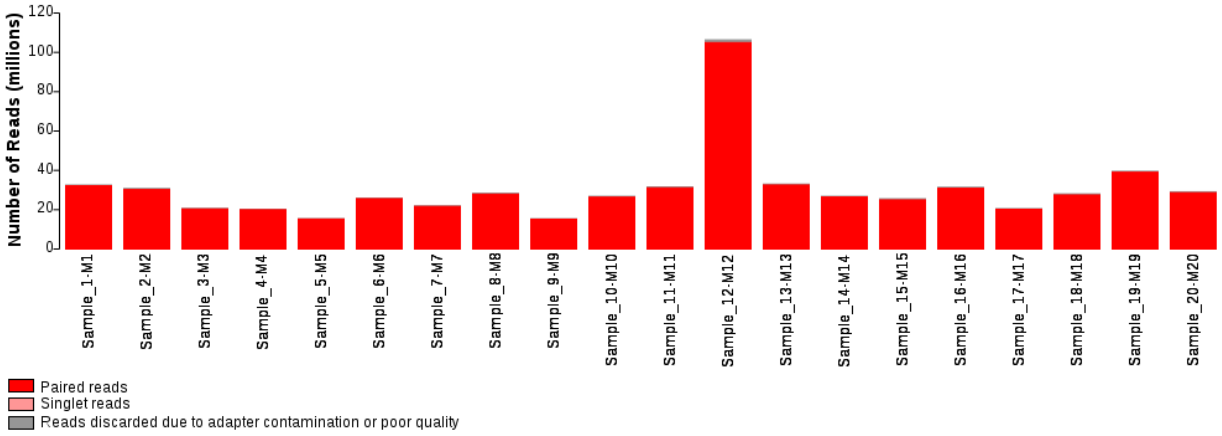


Fig. 5.3.1.1 Diagram illustrating the total number of reads obtained for each sample

Table 5.3.1.3 Summary of raw and trimmed sequence data for reads. The table summarises the read counts before and after adapter and quality trimming.

Sample	Barcode Sequence	Number Raw reads	Trimmed Read Number (% of raw)	Trimmed Read Pairs (R1/R2) Number	Singletons (R0) Number (% of total trimmed reads)
1-M1	CCGCGGTT-CTAGCGCT	32,371,726	31,992,304 (98.83)	15,996,152	178,532 (0.55)
2-M2	TTATAACC-TCGATATC	30,632,276	30,071,710 (98.17)	15,035,855	273,370 (0.90)
3-M3	GGACTTGG-CGTCTGCG	20,524,322	20,277,190 (98.8)	10,138,595	122,685 (0.60)
4-M4	AAGTCCAA-TACTCATA	20,119,158	19,862,506 (98.72)	9,931,253	125,700 (0.63)
5-M5	ATCCACTG-ACGCACCT	15,389,404	15,166,076 (98.55)	7,583,038	108,698 (0.71)
6-M6	GCTTGTCA-GTATGTTC	25,723,292	25,452,526 (98.95)	12,726,263	129,656 (0.51)
7-M7	CAAGCTAG-CGCTATGT	21,813,072	21,578,690 (98.93)	10,789,345	111,165 (0.51)
8-M8	TGGATCGA-TATCGCAC	28,170,194	27,781,260 (98.62)	13,890,630	189,787 (0.68)
9-M9	AGTTCAGG-TCTGTTGG	15,312,506	15,076,354 (98.46)	7,538,177	116,252 (0.77)
10-M10	GACCTGAA-CTCACCAA	26,694,416	26,225,332 (98.24)	13,112,666	230,198 (0.87)
11-M11	TCTCTACT-GAACCGCG	31,407,076	30,944,004 (98.53)	15,472,002	226,241 (0.73)
12-M12	CTCTCGTC-AGGTTATA	105,805,552	104,414,222 (98.69)	52,207,111	676,629 (0.64)
13-M13	CCAAGTCT-TCATCCTT	32,834,850	32,393,702 (98.66)	16,196,851	206,365 (0.63)
14-M14	TTGGACTC-CTGCTTCC	26,691,550	26,301,414 (98.54)	13,150,707	190,848 (0.72)
15-M15	GGCTTAAG-GGTCACGA	25,410,790	24,845,692 (97.78)	12,422,846	241,174 (0.96)
16-M16	AATCCGGA-AACTGTAG	31,233,810	30,720,928 (98.36)	15,360,464	247,126 (0.80)
17-M17	TAATACAG-GTGAATAT	20,424,050	20,089,772 (98.36)	10,044,886	163,855 (0.81)
18-M18	CGGCGTGA-ACAGGCGC	27,897,654	27,342,940 (98.01)	13,671,470	275,76 (1.00)
19-M19	ATGTAAGT-CATAGAGT	39,321,668	38,843,188 (98.78)	19,421,594	236,131 (0.60)
20-M20	GCACGGAC-TGCGAGAC	28,953,840	28,493,470 (98.41)	14,246,735	226,753 (0.79)

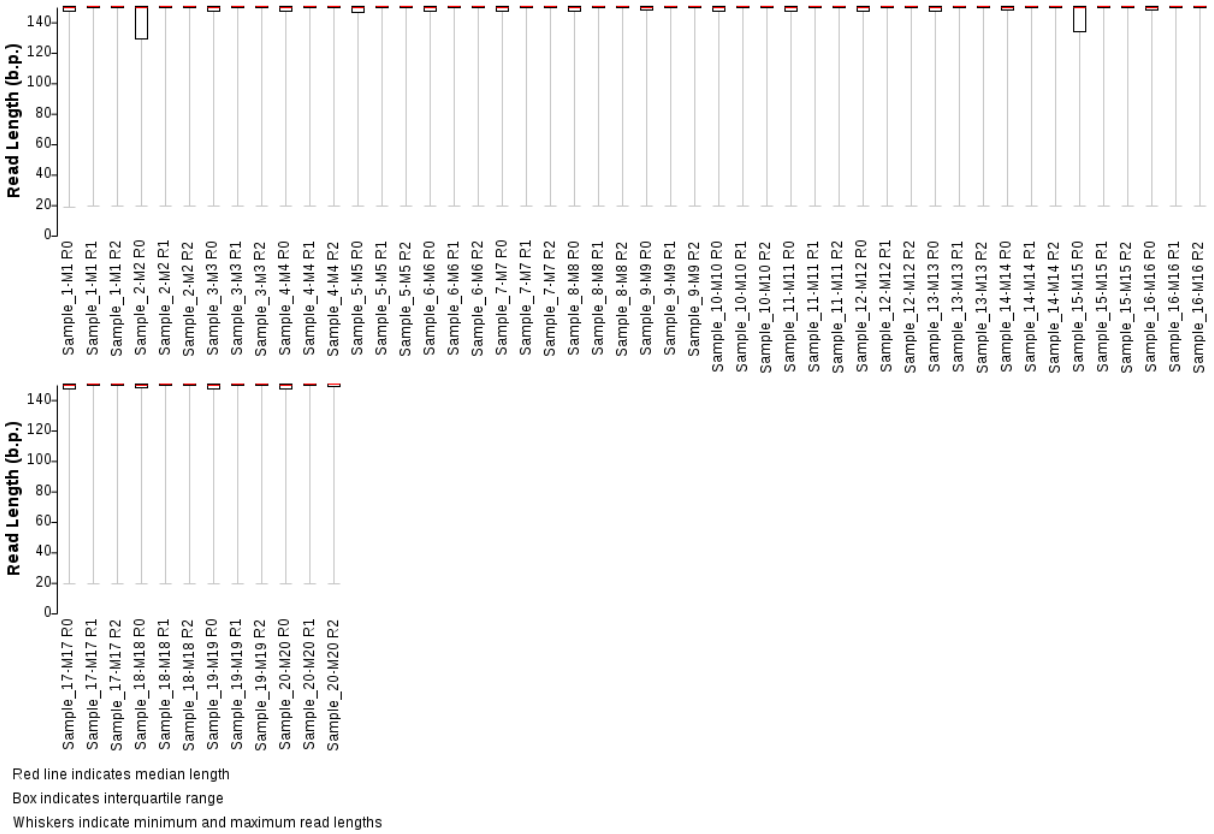


Figure 5.3.1.2 Read length distributions for all samples after adapter and quality trimming of post Illumina DNA sequencing.

Figure5.3.1.2 shows the read length distributions after adapter and quality trimming. Note that R0 (unpaired) reads are trimmed more than paired reads as they more often represent poor quality sequences. The proportion of trimmed reads that are unpaired after trimming was generally low (<1%), indicating that the data are of good quality trimming.

5.3.1.2 Host removal from reads

Host removal from reads led to a low number of retained reads, thus indicating that a high proportion of the reads were associated with host DNA as was to be expected due to the nature of the tissue samples (Table 5.3.1.4).

Table 5.3.1.4 Summary of number host removal on trimmed reads.

Sample	Trimmed Read Pairs (R1/R2) Number	Retained Read Pairs (R1/R2) Number (% of Trimmed read pairs)
1-M1	15,996,152	63,132 (0.395)
2-M2	15,035,855	441,368 (2.935)
3-M3	10,138,595	534,417 (5.271)
4-M4	9,931,253	26,855 (0.270)
5-M5	7,583,038	18,353 (0.242)
6-M6	12,726,263	97,822 (0.769)
7-M7	10,789,345	23,557 (0.218)
8-M8	13,890,630	38,765 (0.279)
9-M9	7,538,177	32,226 (0.428)
10-M10	13,890,630	38,765 (0.279)
11-M11	15,472,002	66,662 (0.431)
12-M12	52,207,111	134,541 (0.258)
13-M13	16,196,851	46,325 (0.286)
14-M14	13,150,707	37,666 (0.286)
15-M15	12,422,846	28,690 (0.231)
16-M16	15,360,464	36,126 (0.235)
17-M17	10,044,886	22,944 (0.228)
18-M18	13,671,470	36,986 (0.271)
19-M19	19,421,594	61,350 (0.316)
20-M20	14,246,735	39,744 (0.279)

5.3.1.3 Taxonomic classification of trimmed reads and abundance estimation of species

Taxonomic classification of short reads DNA was performed using Kraken 2 and investigations into abundance using Bracken. A summary of the numbers of classified reads via Kraken is provided (Table 5.3.1.5).

Table 5.3.1.5 A summary of the number of classified trimmed, host removed, stitched reads found via Kraken 2.

Sample	Host Removed Stitched Read Pairs	Classified Reads (% of Host Removed Stitched Read Pairs)
1-M1	63,132	15,229 (24.12)
2-M2	441,368	193,184 (43.77)
3-M3	534,417	263,378 (49.28)
4-M4	26,855	2,938 (10.94)
5-M5	18,353	790 (4.30)
6-M6	97,822	62,393 (63.78)
7-M7	23,557	1,041 (4.42)
8-M8	38,765	2,183 (5.63)
9-M9	32,226	5,561 (17.26)
10-M10	37,022	1,503 (4.06)
11-M11	66,662	13,126 (19.69)
12-M12	134,541	7,440 (5.53)
13-M13	46,325	4,723 (10.20)
14-M14	37,666	4,305 (11.43)
15-M15	28,690	1,322 (4.61)
16-M16	36,126	1,866 (5.17)
17-M17	22,944	1,387 (6.05)
18-M18	36,986	1,939 (14.87)
19-M19	61,350	9,123 (14.87)
20-M20	39,744	4,656 (11.71)

All samples submitted for shotgun metagenomics (M1-M20) generated DNA sequences from *Staphylococcus aureus* and *Bacillus cereus*. All apart from one healthy sample yielded *Bacteriovorax stolpii* and all apart from one diseased sample had *Turniella parva*. All apart from one diseased sample identified *Salinivirga cyanobacteriivorans*. All healthy samples and 6 diseased samples found *Rhizobium leguminosarum*. *Acinetobacter lwoffii* was present in 7 diseased and 8 healthy samples.

Organisms only present in healthy samples were: *Dietzia sp*. oral taxon 368, *Bradyrhizobium paxllaeri*, *Bradyrhizobium sp*.SK17, *Nocardioides sp*. SB3-45, *Paludisphaera borealis*, *Luteitalea pratensis*, *Rhodoplanes sp*. Z2-YC6860, *Aeromonas schubertii*, *Bradyrhizobium*

icense, *Planctomycetes bacterium EIP*, *Nocardioides sp*. MMS17-SY207-3, and *Pseudolabrys taiwanesis*.

Tissue from a diseased cow with two ITN teats (M2 and M3) and a non-diseased teat (M11) was used to investigate if the microbiome population could potentially differ between affected and non-affected teats on the same animal. While M11 (non-lesioned teat) yielded more retained reads than the other control teats, this sample also had a higher number of reads than five of the ITN teats and was substantially less reads than M2 and M3 by 342,946 and 484,572 respectively. M11 included a proportion of the same bacteria as observed in the ITN teats but in far less numbers. For example, M2, M3 and M11 all had large numbers of *Fusobacterium* sp. detected but M11 levels were 5.1% of the levels of M2 and 4.7% of the levels of M3.

Interactive summary plots of the taxa found via Kraken 2 of all samples were created through Krona (Ondov et al., 2011). The link to the Kraken interactive Krona output of each samples M1-M20 is [Krona - Bacteria (liv.ac.uk) which demonstrates the microorganisms identified in each sample](). An example of the Krona output is provided for M10 (Fig 5.3.1.3).

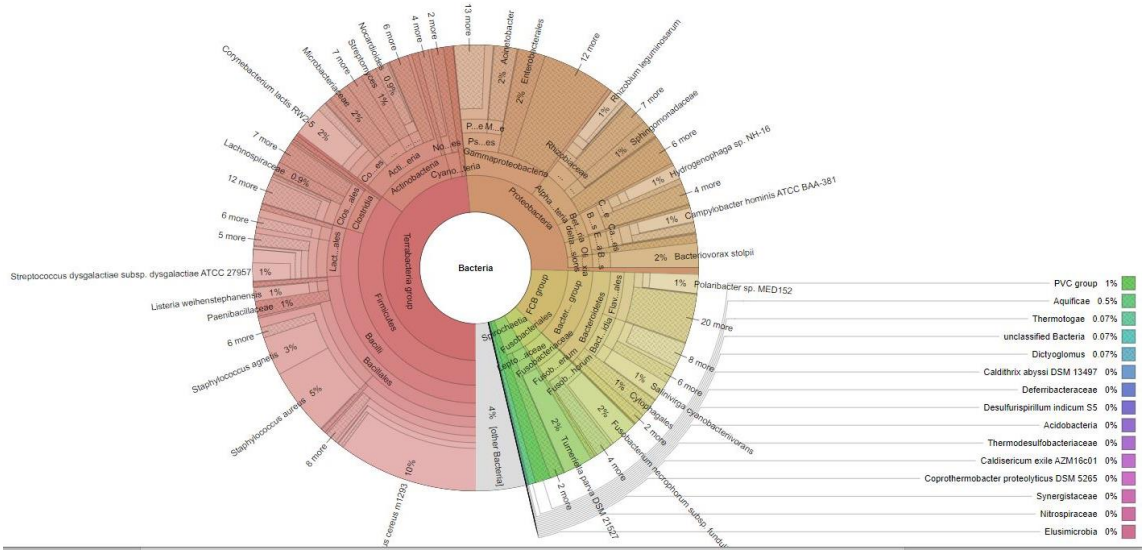


Figure 5.3.1.3 Krona output created via Kraken 2 with the bacteria identified in the sample M10 comprising of 4% of the root.

The output from Kraken 2 was analysed with Bracken to provide an estimate of the abundance of each microorganism present in each sample. A principal component analysis (PCA) was performed to investigate the differences between ITN lesion types and non-ITN teats (Fig. 5.3.1.4). The highest variability in the abundance of the bacteria at genus and species level was for type 3 ITN lesions. The control teats and types 1 and 2 were closely clustered.

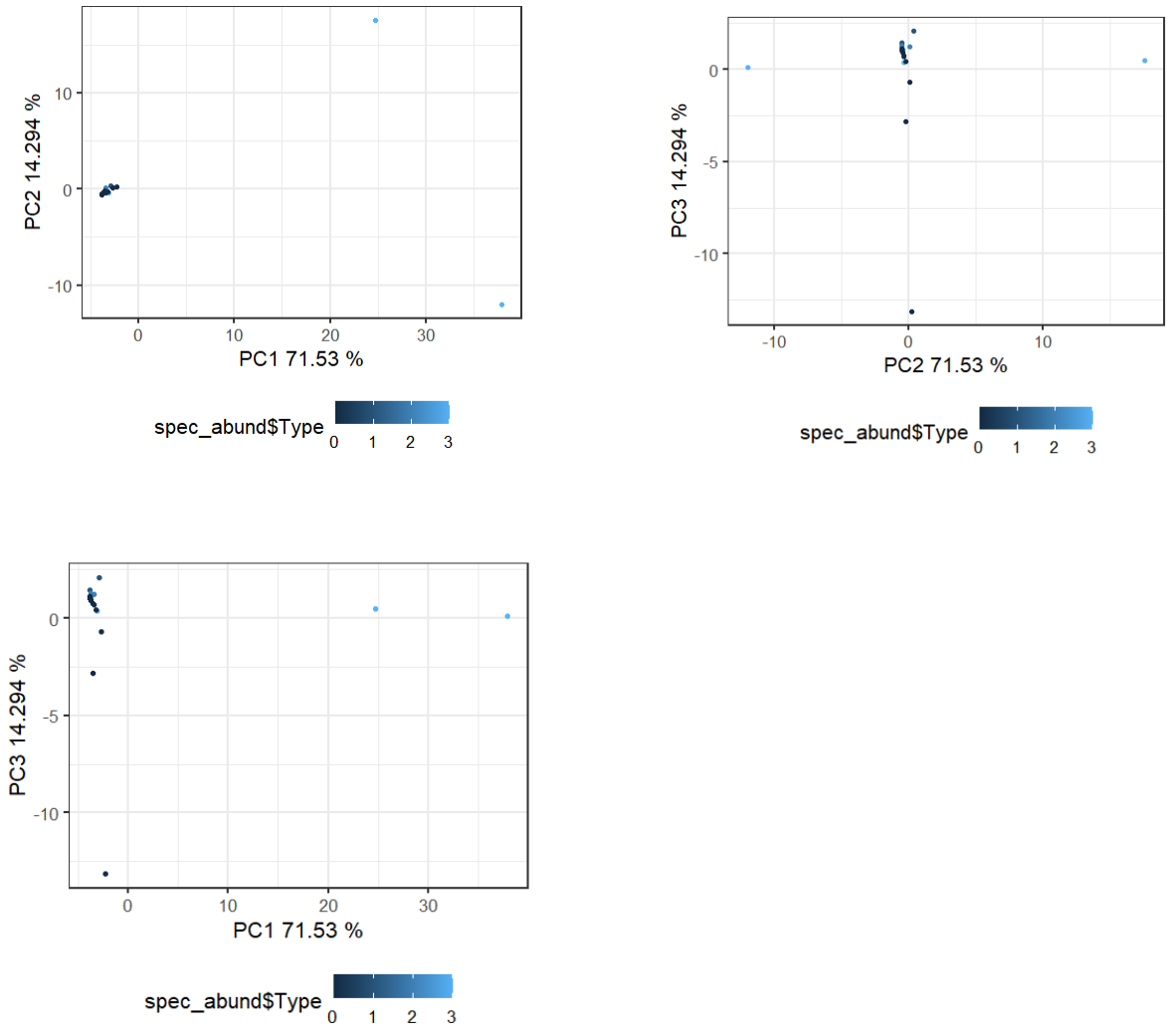


Figure 5.3.1.4. The principal component analysis of the abundance of microorganisms at the levels of genus and species separated by type of ITN where 0 is control, 1 is type 1 lesion, 2 is a type 2 lesion and 3 is a type 3 lesion.

The abundance data at a family level was inputted into a heat map (Fig. 5.3.1.5) to assess similarities or differences in the abundance of microorganisms between diseased and non-

diseased teats. The majority of the diseased samples (excluding M2 and M3) had fewer microorganisms present than the non-diseased teats.

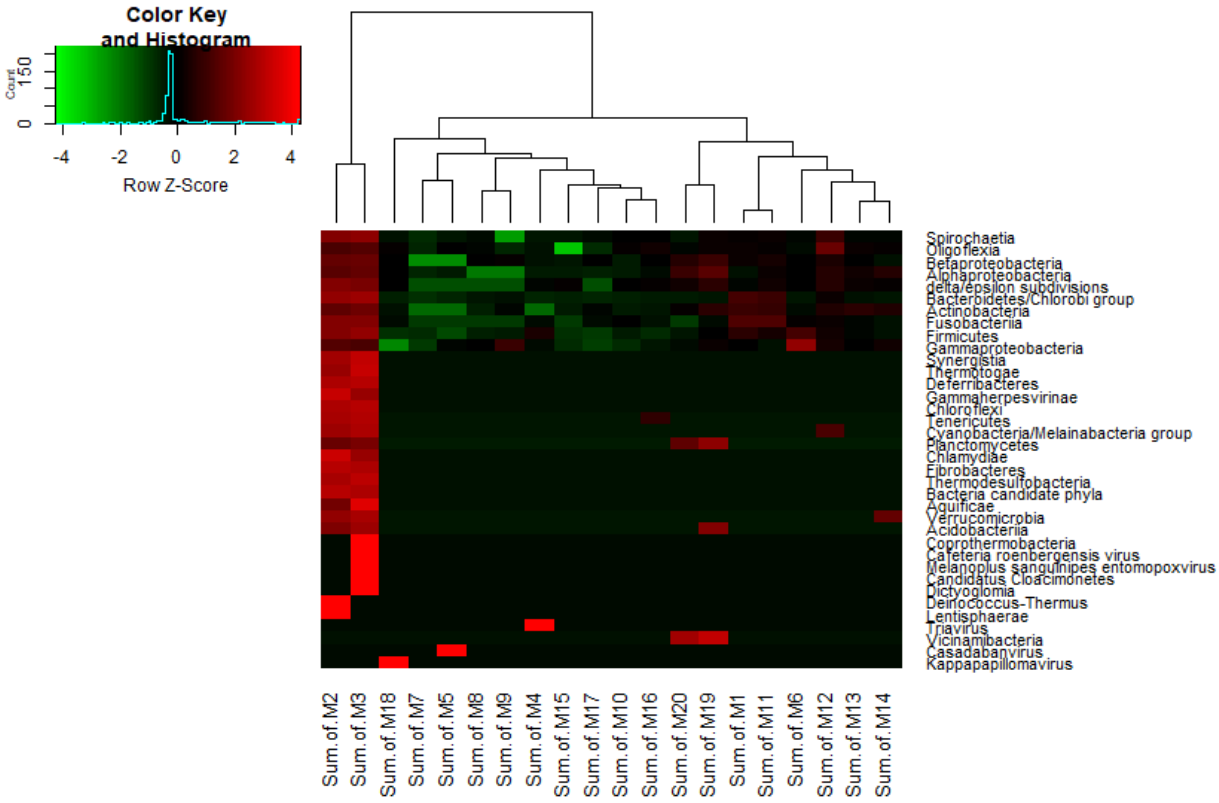


Figure 5.3.1.5 Log transformed family abundance heatmap from Bracken output data. M1-M10 are ITN diseased teats and M11-M20 are healthy control teats. Samples are clustered based on the similarity of the bacterial population present with the bacterial families present. A more intense red demonstrates an increase abundance of the family while green represents a decrease and black shows a similar level in the samples.

After the heat map of the abundance of microorganisms at the family level, the data was converted to a correlation heatmap to assess the similarities and differences between samples (Fig. 5.3.1.6). The control, non-diseased teats were far more similar using the Pearson correlation test than the ITN teats.

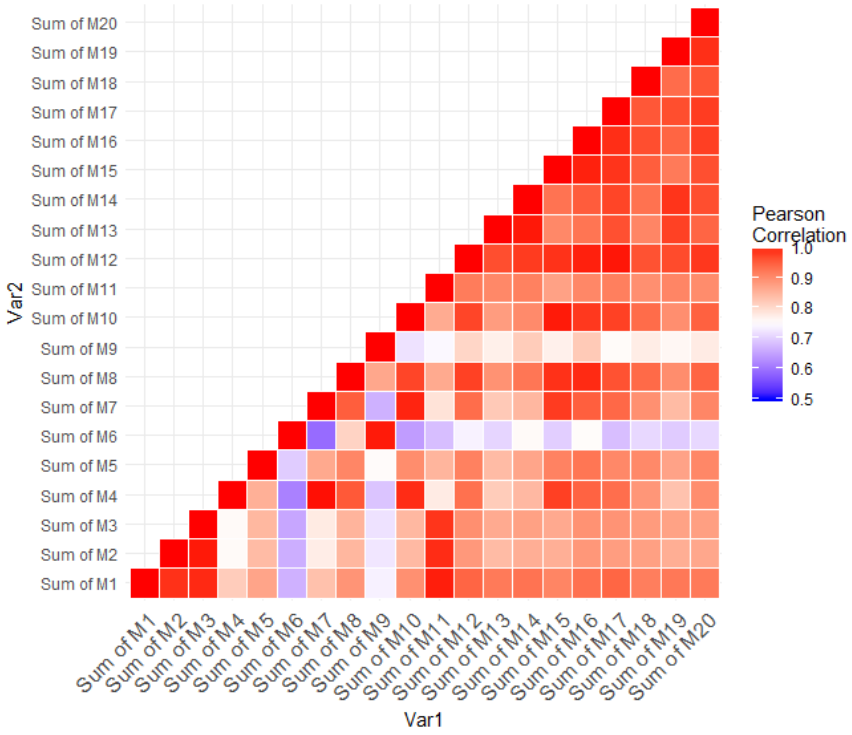


Figure 5.3.1.6 Relative abundance of family half correlation heatmap. Samples M11-M20 are closer to the Pearson correlation score 1.0 and therefore more similar than samples M1-M10, the ITN teats.

HUMAnN2 was used to carry out taxonomic classification as part of the microbial functional profiling. As it uses the data produced from Kraken 2, samples with low classification using Kraken 2 were poorly profiled by HUMAnN2. Two of the pathways HUMAnN2 always produces are “UNMAPPED” and “UNINTEGRATED”, therefore samples with 2 pathways only have these two. Table 5.3.1.6 shows 5 diseased samples with more than 2 pathways and only 1 healthy sample with more than 2 pathways.

Table 5.3.1.6 Summary of the number of pathways discovered for each sample using HUMAnN2.

Sample	Number of Pathways
1-M1	17
2-M2	264
3-M3	311
4-M4	7
5-M5	2
6-M6	370
7-M7	2
8-M8	2
9-M9	2
10-M10	2
11-M11	9
12-M12	2
13-M13	2
14-M14	2
15-M15	2
16-M16	2
17-M17	2
18-M18	2
19-M19	2
20-M20	2

5.3.1.4 Biomarker detection

A small number of biomarkers were detected in the diseased samples which were absent in the healthy samples when analysis was performed with HUMAnN2 (Table 5.3.1.7). For the analysis with Bracken there were more healthy biomarkers detected than diseased.

Table 5.3.1.7 Summary of the number of biomarkers detected for the Bracken and HUMAnN2 outputs.

Comparison	Analysis	Healthy Biomarkers	Diseased Biomarkers
Healthy vs Diseased	Bracken	31	24
Healthy vs Diseased	HUMAnN2	0	7

The bracken biomarkers were analysed by linear discriminant analysis effect size (LefSe) and inputted into a cladogram to map where the diseased ITN teats varied from the control non-lesion teats (Fig. 5.3.1.7). *Pasteurellales* were more abundant in the diseased ITN teats than the control, non-lesion teats.

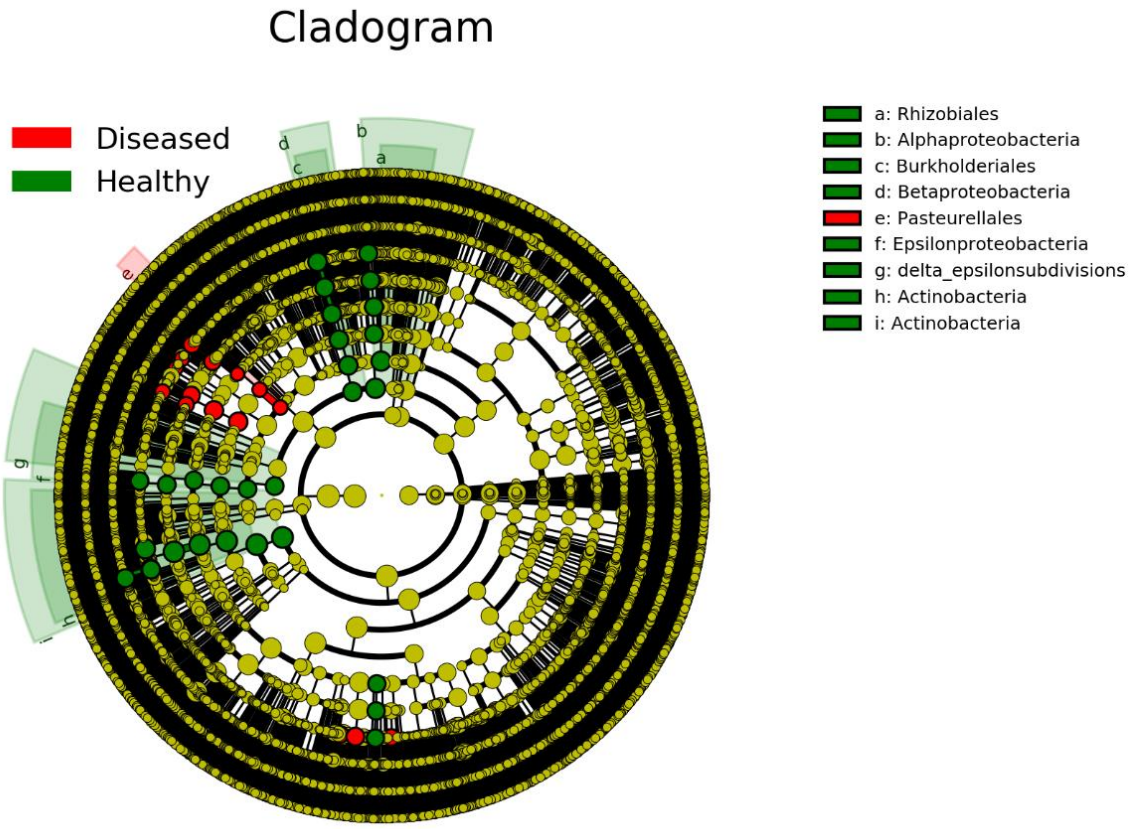


Figure 5.3.1.7 Bracken output cladogram highlighting the taxonomies detected as biomarkers.

Out of 3261 organisms identified on LefSe output, 55 were significant with a P value less than 0.05. Thirty-one of these were healthy, 24 were diseased. The 2 viruses that were significant in diseased samples were bacteriophages (King et al., 2012). The linear discriminant analysis (LDA) values of the taxonomies detected as biomarkers are displayed in Fig. 5.3.1.8.

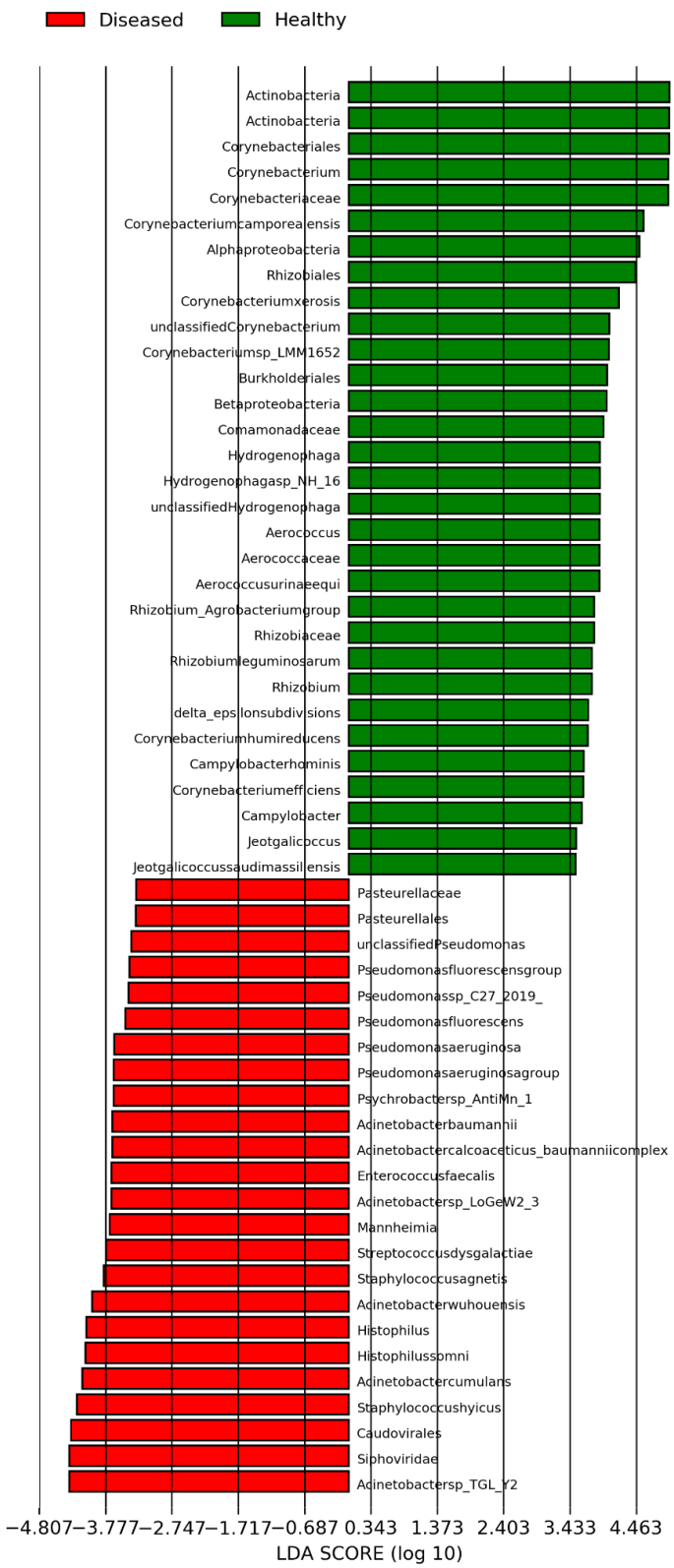


Figure 5.3.1.8 Bar chart showing the LDA values of the taxonomies detected as biomarkers from Bracken data.

There were far fewer, seven, biomarker pathways detected using LefSe on the output from HUMAnN2 analysis and the LDA values are presented (Fig. 5.3.1.9).

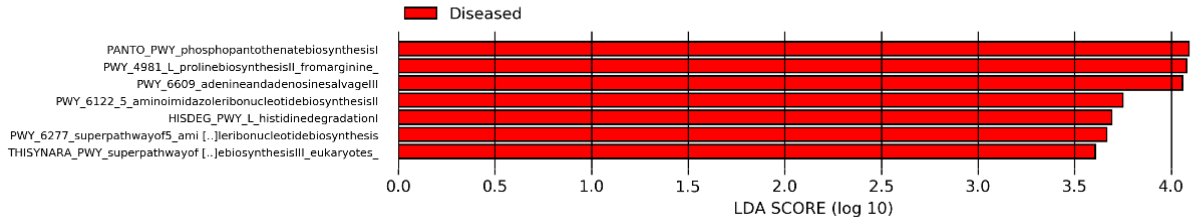


Figure 5.3.1.9 Bar chart showing the LDA values of the pathways detected as biomarkers via HUMAnN2.

5.3.1.5 Resistance gene identification

There were a large number of potential resistance genes identified when looking outside of the model cut-offs (loose hits) for detection of potential new or emerging threats to antimicrobials using the comprehensive antibiotic resistance database (CARD). These were found in teats with and without ITN lesions. When the algorithm applied was limited to the model cut-offs, potential resistance genes were limited to single digits in three ITN teats and one non-ITN teat (Table 5.3.1.8).

Table 5.3.1.8 Number of resistance gene hits for each sample with loose hits included and loose hits excluded.

Sample	Hits to CARD (including loose)	Hits to CARD (excluding loose)
1-M1	863	0
2-M2	25,832	3
3-M3	31,558	6
4-M4	411	0
5-M5	364	0
6-M6	6,084	4
7-M7	389	0
8-M8	573	0
9-M9	312	0
10-M10	637	0
11-M11	643	0
12-M12	1,977	0
13-M13	591	0
14-M14	465	0
15-M15	481	0
16-M16	563	0
17-M17	390	0
18-M18	505	0
19-M19	746	1
20-M20	575	0

Samples M2, M3 and M6 had perfect hits for resistance genes (Fig. 5.3.1.10). Samples M2 and M3 both had *tet*(W/N/W) and *tetQ* resistance genes present with M3 having an additional gene conferring resistance to Pulvomycin. Sample M6 also had the latter resistance gene and also an *Escherichia coli marR* mutant, *fosA6* and *marA* resistance genes.

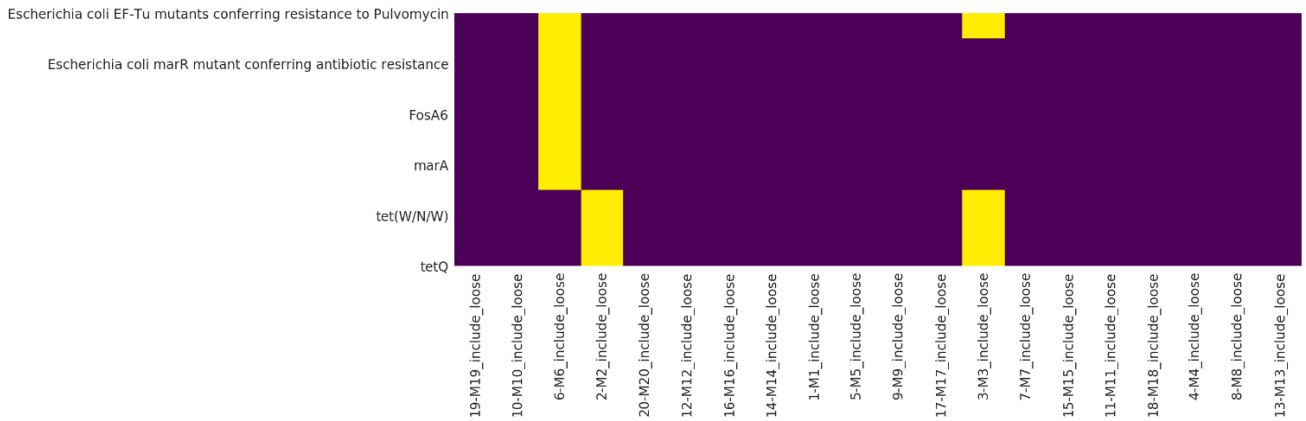


Figure 5.3.1.10 Heatmap of strict and perfect hits for resistance gene identification. Yellow on the heatmap represents a perfect hit, teal represents a strict hit and purple represents no hits.

5.3.2 16S rRNA gene amplicons microbiome investigations using ONT minION

5.3.2.1 Quality control for input DNA for library preparation

Ten different ITN teats and ten different teats without lesions were used to assess the 16S rRNA gene amplicons in an aim to use the most recent samples for assessment of the teat skin microbiome (Table 5.3.2.1). The DNA concentrations were measured using NanoDrop™ (Thermo Scientific™, Waltham, MA, USA) for both extractions using the DNeasy Blood and Tissue Kit and the QIAamp DNA Microbiome kit (Table 5.3.2.2).

Table 5.3.2.1 Sample description with the clinical grade of ITN present on the teat and the cow identifier selected for MinION 16S rRNA gene amplicon sequencing.

Sample	Animal	Clinical grade
MI1	Cow 1	Control
MI2	Cow 2	Control
MI3	Cow 3	Control
MI4	Cow 4	Control
MI5	Cow 5	Control
MI6	Cow 6	Control
MI7	Cow 7	Control
MI8	Cow 8	Control
MI9	Cow 9	Control
MI10	Cow 10	Control
MI11	Cow 11	Type 1
MI12	Cow 11	Type 3
MI13	Cow 11	Type 3
MI14	Cow 12	Type 1
MI15	Cow 12	Type 1
MI16	Cow 13	Type 2
MI17	Cow 14	Type 2
MI18	Cow 15	Type 1
MI19	Cow 16	Type 3
MI20	Cow 16	Type 3
MI21	Zymo Mock Communities (positive control)	

Table 5.3.2.2 DNA quantity and quality from the DNeasy Blood and Tissue extraction kit and with the QIAamp DNA Microbiome kit (assessed by NanoDrop™)

	DNeasy Blood and Tissue extraction kit			QIAamp DNA Microbiome kit		
Sample	**Concentration ng/ul**	**260/280**	**260/230**	**Concentration (ng/µl)**	**260/280**	**260/230**
MI1	44.6	1.92	1.97	15.2	1.92	0.85
MI2	41.4	1.94	1.45	21.1	2.08	0.32
MI3	62.1	1.89	1.99	26.7	1.45	0.32
MI4	45.6	1.91	2.35	5.2	2.30	0.53
MI5	86.3	1.89	2.04	10.4	2.06	1.31
MI6	77.3	1.88	2.07	3.8	1.75	0.02
MI7	39.5	1.93	2.14	3.3	2.24	0.08
MI8	60.0	1.91	2.04	21.0	1.88	0.12
MI9	68.7	1.91	2.24	12.3	2.03	2.34
MI10	52.7	1.90	1.97	11.1	1.83	0.71
MI11	86.7	1.85	1.64	277.6	1.89	2.26
MI12	70.2	1.92	2.15	154.2	1.92	1.73
MI13	22.2	1.82	1.24	46.5	2.01	1.61
MI14	60.7	1.90	1.68	102.8	1.92	2.05
MI15	29.9	1.93	1.43	10.7	2.06	1.25
MI16	31.6	1.96	1.84	116.5	1.90	1.69
MI17	50.8	1.62	0.63	131.1	1.91	1.81
MI18	47.8	1.86	1.51	295.6	1.92	1.86
MI19	15.4	1.94	1.39	2.7	2.23	0.07
MI20	49.7	1.84	1.16	2.5	1.74	0.13
MI21	0.9	2.02	0.14	2.5	1.71	0.09

5.3.2.2 Results of the analysis of MinION 16S rRNA gene amplicon sequencing

When reviewing the number of classified reads via EPI2ME, the DNA extractions performed better using the DNeasy Blood and Tissue kit with 43,202 of classified reads, 209 unclassified reads and an average accuracy of 90%. In contrast, the DNA extracted using the QIAamp DNA microbiome kit only had 1,665 of classified reads and 21 unclassified reads, again with a 90% average accuracy (Table 5.3.2.4).

Table 5.3.2.4 Number of classified reads per sample from the different extraction methods.

Sample	DNeasy Blood and Tissue extraction kit	QIAamp DNA microbiome kit
MI1	6	0
MI2	22	0
MI3	3,366	0
MI4	1,914	0
MI5	40	0
MI6	169	0
MI7	128	2
MI8	128	2
MI9	29	9
MI10	14	7
MI11	3,314	8
MI12	16,013	0
MI13	21	11
MI14	39	46
MI15	9,803	1,144
MI16	67	179
MI17	434	7
MI18	3,773	8
MI19	1,011	9
MI20	138	1
MI21	1,739	17
Negative control	3	0
Unclassified barcode	1,223	97

As such the data from DNA extractions performed from the DNeasy Blood and Tissue kit are presented and can be access via the link https://epi2me.nanoporetech.com/shared-report-356841?tokenv2=8a49a95c-ea01-4084-8d4a-7396ab91d4e9. In general, there were higher numbers if classified reads detected for the diseased ITN teats (MI11-MI20) than for the non-diseased control teats (MI1-MI10) with variations in the top 5 most abundant bacteria per sample (Table 5.3.2.5).

Table 5.3.2.5 Top 5 most abundant taxa in each sample and the number of reads for each.

Sample	Bacteria identified	Number of reads
MI1	*Streptococcus* sp.	1
	Acinetobacter sp.	1
	Jeotgalibaca sp.	1
MI2	*Acinetobacter* sp.	12
	Psychrobacter sp.	5
	Aerococcus sp.	1
	Escherichia sp.	1
MI3	*Psychrobacter* sp.	2,683
	Acinetobacter sp.	340
	Staphylococcus sp.	65
	Jeotgalicoccus sp.	28
	Aerococcus sp.	18
MI4	*Acinetobacter* sp.	1,307
	Psychrobacter sp.	324
	Streptococcus sp.	27
	Lactococcus sp.	13
	Moraxella sp.	4
MI5	*Psychrobacter* sp.	9
	Lactococcus sp.	7
	Acinetobacter sp.	6
	Streptococcus sp.	5
MI6	*Acinetobacter* sp.	79
	Psychrobacter sp.	45
	Streptococcus sp.	7
	Citrobacter sp.	4
	Lactococcus sp.	3
MI7	*Psychrobacter* sp.	33
	Staphylococcus sp.	9
	Paeniclostridium sp.	9
	Aerococcus sp.	8
	Romboutsia sp.	6
MI8	*Staphylococcus* sp.	21
	Streptococcus sp.	15
	Aerococcus sp.	4
	Peptoniphilus sp.	4
	Peptostreptococcus sp.	4
MI9	*Psychrobacter* sp.	7
	Romboutsia sp.	5
	Paeniclostridium sp.	4
	Macrococcus sp.	2
	Aerococcus sp.	1

MI10	*Psychrobacter* sp.	1
	Romboutsia sp.	1
	Paeniclostridium sp.	1
	Lactobacillus sp.	1
	Pantoea sp.	1
MI11	*Mannheimia* sp.	1,675
	Streptococcus sp.	950
	Helcococcus sp.	93
	Fusobacterium sp.	37
	Parvimonas sp.	13
MI12	*Mannheimia* sp.	11,288
	Strepococcus sp.	1,955
	Fusobacterium sp.	39
	Helcococcus sp.	32
	Pasteurella sp.	23
MI13	*Mannheimia* sp.	11
	Streptococcus sp.	5
MI14	*Mannheimia* sp.	25
	Staphylococcus sp.	8
	Streptococcus sp.	1
MI15	*Mannheimia* sp.	7,900
	Streptococcus sp.	396
	Actinobacillus sp.	25
	Haemophilus sp.	8
	Photobacterium sp.	4
MI16	*Streptococcus* sp.	24
	Staphylococcus sp.	14
	Mannheimia sp.	7
	Fusobacterium sp.	3
	Paeniclostridium sp.	1
MI17	*Streptococcus* sp.	145
	Staphylococcus sp.	73
	Helcococcus sp.	31
	Fusobacterium sp.	11
	Parvimonas sp.	8
MI18	*Staphylococcus* sp.	2,251
	Streptococcus sp.	516
	Bacillus sp.	28
	Fusobacterium sp.	20
	Helococuccus sp.	15
MI19	*Lactococcus* sp.	376
	Serratia sp.	105

	Aeromonas sp.	63
	Buttiauxella sp.	45
	Carnobacterium sp.	10
MI20	*Aeromonas* sp.	42
	Serratia sp.	27
	Lactococcus sp.	17
	Buttiauxella sp.	7
	Romboutsia sp.	5
Mock communities	*Listeria* sp.	1,107
	Pseudomonas sp.	381
	Bacillus sp.	74
	Salmonella sp.	17
	Carnobacterium sp.	3
Negative control	Lactobacillus sp.	2

The microbes identified in the mock community standards are consistent with those stated on the datasheet. When looking at the main changes between the non-diseased control teats and the ITN teats the main differences were the presence of *Mannheimia* sp. and the absence or severe reduction of *Acinetobacter* sp. and *Psychrobacter* sp. (when the minimum abundance cut-off value was set to 1%) in ITN teats (Fig 5.3.2.1). In all diseased ITN teats, apart from MI20, *Mannheimia* sp. was present, sometimes in lower numbers compared to other bacteria. *Mannheimia* sp. were the most prominent bacteria in all samples and responsible for 21,408 of the 43,202 classified reads (49.6% of classified reads). *Mannheimia* sp. was detected in three non-diseased teats but in very low numbers, only 1-3 reads. Five *Mannheimia* species were detected across the samples (Table 5.3.2.6).

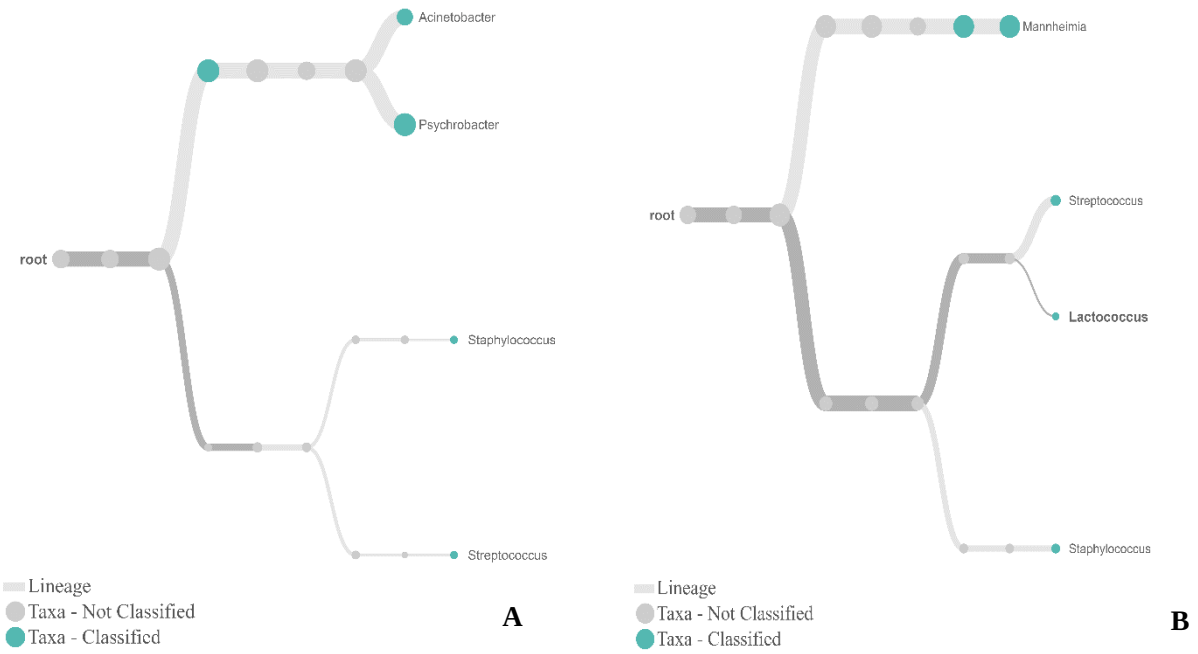


Figure 5.3.2.1 Phylogenetic tree summarising the main taxa observed form the samples of non-diseased healthy teats (A) and diseased ITN teats (B).

Table 5.3.2.6 The different Mannheimia species identified and the number of reads.

***Mannheimia* species**	**Number of reads**
M. varigena	12,847
M. granulomatis	7,532
M. ruminalis	846
M. haemolytica	111
M. glucosida	72

5.4 Discussion

As the aetiology for ITN is currently unknown the studies in this chapter utilised NGS including shotgun metagenomics and 16S rRNA gene amplicon analysis in an attempt to identify potential aetiological candidates for further investigation. The results for this chapter are not clear-cut with no clear potential aetiological agent becoming apparent. Traditional microbiological techniques identified bacteria that are frequently found as commensals or within faeces and therefore it did not seem worthwhile to further pursue classic time-consuming methods for detection of aetiological agents (data not shown). Also, the results from Chapter 3 suggested there may have been an initial DNA viral involvement, therefore NGS with shotgun metagenomic sequencing was an important step to take. Shotgun metagenomic approaches are also useful to attempt to identify if potential aetiological agents can have a symbiotic affect (Sharpton, 2014). This type of method has previously been used to identify the microbiome in dairy cattle DD (Zinicola et al., 2015) and UCD (Ekman et al., 2020) and CODD in sheep (Duncan et al., 2021).

The shotgun metagenomic data highlighted many interesting findings including the low abundance of spirochetes in all samples with control samples frequently having a higher proportion of spirochetes than ITN samples. This is supportive of the data in earlier chapters that suggest that DD associated Treponemes may not be an important aetiological agent in the initiation of ITN lesions. When comparing the diseased and non-lesion teats, there were more similarities between the non-lesioned teats in terms of microbiome than there were between the ITN teats. This suggests that the healthy teat has a broad range of bacteria present in similar proportions compared to the diseased teat which may have overgrowth of several bacterial species, relatively speaking, and therefore a decrease in biodiversity of the microbiome in the diseased tissue. For example, many of the ITN samples have a much higher proportion of either

Fusobacteriaceae or *Staphylococcus*. The control, non-diseased teats were far more similar using the Pearson correlation test than the ITN teats. This suggests that the microbiome was more similar for the healthy teats than the microbiome of the diseased ITN teats. It is therefore possible that the ITN may not have a single common aetiological agent, but actually presents as a disease ultimately of opportunistic infections subsequent to a previous infective or non-infective episode. This has been observed in skin lesions in multiple species including CODD in sheep (Duncan et al., 2021), DD in cattle (Krull et al., 2014; Caddey and De Buck, 2021), UCD in dairy cattle (Ekman et al., 2020), atopy in dogs (Pierezan et al., 2016) and diabetic foot ulcers in humans (Schmidt et al., 2021). Interestingly, the dysbiosis observed in the ITN samples compared to the control samples mimics the findings of a shotgun metagenomic study in UCD (Ekman et al., 2020) another skin lesion affecting the dairy cow udder.

This reduction in biodiversity of the microbiome is frequently referred to as a dysbiosis. In humans, skin microbiome dysbiosis has also been linked to issues of an imbalanced microbiome in the gut and raised the theory of the skin-gut axis (Pessemier et al., 2021). This hypothesis also seems have crossed over to veterinary species with preliminary data suggesting similar findings in dogs (Rostaher et al., 2022). As such, further studies of the gut microbiome alongside skin diseases such as ITN are required.

When using LefSe to investigate biomarker pathways, there were more *Pasteurellaceae* in the diseased teats than the non-lesioned teats. This is an interesting finding. Frequently in cattle *Pasteurellaceae* are found as commensal organisms in the nasopharynx and mouths of calves and cows (Dabo et al., 2007). Many of the cows with ITN lesions will lick at their teats; therefore, it may be possible that the act of licking introduces commensal organisms from the mouth into a new environment that is suitable for proliferation as an opportunistic pathogen. In fact, many pneumonias in cattle are caused by these agents gaining access to the lungs when innate immune defences are reduced, often due to stress or viral infection (Panciera and Confer,

2010). When considering typical *Pasteurellaceae* induced pneumonias in cattle, such as *Mannheimia haemolytica*, these bacteria have leukotoxin (LKT)exotoxin that is able to target blood vessels and are proinflammatory (Rice et al., 2007; Panciera and Confer, 2010). From Chapter 3, the histopathology of the ITN tissues showed a strong inflammatory reaction and some blood vessels were affected by a vasculitis and thrombosed. As discussed in Chapter 4 cross suckling can be a problem in dairy herds and there is the possibility that these bacteria may be introduced to the microbiome of the skin of the teat at a young age and reside until an opportunity, such as the stress, hormonal changes and mechanical compression of the teat during early lactation, provides a suitable environment for such agent. One study found negative effects of cross suckling and that the teats were elongated and these cows were more prone to mastitis (Mahmoud et al., 2016). However, another study did not find these negative effects (Vaughan et al., 2016).

Another finding from the shotgun metagenomic data was the identification of low numbers of resistance genes present in a small number of ITN teats and in a single teat without ITN. This is concerning especially as one of the resistance genes was to tetracycline. This may reflect farmers commonly using tetracycline spray (blue spray) for any skin lesion as a first point of call and may indicate that the normal microbiome in some animals already harbours AMR genes.

One of the major limitations of this study was the methods of tissue sampling and DNA extraction which inevitably caused a high level of host contamination and a low sequencing depth to the microbiome (Sharpton, 2014). Subsequently, during analysis, in a large proportion of samples, less than 0.5% of the reads were retained after host DNA removal. Consequentially a relatively low number of sequences classified using Kraken 2 were available for downstream analysis, such as use by LefSe for the identification of biomarker pathways and for the outputs of both Bracken and HUMAnN2. As such the biomarker data required careful interpretation

due to the overall low number of classified sequences and low numbers of pathways found in non-lesion, control teats. As host contamination proved a major limitation to shotgun metagenomics, different methods were explored in an attempt to reduce the host contamination (Bjerre et al., 2019). However, such depletion techniques can potentially bias bacterial detection (Ganda et al., 2021). After consideration, it was decided to change the DNA extraction method to target the microbiome and include a bead beating step (Wiscovitch-Russo et al., 2022) as is frequently done in human sputum samples (Oriano et al., 2018) and to be more targeted with the sequencing approach by focusing on the bacterial community and the 16S rRNA housekeeping gene. As the recent improvements in specificity and sensitivity around ONT and for the rapid analysis of data, further samples were sequenced using a MinION Mk1C (ONT, Oxford, UK). The results demonstrated that although the host depletion steps during DNA extraction were successful, the overall DNA yield was poor. Subsequently the DNA extraction method used for the shotgun metagenomics (using the DNeasy blood and tissue kit) provided far higher classified 16S rRNA gene amplicon reads when using the ONT device than the microbiome kit designed to deplete host DNA with 43,202 classified reads compared to 1,665 classified reads respectively.

Although there was the ability to perform adaptive sampling with the ONT device to deplete the host DNA during sequencing, due to the approach of targeting the 16S amplicons, adaptive sampling was deemed unnecessary. The main findings from this experiment was the severe reduction of *Acinetobacter sp*. and *Psychrobacter sp*. in the ITN teats and the presence of *Mannheimia sp*. This is similar to the shotgun metagenomic findings of an increase in *Pasteurellaceae* in the diseased teats. All but one ITN teat had some reads classified as *Mannheimia sp.* using the ONT. Five *Mannheimia* species were identified including *M. varigena*, *M. granulomatis*, *M. ruminalis*, *M. haemolytica* and *M. glucosida* all of which were previously considered part of the *Pasteurella haemolytica* complex (Angen et al., 1999). As

the species classification accuracy for the ONT device averaged 90% there maybe reads that were misclassified especially considering the similarities between the *Mannheimia* species.

Mannheimia species are opportunistic pathogens that frequently inhabit the ruminant forestomach and mucous membranes (Bisgaard, 1993) and can be isolated from asymptomatic carrier. As previously described, *M. haemolytica* has long been associated with severe pneumonia in cattle and vaccination has been shown to aid in reduction of *M. haemolytica* pneumonia cases (Rice et al., 2007). *M. varigena* has previously been isolated from cases of bovine pneumonia, mastitis and septicaemia (Bisgaard, 1993; Blackall et al., 2002) and can induce gross lung lesions similar in appearance to those induced by *M. haemolytica* (Harhay et al., 2014). Similarly, *M. granulomatis* has been isolated from mastitis in cattle and pneumonia in cattle, hares and roe deer (Britton et al., 2017) and *M. glucosida* has also been associated with mastitis in sheep (Omaleki et al., 2012). Although, *Mannheimia* species were not identified in one study investigating the microbiome of UCD lesions (Ekman et al., 2020) suggesting that the two diseases may not involve the same microorganisms. Therefore, further studies investigating *Mannheimia spp.* presence in ITN lesions, such as immunohistochemistry targeting the *Mannheimia* leukotoxin, could be interesting to pursue for ITN cases.

The differences in the outputs from the shotgun metagenomics with Illumina sequencing and the 16S amplicon sequencing using ONT MinION are likely due to a combination of limitations associated with the two techniques. For ONT, primer bias with the 16S rRNA amplicon sequencing may fail to detect a proportion of bacterial species. Although sequencing of near to the entire 16S rRNA gene is considered the gold standard for taxonomic designation using a single gene (Stackebrandt and Goebel, 1994). Whilst the breadth of sequencing was limited to only the 16S rRNA gene with ONT, due to the longer reads and the ability to sequence the whole 16S rRNA gene, with an average of 1,500 base pair (bp) sequencing length, this is considered a reliable way to identify the bacteria to the species level. High throughput genome

sequencing entries for the generation of entire genomes requires a high enough sequencing depth. As the Illumina data was severely affected by host contamination, whilst a substantial amount of DNA was sequenced this was inadequate for entire genomes to be produced, thus limiting the microbial identification. In addition, Illumina produces short reads (around 400 bp) that potentially allow for annotation artefacts to occur. Consequently, for this study the 16S rRNA gene amplification results are considered more reliable. Whether, different samples used for the two studies, could be responsible for the differences should be considered, although this was required due to the time delay between the two experiments. However, given similar sampling strategies were used and the results do collectively indicate Pasteurellaceae from the Illumina, it is considered that the ONT data use of the near entire 16S rRNA gene allows for better taxonomic resolution to the implication of *Mannheimia spp*. (*M. varigena, M. granulomatis, M. ruminalis, M. haemolytica, M. glucosida*) that is the cause of the difference and that the two datasets are not conflicting.

The onset of ITN lesions is probably a complex and multifactorial process. From the findings presented in this chapter, ITN appears as a dysbiosis in the teat skin microbiome and is likely to be yet another disease that is polymicrobial in its aetiopathogenesis. Polymicrobial diseases, such as footrot in sheep and DD in cattle, can be treated and controlled. Footrot in sheep is multifactorial with the bacterium *Dichelobacter nodous* considered the main causative agent (Blanchard et al., 2021). With this information, a vaccine developed against *Dichelobacter nodosus* antigens (Footvax®, MSD Animal Health UK Ltd, Milton Keynes, UK) has long been reported to reduce the number of cases of not only footrot but also CODD in affected flocks (Duncan et al., 2012). Similarly, whilst DD is now considered polymicrobial, the antibiotics identified as efficacious against the treponemes (Evans et al., 2008, 2012; Duncan et al., 2014), considered the major aetiological agent , have also been shown to be efficacious in field trials of sheep with CODD (Duncan et al., 2012).

In line with polymicrobial diseases having important taxa which are key to the disease pathogenesis, here in addition to the dysbiosis, there is mounting support for *Mannheimia spp*. as a potential causal agent of ITN. Targeting *Mannheimia spp*. in the future could allow for treatment or control of ITN and it should be clarified whether *Mannheimia spp.* are more abundant in different stages of the lesion. Such work is required to ascertain if *Mannheimia spp*. presence are causative for ITN lesions or if these bacteria are found due to the effect of animals licking their teats.

In summary, the main findings from the studies presented in this chapter are that ITN lesions appear to be present as a dysbiosis with evidence towards the involvement of *Pasteurellaceae* and more specifically *Mannheimia spp*. in the aetiopathogenesis which require further investigation and may allow for control and treatment options to be implemented.

Chapter 6: General Discussion

Multiple approaches to disease investigations are essential to attempt to understand causes and risk factors for all manner of diseases in human, animal and plant populations. Key aspects of disease investigations are to understand the population at risk, the potential risk factors for disease and the possible aetiologies when an infectious agent is considered likely (Hitchcock et al., 2007; Fricker and Rigdon, 2020). For production animals particularly, the economic impact of infectious diseases must be considered as these animals also provide a livelihood for farmers and if ensuing impacts are substantial enough, farms may prove uneconomical and go out of business. This has an immediate impact on the farm workers and families but also on the community for which the farm provides produce (FAO, 2016; Barratt et al., 2019). Bovine ischaemic teat necrosis has been demonstrated to affect around half of the GB dairy farms surveyed, has only been described in relatively recent times and has clearly been emerging in recent years. Due to the increasing number of farmers reporting cases for the first time, an infectious aetiology was suspected and underpinned the work described in this thesis.

6.1 Understanding the aetiology of ITN

During this study, a multifactorial approach has been applied to understand the potential pathogenesis, aetiological agents and risk factors that may induce ITN in lactating cows. This included a farmer-reported questionnaire, pathological screening and microbiological studies. A previous small study of 12 animals with ITN lesions found that a large proportion, 91.7% of lesions, had DD associated treponemes detected via PCR (Clegg et al., 2016b); however, this finding was not repeated in this study.

Only 31% of ITN lesions detected DD associated treponemes in 113 ITN lesions when screened using the same PCR methods. Furthermore, the farmer reported questionnaire did not find an association of farms with ITN cases reporting issues with DD in the milking herd. Also,

upon interviewing farmers and veterinary surgeons that submitted cases of ITN for this study, many reported that there was no DD currently on the farm or that a previous DD problem had resolved at the time of the ITN case development. In fact, the shotgun metagenomic study found that the control teats without lesions had a higher abundance of spirochaetal bacteria present than the affected teats. This is interesting and suggests that treponemes on the bovine udder may be part of the normal flora and potentially present as an opportunistic agent. Although treponemes can be found throughout the dairy environment (Evans et al., 2012), DD treponemes that are considered pathogenic appear restricted to disease manifestations or are occasionally present in the recto-anal junction and oral mucosa (Bell, 2017). The immunohistochemical (IHC) study assessing the presence of DD associated treponemes within tissue sections also failed to show treponemes in the ITN lesions further supporting the new hypothesis that DD associated treponemes are no longer a key aetiological agent for ITN but may present as an opportunistic agent further complicating a subset of cases.

As DD associated treponemes no longer seemed to be the major pathogen involved with ITN, a new hypothesis was required. Next generation sequencing methods have been used in many previous studies for hypothesis development (Ekman et al., 2020; Duncan et al., 2021; Schmidt et al., 2021; Rostaher et al., 2022). In this study, NGS methods were utilised to gain insight into other potential infectious agents that may be present in ITN teats that are missing from teats without lesions, which is one of the first steps in Koch's postulates. No key common viral or fungal agents were detected between ITN samples that were not present in the non-diseased teats on NGS. As such, viral and fungal agents were deemed less likely to be the aetiological agents of ITN. Both shotgun metagenomics and 16S rRNA amplicon analysis suggested there was an increased relative abundance of *Pasteurellaceae* within the ITN teats compared to those without lesions. The ITN teats also had reduced numbers of bacteria that are considered to make up most of a healthy microbiome such as *Acinetobacter sp*. and *Psychrobacter sp*. In

addition, there was often a reduction in the biodiversity of the bacterial population present on the ITN teats compared to the non-lesioned teats that may indicate that a general dysbiosis is an important aspect of disease instigation or progress.

The farm livestock skin microbiome is, inevitably, composed of many microorganisms, which when balanced aid in protecting the body against invading pathogens and are also important in training the healthy cutaneous immune system (Byrd et al., 2018). There are many skin diseases that have been reported in both humans and animals that are now considered to be due to an alteration in the microbiome, with an increased presence of some bacterial species and a decrease in others, frequently referred to as a dysbiosis (Pierezan et al., 2016; Liang et al., 2021; Schmidt et al., 2021; Rostaher et al., 2022). Many things can cause a dysbiosis including but not limited to topical antimicrobials, topical chemical use, hormonal changes and nutritional changes (McLoughlin et al., 2022). It was demonstrated in Chapter 5 that the main consistency between ITN teats and teats without lesions was the decrease in the biodiversity of the microbiome on the ITN teats rather than the presence of a single aetiological agent. Increasingly, this is a common finding in ruminant skin diseases that have previously been thought to be the sole responsibility of one agent (Krull et al., 2014; Ekman et al., 2020; Caddey and De Buck, 2021; Duncan et al., 2021). Interestingly, the dysbiosis observed in the ITN samples compared to the control samples in many ways mimics the findings of a shotgun metagenomic study in UCD (Ekman et al., 2020). In addition, in Chapter 2, the presence of UCD in the milking herd was found to be a farm level risk factor for ITN. This brings to question if there something happening on these farms during milking or other managements that is causing a critical change in the environment in the teat that is suitable for dysbiosis to develop. Another aspect that needs to be considered is the potential for an immune-mediated process to be involved in the pathogenesis of ITN either primarily or secondarily. One study found that atopic dogs that were exposed to allergens also presented with a dysbiosis (Pierezan

et al., 2016). There are also human diseases that are considered immune-mediated and associated with a dysbiosis such as psoriasis (Liang et al., 2021). The histopathology in some of the ITN teats indicated that there was a vasculitis which could indicate a hypersensitivity reaction or viral infections (Smoller et al., 1990; Maxie, 2016). However, a vasculitis can also be induced by bacterial toxins (Smoller et al., 1990). It has been reported particularly in Channel Island cattle breeds, where any retention of milk within the udder can cause cows to develop an autoallergy to the casein in their own milk (Moroni et al., 2018). Although there were differences in the breeds of cattle affected in this study with Holsteins to Jerseys affected, investigating a potential genetic component to a hypersensitivity development may be warranted.

The histopathology analyses also provided potential insights as to why some ITN teats were removed by the animal, when others were not. The type 3 lesions often had large numbers of eosinophils present. Eosinophils and mast cells in tissue can release histamine when they degranulate and can cause an itchy sensation (Shim and Oh, 2008). These inflammatory cells are often found in hypersensitivity reactions or as a response to a parasitic infection. As no parasites were observed within any of the histological sections this seems unlikely and instead exploring potential allergens in ITN cases could be useful.

One of the more unique presentations of ITN is that the cow often exhibits signs of pruritis in the affected teats and are frequently observed licking the teats. This may be a way of the bacteria being introduced to this anatomical location and why certain microbial species are found in ITN teats compared to teats without these lesions. Interestingly, the 16S rRNA gene amplicons study found there to be an association with an increased abundance of *Mannheimia spp.* in ITN cases with this genus only found in very low numbers in a few non-ITN teats. The long-read approach by the ONT allowed for sequencing the whole 16S rRNA gene which clarified that there were only five different *Mannheimia spp.* found in the ITN teats.

Mannheimia spp. are frequently found within the nasopharynx of cattle and are known opportunistic pathogens of the lower respiratory track but have also been reported to cause mastitis in ruminants (Omaleki et al., 2012). *Mannheimia spp.* is one such genus of bacteria that release LPS endotoxins and exogenous leukotoxins as part of the pathogenic response. In respiratory infections, a similar process has been demonstrated to induce a vasculitis and cytotoxic effects on infiltrating leukocytes (Panciera and Confer, 2010). The pathogenic effects of these bacteria may be secondary to an initial teat damage, which if tackled might prevent the bacterial infection. What causes cows to lick their teats to introduce this potential aetiology in the first place?

From the clinical histories provided with cases and from the farmer reported questionnaire, it has become apparent that the animals most frequently reported to have cases of ITN are first lactation heifers in the first 90 days in milk. This is a time of great stress to the dairy cow. There are numerous physiological and hormonal changes that the cow undergoes in this time, including those associated with udder development and onset of lactation, negative energy balance, to social aspects of being introduced into the milking herd and the new sensation of milk let down and the use of a milking machine (Pascottini et al., 2020). Having experienced parturition and lactation myself during this PhD I can assure you that these are no minor events and are a cause of great stress. In addition, it has been demonstrated in humans that the skin microbiome is overhauled at the time of puberty and the relative abundance of bacteria present changes due to the influence of hormones (Byrd et al., 2018). The peri-parturient cow has to deal with hormonal changes and negative energy balance due to decreased rumen capacity from the calf occupying increasing space in the abdomen with dry matter intake often remaining low a few days after parturition (Thatcher et al., 2010). This can put the cow at risk of developing ketosis. In addition, a decreasing calorie intake can dysregulate the cell mediated immune system and neutrophil function has been shown to decline in cows with the onset of

lactation (Thatcher et al., 2010). This lowers the capacity of the immune system to respond to potential pathogens. It has been reported that feed restriction early in lactation can cause an increase in cortisol detected in the milk (Gellrich et al., 2015). In lactating ewes, a reduction in immune competence was observed in animals with high levels of cortisol (Caroprese et al., 2010). In addition, ruminants may become hyperglycaemic around the peri-parturient period due to development of insulin resistance to ensure a high enough supply of glucose is present for the milk production (Mair et al., 2016). Furthermore, a study in humans found that diabetic patients with hyperglycaemia had an increased susceptibility to ischaemic necrosis (Lévigne et al., 2013); as such, it would be interesting to investigate the glucose levels in ITN affected animals.

A further issue that may arise in cows around the time of parturition is the development of udder oedema, although the exact pathogenesis of development of udder oedema is not fully understood (Moroni et al., 2018). It has been shown that udder oedema can be a risk factor for the development of ITN cases (Manning, 2016). Furthermore, the onset of lactation itself produces an unusual sensation that may cause cows to first lick their teats after milking and a possible way of introducing bacteria from the nasopharynx. This could especially be an issue if the animal had previously been a cross/self-suckling animal (Mahmoud et al., 2016).

Another finding from the questionnaire was that the presence of chapped teats on the farm were a major risk factor for development of ITN cases. These teats will have alteration in the protective skin barrier that could allow a site of entry for bacteria and then ITN ensues; or alternatively this loss of skin integrity could initiate the dysbiosis process. It was demonstrated in Chapter 2 that more cases of chapped teats were reported in farms that used peracetic acid in a pre-milking teat preparation and also in farms that used an automated flushing system. Potentially, peracetic acid use may not only reduce the overall bacterial load but also alter the microflora of the teat. In humans, skin care products have been shown to alter the bacterial

diversity on the skin (Bouslimani et al., 2019). Peracetic acid is a common disinfectant used in the dairy industry and has not been linked to any major hypersensitivities or dermatitis in animals or humans unless used at high concentrations for prolonged periods (Müller et al., 1988; Laven and Hunt, 2002; Bore and Langsrud, 2005; Pechacek et al., 2015; Megahed et al., 2019) therefore, farmers may have to consider the concentrations of products used, with highly concentrated products having the potential to damage to teat skin.

6.2 Potential areas for control and intervention

When it comes to attributing disease to specific infectious agents, it is frequently not possible to fulfil all of Koch's postulates. Consequently, other approaches need to be taken to understand the involvement and role of identifiable microorganisms in disease aetiology and pathogenesis with a view to prevention or effective treatment.

Overall good health of the skin of the teat and the udder throughout the milking herd is essential. If the skin integrity on the teats or the udder is disrupted then there is the potential for pathogens to enter (Moroni et al., 2018). Ensuring the teat is moisturised, to prevent drying and chaffing, should be a key component in disease prevention, but careful consideration of product suitability is required to try to prevent any alteration in the microbiome. Maintaining the milking machine and teat liners to prevent injury to the teats is essential not only for ITN but for mastitis (Gleeson et al., 2004; AHDB, 2022c). If a farm is having issues with other teat lesions, such as teat end hyperkeratosis and teat oedema after milking then performing a dynamic milking parlour test should be considered to ensure the appropriate vacuum is applied to the teat (Farming Connect, 2022).

Given the microbiological findings suggesting the possibility of involvement of *Mannheimia spp.* it would seem important to consider the bacterial pneumonia incidence in the herd. This was not considered as part of this study but contemplating that *Pasteurellaceae* frequently

cause pneumonia in calves and occasionally pneumonia in adult cattle (Rice et al., 2007; Dorso et al., 2021), it would be worthwhile investigating the causes and numbers of pneumonia cases on farms with ITN. In addition, if pneumonia due to *Mannheimia spp*. is an issue in the herd, there are vaccines available that can be introduced in to the herd to reduce incident of respiratory disease, and hopefully therefore ITN (Larson and Step, 2012).

Histopathology suggested that a hypersensitivity response may be a factor in disease onset. In addition, the questionnaire data suggested that chapped teats were a possible risk factor for ITN. Subsequently, peracetic acid use increased incidence of chapped teats in the herd, then is it possible that peracetic acid use may induce a hypersensitivity in some animals. This has not been reported in cattle and may be a cofounding factor that requires further investigation. Nevertheless, if a herd is having a problem with ITN and chapped teats and is using peracetic acid, it may be worth investigating if the use of a different product could reduce the incidence in the herd. Or alternatively checking the concentration used and how it is applied. If the product is applied using faecal contaminated teat cups then there may be more of an issue with general cleanliness than with chemical usage. Aside from peracetic acid, there are many other potential chemicals and products that are frequently used on a dairy farm that may induce a hypersensitivity. There are common skin allergies in humans to latex (Binkley et al., 2003) and chlorhexidine (Chiewchalermsri et al., 2020). Some disposable gloves are composed of latex and can induce skin irritations in humans (Binkley et al., 2003). Although not reported in cattle, if the milker handles the cows' teats with such gloves at every milking there is the potential for hypersensitivity to develop and would be worth considering in any further farm investigations with the possibility to change to nitrile gloves if necessary. However, glove use was not identified as a factor for ITN development in the questionnaire responses. Chlorhexidine use was reported in several farms but was not demonstrated to be a potential risk factor. A review of chemicals used (and their concentrations) in the milking process for any that may potentially

induce a skin irritation could be relevant to disease control. Indeed, removing a chemical(s) from the flushing system might be beneficial to teat health as it has been demonstrated that flushing with water alone was beneficial at reducing bacterial load on the teats before milking (Skarbye et al., 2020). Furthermore, care must be taken to maintain the milking machine and especially teat liners. If the teat liners are old or perished this could potentially harbour bacteria that may be transmitted from one cow to another (AHDB, 2022c). In Chapter 4 it was demonstrated that in a small subset of cases it was possible to detect DD associated treponemes (by PCR) from swabs of the teat liners after milking an ITN teat. As such, it may be possible for the milking machine to act as an infection reservoir for DD with the treponemes then moving over the skin to areas with suitable conditions to induce disease. Maintenance of the milking machine to prevent other teat lesions, such as those induced by excessive vacuums, should also be considered in a control plan. While it was not investigated in this study, ischaemic necrosis of the skin can be caused by a focal repeated trauma or a constant pressure (Ressel et al., 2016); an example of which would be pressure sores or decubitus ulcers. Furthermore, studies in greyhounds suggest that intermittent focal vascular occlusion not only causes injury due to ischaemia but can lead to further damage from reperfusion of the ischaemic site (Mauldin and Peters-Kennedy, 2015). The suction process of a mechanical milking machine may mimic this intermittent vascular occlusion. As such, the milking machine setup should be part of any further investigations into ITN aetiopathogenesis.

While treatment trials were not attempted as part of this investigation, reports from farmer and veterinary surgeon interviews and from the questionnaire data suggested that there was no one successful treatment but that often animals would either be culled close to disease onset or the case managed in the herd until the end of the lactation. A common finding was that response to topical antimicrobials was poor. This is similar to the findings by Manning (2016) that reported topical antimicrobials were of little use. The shotgun metagenomic data even found

evidence of genes conferring antimicrobial resistance (AMR) to tetracyclines, commonly used in dairy farms as a first response to skin disease, in some of the ITN teats. Therefore, topical antimicrobial may not be the best response to ITN cases. In fact, a study in North America and another in Europe found high levels of resistance to tetracycline in Pasteurellaceae (El Garch et al., 2016; Timsit et al., 2017) with further work required to fully investigate other treatments. This should be an important take home message for dairy farmers and veterinary surgeons alike that while the use of such a topical antimicrobial treatment may not appear to do any harm, there was already evidence of AMR in the microbiome of ITN teats.

6.3 Future work

Many areas that require further work and understanding have been alluded to earlier. One of the next essential steps is to identify farms that could be monitored and used for longitudinal studies. These studies could be used to assess the timescale from disease onset to loss of the teat and detail any different clinical features. Such a study may be able to provide insight as to why one animal may recover uneventfully, while another animal presents with rapid deterioration and loss of the teat, and another will apparently be relatively inert for a time before progressing further. In addition to monitoring disease progression, longitudinal studies would allow for animals to be assessed for hormonal levels, particularly the stress hormone cortisol at a key time for suspected disease onset (such as the first 90 days in milk). Furthermore, cow teat skin could be swabbed at various time points throughout the lactation to analyse the microbiome through the stages of the production cycle. It would also be useful to assess how certain hormones, such as oestrogen, could affect the teat skin microbiome. Not only could the teat skin be assessed in these studies but also the microbiome present in the nasopharyngeal region to see if cases of ITN do have similar species of bacteria present in both the nasopharynx and affected teat tissue. This data could be used alongside any evidence of pneumonia cases in the herd. Another interesting area would be to follow female calves through to first lactation

and note any cases of disease or behavioural abnormalities, such as cross/self-suckling, to see if these animals are more at risk of developing ITN when lactating. Longitudinal studies would also allow for genetic typing of the animals and then investigating if there are any specific nucleotide polymorphisms more associated with ITN animals than those that do not present with these lesions. This type of data could also be used to assess if there was a common genotype or ancestor that connects ITN cases, with potential for selective breeding out such a trait.

Another benefit of a long-term study is that it could allow for careful assessment of the milking protocol and any effects of the milking machine within the herd. This could include assessment of teat preparation products on the overall health of the teat skin. In addition, the potential associated farm level risk factors found in this study of the presence of UCD and chapped teats in the milking herd needs a more careful assessment and further investigations in to their risk factors.

As this study found *Mannheimia spp*. in ITN teats it would be useful to use further techniques to assess if these are likely to be causing the lesion. One step towards this would be to utilise immunohistochemical techniques to assess for either the presence of *Mannheimia sp*. within the infected tissues or the presence of *Mannheimia* leukotoxin within the lesions. A further relevant technique to further dissect this putative aetiology by visualising the tissue distribution of the bacteria would be the use of *in situ* hybridisation.

While further studies into the aetiologies and pathogenesis of the disease are clearly required, there is also the need for monitoring the disease throughout Great Britain and the rest of the World and for increased awareness of ITN. This is particularly important as all the available evidence is that ITN is increasing in incidence with reports also being received of ITN cases from North America, New Zealand, the Netherlands and Finland. A good step would be to

contact national surveillance groups such as the government group Animal and Plant Health Agency (APHA) and the National Animal Disease Information Service (NADIS) to encourage monitoring of ITN. Developing information guides for a key knowledge exchange organisation for the dairy industry, AHDB, would also be paramount not only to readily disseminate some of the findings on this study but to increase awareness of ITN and potentially enrol farms for further studies.

Another area that could be a focus of further investigation would be to assess the potential for teat liners to act as a reservoir for DD associated treponemes. While, treponemes are no longer deemed the primary infectious agent involved in ITN, the detection of these bacteria in teat liners after milking as subset of ITN positive teats is important to investigate further. Teat liners may act as a potential transmission route to allow for opportunistic agents to cause teat skin disease.

In this study it has been shown that ITN mostly affects first lactation cows in the first 90 days in milk. There are many farmers who have already experienced a case of ITN and the number of farmers reporting the first case on their farms is increasing and as such ITN can be considered an emerging disease. Since a high proportion of ITN cases have to be culled due to the disease, not only is this disease a welfare issue but also poses a financial threat to the dairy industry through production losses. In addition, the pathological study has demonstrated multiple areas for further investigations to clarify the aetiopathogenesis of this disease. From the microbiome investigations, ITN appears to be caused by a dysbiosis in which *Mannheimia spp*. could be a potential causative agent.

Given the recent increase in the number of ITN cases alongside the poor recovery and high cull rate of affected animals, these findings are timely and provide evidence-based suggestions for control and treatment options such as close monitoring of early lactation animals and increasing

the health of the teat and udder skin. This study has also highlighted the potential involvement of a bacterial aetiology, which in the future, could be targeted to identify effective vaccination and antimicrobial strategies to prevent the spread of disease within or between farms.

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APPENDIX A: Supplementary material relating to Chapter 2

A.1.1 Farmer Questionnaire

Bovine Ischaemic Teat Necrosis- A New and Increasing Disease Threat!

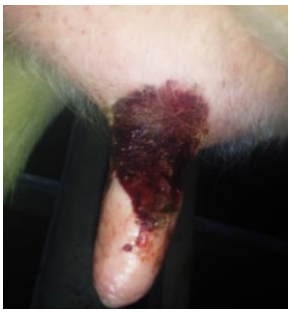


Figure 1. *Extensive lesion at base of the teat and extending half way down the teat.* *Photograph courtesy of Al Manning, Royal Veterinary College.*

We would like to tell you about a new disease that is increasingly being found on UK dairy farms. This is a disease that affects the skin of the teat base where the teat meets the udder. It causes skin sores that are highly irritable to the cow, often causing her to lick her teats and in the most extreme cases she may remove her own teats by constant licking (please see the pictures below and in the questionnaire figures). Some farms have reported ***losing up to 20% of their heifers each year.*** *At the moment very little is known about what causes this disease or what the risk factors are, and currently we do not know how common this problem is in the UK.* ***We need your help to unravel this disease****.*

This is a study run by vets at the University of Liverpool Vet School, we will ensure that all answers supplied are kept ***fully confidential****. Our aims are to find out the cause of the disease, what the risk factors are and attempt to work out where in the UK the disease is. With this information we will be able to develop preventative measures and treatment plans that will hopefully prevent these animals losing their teats which we will deliver back to you, the farmers.*

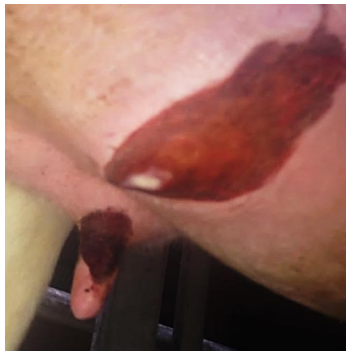


Figure 2. *The teat nearest the camera has been removed due to ITN and a teat shown in the background is the same as figure 1.* *Photograph courtesy of Al Manning, Royal Veterinary College.*

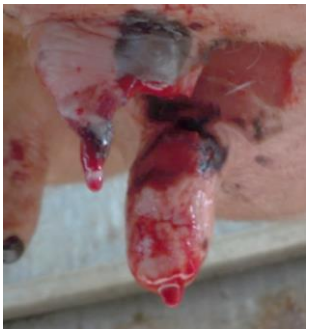


Figure 3. *The teat nearest the camera has been removed by the cow. The teat furthest from the camera has early lesions at the base of the teat extending down the teat and onto the udder.* *Photograph courtesy of Al Manning, Royal Veterinary College.*

This is a voluntary study and should you wish to withdraw from this study at any time, please contact Nick Evans (evansnj@liverpool.ac.uk).

Thank you in advance for taking part in this survey and please fill in as many answers as you are able. If you are unsure on an answer feel free to leave it blank. The survey will take about 20 minutes to complete and your responses will greatly benefit the wider UK dairy industry. Returning the survey is taken as consent to use the data provided for research purposes only. Returned surveys will be entered into a prize draw for the chance to win a £100 Amazon voucher.

If you would rather fill this questionnaire out via the telephone please contact vet Hayley on hcrosby@liverpool.ac.uk or +44(0)7765456529. Hayley will be working full time on this project and is willing to look at any pictures of teats that you may be worried about. Link to online version of this questionnaire is: https://www.surveymonkey.co.uk/r/BovineITN

The Disease- Bovine Ischaemic Teat Necrosis

We are first going to ask you some specific questions with regard to the disease. ***We are really interested in your answers even if you have not seen the disease.***

1. Have you had any cases of cattle constantly LICKING their teats? (Please circle)

Yes there is a current case Yes I have seen it in the last month

Yes I have seen in the last year Yes but it was more than one year ago

No, I have never seen this Don't know

2. Have you ever seen sores such as Fig. 1-4 (see Picture Guide provided) **in your dairy cattle?** (Please circle. If no or don't know, proceed to question 3 on page 4).

Yes No Don't know

We call these sores Bovine Ischaemic Teat Necrosis and from now on we will refer to them as ITN.

2a. Do you call these sores by any other name? (if so, please specify below)

……..

2b. Approximately, when did you FIRST SEE a case of Bovine ITN on your farm?

…….. month/year (please write approximate date)

2c. Roughly, how MANY TIMES in the last 5 years have you seen ITN lesions?

……..

2d. When the disease was at its worst roughly how MANY cases did you have at any one time?

……

2e. What LACTATION were the animals when you noticed the disease? (Please circle all that apply).

Pre-calving Heifers 1st Lactation 2nd Lactation

3rd Lactation 4th Lactation 5th Lactation 6th Lactation

Other, please specify…… Don't know

2f. How far into lactation were the animals when you first noticed the disease? (Please circle)

1-30 days 30-90 days 90-200 days 200-300 days >300 days Dry Don't know

2g. If you have seen ITN, have you tried ANY treatments? (Please circle).

Yes (please specify all treatments attempted in **table** below and if you think they worked)

No (please move to question 2h)

	Intramammary tube	**Intramuscular injection**	**Subcutaneous injection**	**Administration on to skin** eg. Creams, sprays, washing etc.	**Other** eg. bull rings in nose, rubber rings around the neck etc
Product used					
Did this work? (yes/no/don't know)					
What method worked the best? (please tick/state)					

2h. What happened to the cows affected by ITN? (Tick for each case, this is a tally box, you can tick the box multiple times if relevant).

Recovered	**Retained the teat**			**Lost the teat**			**Culled**	**Other, please specify……**
	Milked on 3	**Did not get mastitis**	**Got mastitis**	**Milked on 3**	**Did not get mastitis**	**Got mastitis**		

2i. In which months have you EVER seen cases of ITN? (Please circle all that apply)

Jan Feb Mar April May June July Aug Sept Oct Nov Dec Don't know

2j. What time of year do you see the MOST cases of ITN? (Please circle all that apply)

Jan Feb Mar April May June July Aug Sept Oct Nov Dec Don't know

If you have any more specific information relating to cases of ITN and would be willing to share it, please if you might provide this information within the table on page 13.

The Udder

We are now going to ask some more general questions about the udders of your cows.

3. **In the last year HOW MANY cases of clinical mastitis** (e.g. hard udder, clots in milk) **have you had in the milking herd?** (a case is defined as one quarter affected once)

……

4. **Do you know what agents have caused mastitis on your farm in the last year?** (i.e. has any milk testing done. Please write below if know the agent(s). You may write more than one).

……

5. **How do you usually <u>TREAT</u> your mastitis cases?** (You many have more than one approach).

……

……

……

……

6. **What is your average <u>Somatic Cell Count (SCC)</u> for the last year?**

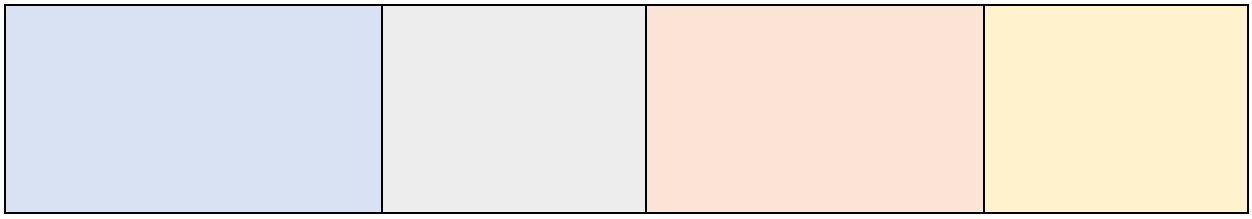
   ……

7. **In the last year approximately how many of the following lesions have you seen?** (Please use picture guide provided).

Lesion	Description	Number of cases	Current case? (Yes/No)
Bovine Warts (papillomavirus)	Wart or frond like lesions on the teat (**Fig. 5**)		
Bovine Ulcerative Mammillitis (Bovine herpesvirus 2)	Fluid filled blisters to large ulcerated regions along teats and on to udder skin (**Fig. 6-8**)		
Udder Cleft Dermatitis	Moist, pungent ulcerated areas on udder skin between the front quarters and between the udder and abdomen (**Fig. 9**)		
Pseudocowpox/Milker's nodules (parapox virus)	Red scabs that last 7-10 days and fall off to leave a **horseshoe/ring shape**. Causes milker's nodule in humans		
Dermatitis/Udder acne	Multiple raised scabs over the skin of the udder sometimes involving the teat (**Fig. 10**)		
Chapped/dry skin on teats	Dry skin on teats that is easily traumatised		

8. **Approximately <u>HOW MANY</u> cows in the herd have the following <u>after milking</u>?** (Please place a number in each column of the table).

Discolouration of the teat	Swelling/ringing of the teat	Teat end hyperkeratosis	Teat end eversion
Any discolouration of the teat (blue/pink/red) (**Fig. 11**)	Swelling of the teat of any kind (**Fig. 12**)	Raised/smooth roughened rings around the teat end (**Fig. 13**)	Streak canal everted after milking



9. What is your usual <u>DRY COW protocol</u>?

……

……

……..

10. How <u>LONG</u> is your average cow's <u>dry period</u>?

……days

11. What are your main <u>METHODS</u> for detecting clinical cases of <u>mastitis</u>? (Please circle all that apply).

Clots in the milk	Other changes in milk	California Milk Testing (CMT)
Changes in the udder	Behaviour of cow	Hard quarter
Reduced milk yield	High/low temperature	Milk conductivity results
Being last/order come into the parlour		Other, please specify…….

General questions about the cows on your farm

The following questions are related to the general health status of your dairy cattle.

12. Have you ever seen Digital Dermatitis (also known as Digi, hairy heel warts, Mortellaro's disease. See Fig. **14-15**) **in your cattle?** (Please circle).

Yes, there are current cases Yes I have seen it in the last month

Yes I have seen in the last year Yes but was more than one year ago

No, I have never seen this (Please move to 13.) Don't know (Please move to 13.)

12 a. Approximately, when did you FIRST SEE a case of Digital Dermatitis?

……month/year (Please provide an approximate date)

12 b. When the disease was at its worst roughly how MANY cases did you have at any one time?

.....

12 c. In which months have you EVER seen cases of Digital Dermatitis? (Please circle all that apply).

Jan Feb Mar April May June July Aug Sept Oct Nov Dec Don't know

12 d. What time of year do you see the MOST cases of Digital Dermatitis? (Please circle all that apply)

Jan Feb Mar April May June July Aug Sept Oct Nov Dec Don't know

13. Have you had any animals persistently infected (PI) with Bovine Viral Diarrhoea Virus (BVDv) removed from the herd within the last year? (Please circle).

Yes No Don't know

14. Do you VACCINATE your herd against BVDv and if so, WHAT PRODUCT do you use?

.....

15. Have you had any cases of tuberculosis (TB) in the last 12 months? (Please circle).

Yes, confirmed case Yes, reactor with no disease No Don't know

Milking Routine

You are doing great. Now we would like ask you a little about your milking routine and machine.

16. How many times a day do you milk your cows? (Please circle a letter).

A All cows are milked 2 times a day

B All cows are milked 3 times a day

C Cows are milked on a voluntary basis by an automated system (e.g. robot)

D Other, please specify……..

17. Do you use a pre-milking teat dip/spray? (Please circle).

Yes No Don't know Other, please specify……

17a. If you use a pre-milking teat dip/spray, WHAT do you use?

……

17b. How do you APPLY the product? (Please circle).

Spray Dip Foam cup Other, please specify……..

18. Do you FOREMILK your cows (i.e. express milk before applying the cluster)? (Please circle).

Yes, always Most of the time Occasionally

Only if suspect mastitis No Don't know

19. How much TIME do you leave between preparing teats & attaching the cluster? (Please circle).

No time (clusters are attached immediately)

Less than 30 s 30 s to 1 min 1-2 min more than 2 mins

20. Do you wear disposable GLOVES when milking? (Please circle).

Yes, always Most of the time Occasionally No Don't know

21. Do you use paper towel to DRY the teats before milking? (Please circle a letter).

A Yes, new towel for each cow B Yes, but the same towel for multiple cows

C No, I let them dry naturally

D I use another method to dry the cow's teats. Please specify……

22. In what ORDER do you milk the freshly calved cows? (Please circle a letter).

A Before the rest of the milking herd

B After the milking herd but before cows with clinical mastitis

C After the rest of the milking herd

D They are milked with the milking herd in any order

E Other, please specify……..

23. In what ORDER do you milk the cows with clinical mastitis? (Please circle a letter).

A Before the rest of the milking herd

B After the rest of the milking herd

C They are milked with the milking herd, into a dump bucket with the same cluster

D They are milked with the milking herd, into a dump bucket using a separate cluster

E Other, please specify……..

24. What type of PARLOUR do you use? (Please circle).

Herringbone Rotary Swing Side by side Rapid exit Robotic

Other, please specify…….

25. Do you use an Automatic Cluster Removal (ACR) system? (Please circle).

Yes, it cuts out at……. No Don't know Other, please specify……

26. Do you use an Automated Dipping & Flushing (ADF) system? (Please circle).

Yes No (Please move to 27.) Don't know (Please move to 27.)

26 a. If yes, what flushing fluid do you use? (Please circle).

Water Peracetic acid Other, please specify……

27. Do you DISINFECT the clusters between cows? (Please circle).

Yes, between every cow Yes, but only if a cow has a case of mastitis

No Don't know Other, please specify…….

27a. If yes, what disinfectant do you use? (Please state below).

……

28. What material are your TEAT LINERS made of? (Please circle).

Rubber Silicon Don't know Other, please specify…….

If possible, please can you provide the make and serial number……..(this will allow us to determine the shape and size of the liners).

29. On average, after how <u>many</u> milkings/months do you <u>CHANGE</u> your teat liners?

……milkings/months (please delete as appropriate)

30. How often is your milking machine <u>SERVICED</u>? (Please circle).

Every 1-5 months Every 6-9 months Every 10-12 months

Every 12-18 months When it needs it Never service the machine

31. Have you had a dynamic parlour test? (Please circle).

Yes, in the last 6 months Yes, in the last year Yes, but more than a year ago

No Don't know

31a. If Yes - Who performed your dynamic parlour test? (Please specify).

…….

32. <u>WHO</u> milks the cows? (e.g. Yourself only, relief milkers, students, family members, hired staff)

……

33. Do cows <u>LEAK</u> milk before/after milking? (Please circle a letter).

A Yes there are cows that leak milk <u>before & after</u> milking (approximate number……..)

B Yes there are cows that leak milk <u>before</u> they are milked (approximate number……..)

C Yes there are cows that leak milk <u>after</u> they are milked (approximate number……)

D <u>None</u> of the cows leak milk before or after they are milked

E Don't know

Farm Environment

Nearly there! We just have a few more questions regarding your farm in general.

34. Do you keep any <u>other animals</u> on your farm? (i.e. Beef cattle, sheep, pigs, horses, dogs, and cats) (Please circle).

Yes, please specify……

No, we keep only dairy cattle (please go to question 35.)

34 a. If you have sheep have you ever had a case of Contagious Ovine Digital Dermatitis (CODD) (Please see **Fig. 16** in the picture guide)?

Yes, currently Yes, in the last 12 months Yes but over 12 months ago

No Don't know

35. Do you <u>BUY IN</u> any replacement dairy cattle? (Please circle a letter).

A Yes, directly from the <u>same farms</u> every time

B Yes, from <u>auction market</u>

C No, all replacements are <u>bred on farm</u> (please proceed to question 36)

D Other, please specify……

35 a. Do you <u>ISOLATE</u> in a separate pen/field any dairy cattle brought on to the farm before introducing them into the herd? (Please circle).

Yes for…….. days/weeks/months (please delete as appropriate)

No Don't know

36. Do you rear your heifers on site? (Please circle one or more letters).

A Yes, they are reared <u>on the same site</u> as the milking herd

B Heifers are reared on the <u>same farm</u> but on a <u>different site</u> to the milking herd

C Heifers are reared on a <u>separate farm</u>

D Don't know

E Other, please specify……

37. Are calving pens <u>shared</u> with sick or lame cows? (Please circle).

Yes No I don't know Other, please specify……

38. Are freshly calved cows kept <u>separate</u> from the milking herd? (Please circle).

Yes for ……..days/weeks No Don't know

39. Please fill in the table below. (Tick all that applies to each group of cows and fill in type of bedding if applicable).

	Lactating cows	Freshly calved cows	Dry cows	Heifers
Access to pasture for some of the year				
Housed at night				
Housed in winter only				
Housed year round				
Type of housing				
Cubicle houses				
Loose housing				
No housing				
Type of bedding material				
Please specify in each box				

40. What <u>CATTLE BREEDS</u> are currently in your milking herd?

……

41. How <u>LONG</u> do calves stay with dams?

.....hours/days (delete as appropriate)

42. Do you use <u>artificial insemination (AI)</u>, <u>embryo transfer (ET)</u>, <u>bull</u> or a <u>mixture?</u> (Please circle).

AI ET Bull Mixture, please specify……

42 a. What are your current priorities when selecting an AI bull?

……

43. When do you calve? (Please circle).

Spring only Spring and autumn Autumn only Year round

Other, please specify……..

43a. If you calve <u>year round</u>, what is your average <u>NUMBER</u> of cows in milk and dry?

Cows in milk…….. Cows dry……

44. How <u>MANY</u> cattle do you have and what is the <u>SIZE</u> of your milking herd? (Please state number in each category).

Total cattle……. (including beef cattle, dairy bull calves etc.)

Total milking herd……..

45. What is your cows' average <u>LENGTH of lactation</u>? (Please state number).

……days

46. What is your average <u>MILK YIELD</u> per cow <u>per lactation</u>?

……

47. And lastly what <u>county/counties</u> is your farm in?

……

Please use the space below for anything you would like to tell us that you feel is important for us to consider, or if you feel there is anything we may have overlooked.

……
……
……
……
_____

If you wish to be entered into a prize draw for the chance to win a £100 Amazon voucher and to receive the results of this national survey please fill in your name and contact details below:

Name:

Address:

Postcode:

Email:

Telephone:

Would you be happy for us to contact you in the future about your questionnaire answers? (Please circle your response). Yes No

<u>THANK YOU FOR YOUR TIME IN PARTICIPATING IN THIS QUESTIONNAIRE!</u>

<u>Please note that your response has potentially helped hundreds of other dairy farmers and thousands of cattle throughout the UK!</u>

If you are willing to share any specific information on cows that have had ITN please fill in the table below.

<u>Date Calved</u>	<u>Date first saw the lesion</u>	<u>Lactation number</u>	<u>Details of the case</u>
E.g 29/08/2017	14/09/2017	1	Sore on teat, constantly licking, lost teat. Tried udder cream, no response.

A.2.1.1 The number of farmers reporting ischaemic teat necrosis (ITN) presence on the farm against each variable with the numbers that responded and the numbers that didn't respond to each question.

Variable name	Code used in the model	ITN Positive farms	ITN negative farms	Total number of responders	Non-responders
Country				225	3
England	1	86 (38.2%)	75 (33.3%)		
Wales	2	18 (8.0%)	23 (10.2%)		
Scotland	3	13 (5.8%)	10 (4.4%)		
Disease factors					
The presence of teat licking on the farm				224	4
No teat licking	0	8	100		
Have teat licking	1	88	28		
Presence of Ischaemic Teat Necrosis (ITN) on the farm				228	0
No ITN	0		109		
Have ITN	1	119			
Presence of bovine papillomas on farm				217	11
No bovine papilloma	0	49 (22.6%)	66 (30.4%)		
Have bovine papilloma	1	61 (28.1%)	41 (18.9%)		
Presence of Bovine Herpes mammillitis (BHM)				217	11
No ulcerative mammillitis	0	104 (47.9%)	107 (49.3%)		
have ulcerative mammillitis	1	6 (2.8%)	0		
Presence of chapped teats on farm				217	11
no cases of chapped teats	0	90 (41.5%)	103 (47.5%)		
cases of chapped teats	1	20 (9.2%)	4 (1.8%)		
Presence of udder acne on farm					
No udder acne	0	103 (47.5%)	105 (49.5%)		
Udder acne	1	7 (3.2%)	2 (0.92%)		
Presence of pseudocowpox on the farm				217	11
No cases of pseudocowpox	0	109 (50.2%)	106 (48.8%)		
cases of pseudocowpox	1	1 (0.46%)	1 (0.46%)		
Presence of Digital dermatitis (DD) on the farm				225	3
No DD on farm	0	8 (3.6%)	10 (4.4%)		
Farms with DD	1	109 (48.4%)	98 (43.6%)		
Presence of DD in spring farm level				212	6
Farms never had DD in spring	0	37 (17.5%)	42 (19.8%)		
Farms with DD in spring	1	72 (34.0%)	61 (28.8%)		
Presence of DD summer farm level				212	6
Farms never had DD in summer	0	50 (23.6%)	64(30.2%)		
Farms with DD in summer	1	59 (27.8%)	39 (18.4%)		
Presence of DD in autumn farm level				212	6
Farms never had DD in autumn	0	21 (9.9%)	34 (16.0%)		
Farms with DD in autumn	1	88 (41.5%)	69 (32.5%)		
Presence of DD in winter farm level				212	6
Farms never had DD in winter	0	15 (7.1%)	15 (7.1%)		
Farms with DD in winter	1	94 (44.3%)	88 (41.5%)		
DD worst in spring farm level				197	31
DD not worst in spring	0	77 (39.1%)	77 (39.1%)		
Farms with DD worst in spring	1	25 (12.7%)	18 (9.1%)		
DD worst in summer farm level				197	31
DD not worse in summer	0	96 (48.7%)	89 (45.2%)		

Farm with DD worst in summer	1	6 (3.0%)	6 (3.0%)		
DD worst in the autumn farm level				197	31
DD not worst in autumn	0	67 (34.0%)	76 (38.6%)		
farms with DD worst in autumn	1	35 (17.8%)	19 (9.6%)		
DD worst in the winter farm level				197	31
DD not worst in winter	0	28 (14.2%)	21 (10.7%)		
Farms with DD worst in winter	1	74 (37.6%)	74 (37.6%)		
Any Bovine viral diarrhoea virus (BVDv) persistently infected (PI) animals removed from farm				212	16
No BVDv PI removed from farm in last year	0	97 (45.8%)	94 (44.3%)		
BVDv PI removed from farm in last year	1	9 (4.2%)	12 (5.7%)		
Have you had any cases of tuberculosis (TB) on farm				225	3
No cases of TB in last year	0	87 (37.0%)	85 (36.2%)		
Confirmed cases of TB	1	23 (9.8%)	13 (5.5%)		
Reactor, but no lesions on post mortem	2	6 (2.4%)	11 (4.7%)		
Type of mastitis on the farm				145	83
No testing for mastitis	0	22 (14.5%)	38 (25.0%)		
Environmental mastitis	1	26 (17.1%)	25 (16.4%)		
Contagious mastitis	2	4 (2.6%)	6 (3.9%)		
Mixed environmental & contagious	3	9 (5.9%)	11 (7.2%)		
Test but don't specify	5	1 (0.66%)	3 (2.0%)		
Any contagious ovine digital dermatitis (CODD) in sheep				192	36
Farms without CODD	0	83 (43.2%)	79 (41.1%)		
Farms with CODD	1	15 (7.8%)	15 (7.8%)		
Average herd somatic cell count (SCC) for last year				214	14
< 100,000 cells	1	15 (7.0%)	20 (9.3%)		
101-150,000 cells	2	55 (25.7%)	43 (20.1%)		
151-200,000 cells	3	29 (13.6%)	37 (17.3%)		
> 200,000 cells	4	8 (3.7%)	7 (3.3%)		
Chemical factors					
Any pre milking teat product used (pre-dip)				224	4
No pre milking teat product used	0	42 (18.8%)	42 (18.8%)		
Pre milking teat product used	1	73 (32.6%)	67 (29.9%)		
Iodine in pre-dip?				111	117
No iodine in pre-dip	0	38 (34.2%)	38 (34.2%)		
Iodine in pre dip	1	20 (18.0%)	15 (13.5%)		
Chlorhexidine in pre-dip?				111	117
No chlorhexidine in pre-dip	0	49 (44.1%)	38 (34.2%)		
Chlorhexidine in pre-dip	1	9 (8.1%)	15 (13.5%)		
Peracetic acid in pre-dip?				111	117
No peracetic acid in pre dip	0	48 (43.2%)	49 (44.1%)		
Peracetic acid in pre-dip	1	10 (9.0%)	4 (3.6%)		
Chloride dioxide in pre-dip?				111	117
No chloride dioxide in pre dip	0	52 (46.8%)	47 (42.3%)		
Chloride dioxide in pre-dip	1	6 (5.4%)	6 (5.4%)		
Lactic acid in pre-dip?				111	117
No lactic acid in pre-dip	0	40 (36.0%)	35 (31.5%)		
Lactic acid in pre-dip	1	18 (16.2%)	18 (16.2%)		
Other active ingredients in pre-dip?				111	117
No other things in teat pre-dip	0	53 (47.7%)	49 (44.1%)		
Other things in teat pre-dip	1	5 (4.5%)	4 (3.6%)		
How are the teats cleaned				138	90

Spray teat dip	1	24 (10.7%)	21 (9.4%)		
Dip teat	2	14 (6.3%)	8 (3.6%)		
Foam cup	3	20 (8.9%)	30 (13.4%)		
Cloth/wipe	4	9 (4.0%)	3 (1.3%)		
Teat scrubber/ brush	5	6 (2.7%)	3 (1.3%)		
Which disinfectant used in pre milking preparation				116	112
Clean water	1	1 (0.54%)	2 (1.1%)		
hypochlorite	2	5 (2.7%)	7 (3.8%)		
Iodine	3	2 (1.1%)	2 (1.1%)		
Peracetic acid	4	52 (28.0%)	39 (21.0%)		
Others	5	5 (2.7%)	1 (0.54%)		
Use of an automatic dipping and flushing (ADF) system				213	15
Don't use ADF	0	74 (34.7%)	82 (38.5%)		
Do use ADF	1	37 (17.4%)	20 (9.4%)		
What is used in the flushing part of ADF				52	176
Flush with water	1	6 (2.9%)	5 (2.4%)		
Flush with peracetic acid	2	30 (14.4%)	11 (5.3%)		
If clusters are flushed, what are they flushed with?				116	112
Don't use water to flush	0	64 (55.2%)	49 (42.2%)		
Use water to flush	1	1 (0.86%)	2 (1.7%)		
If clusters are flushed, what are they flushed with?				116	112
Don't use hypochlorite to flush	0	60 (51.7%)	44 (37.9%)		
Use hypochlorite to flush	1	5 (4.3%)	7 (6.0%)		
If clusters are flushed, what are they flushed with?				116	112
Don't use iodine to flush	0	63 (54.3%)	49 (42.2%)		
Use iodine to flush	1	2 (1.7%)	2 (1.7%)		
If clusters are flushed, what are they flushed with?				116	112
Don't peracetic acid to flush	0	12 (10.3%)	11 (9.5%)		
Peracetic acid to flush	1	53 (45.7%)	40 (34.5%)		
If clusters are flushed, what are they flushed with?				116	112
Don't use hydrogen peroxide to flush	0	64 (55.2%)	50 (43.1%)		
Use hydrogen peroxide to flush	1	1 (0.86%)	1 (0.86%)		
If clusters are flushed, what are they flushed with?				116	112
Don't use other ingredient to flush	0	61 (52.6%)	51 (44.0%)		
Use other ingredients to flush	1	4 (3.4%)	0 (0.0%)		
Management and milking machine factors					
Teat discolouration after milking farm level				168	60
no teat discolouration	0	59 (35.1%)	66 (39.3%)		
cases of teat discolouration	1	26 (15.5%)	17 (10.1%)		
Teat ringing after milking farm level				169	59
no teat ringing	0	53 (31.4%)	65 (38.5%)		
cases of teat ringing	1	32 (18.9%)	19 (11.2%)		
Presence of teat end keratosis farm level				169	59
no teat end keratosis	0	36 (21.3%)	56 (33.1%)		
cases of teat end keratosis	1	49 (29.0%)	28 (16.6%)		
Presence of teat end eversion				167	61
no teat end eversion	0	70 (41.9%)	76 (45.5%)		
cases of teat end eversion	1	14 (8.4%)	7 (4.2%)		

Dry cow therapy practice				218	10
no dry cow therapy	0	4 (1.8%)	8 (3.7%)		
Selective Dry Cow Therapy (SDCT)	1	56 (25.7%)	52 (23.9%)		
Blanket Dry Cow Therapy (DCT)	2	51 (23.4%)	43 (19.7%)		
Other DCT	3	2 (0.9%)	2 (0.9%)		
Do you vaccinate against Bovine viral diarrhoea virus (BVD)				212	16
No vaccination against BVD	0	39 (18.4%)	49 (23.1%)		
Vaccinate against BVD	1	69 (32.5%)	55 (25.9%)		
Which vaccine do you use				112	116
Use Bovillis	1	35 (17.3%)	25 (12.4%		
Use Bovella	2	13 (6.4%)	10 (5.0%)		
Use Bovidec	3	16 (7.9%)	13 (6.4%)		
How many times a day the cows are milked				225	3
Milked voluntarily (robot)	0	6 (2.7%)	3 (1.3%)		
Milked twice daily	2	99 (44%)	102 (45.3%)		
Milked three times daily	3	8 (3.6%)	3 (1.3%)		
Milked other	4	3 (1.3%)	1 (0.44%)		
Foremilking on the farm				224	4
Don't foremilk	0	9 (4.0%)	22 (9.8%)		
Always foremilk	1	42 (18.8%)	29 (12.9%)		
Foremilk most of the time	2	12 (5.4%)	14 (6.3%)		
Foremilk occasionally	3	14 (6.3%)	17 (7.6%)		
Foremilk if suspect mastitis	4	38 (17.0%)	27 (12.1%)		
Time between preparing the teats and attaching the cluster				221	7
no time between preparing teats and attaching clusters	0	8 (3.6%)	8 (3.6%)		
<30 s	1	19 (8.6%)	22 (10.0%)		
30 s to 1 min	2	50 (22.6%)	47 (21.3%)		
1 to 2 mins	3	30 (13.6%)	23 (10.4%)		
more than 2 mins	4	7 (3.2%)	7 (3.2%)		
Glove use when milking				220	8
Don't use gloves	0	14 (6.4%)	16 (7.3%)		
Use gloves	1	99 (45%)	91 (41.4%		
Paper towel use to dry teats				219	9
no paper towel	0	15 (6.8%)	12 (5.4%)		
yes, new paper towels each cow	1	54 (24.4%)	49 (22.1%)		
same towel multiple cows	2	23 (10.4%)	32 (14.5%)		
wet wipes	3	6 (2.7%)	8 (3.6%)		
washable cloths	4	10 (4.5%)	5 (2.3%)		
robots	11	3 (4.5%)	2 (0.9%)		
When are freshly calved cows milked in relation to rest of the herd				222	6
fresh cows milked before the herd	1	18 (8.1%)	12 (5.4%)		
fresh cows milked after the herd, before mastitis	2	18 (8.1%)	12 (5.4%)		
fresh cows milked after the herd	3	28(12.6%)	29 (13.1%)		
fresh cows milked with the herd	4	50(22.5%)	53 (23.9%)		
other	5	1 (0.45%)	1 (0.45%)		
When are mastitis cows milked in relation to the rest of the herd				225	3
mastitis cows milked before herd	1	1 (0.44%)	1 (0.44%)		
mastitis cows milked after the herd	2	32 (14.2%)	27 (12%)		
milked with the herd, same cluster	3	48 (21.3%)	46 (20.4%)		
milked with the herd separate cluster	4	33 (14.7%)	33(14.7%)		
other	5	3 (1.3%)	1 (0.44%)		

Use of an automatic cluster release (ACR)				222	6
Don't use ACR	0	17 (7.7%)	27 (12.2%)		
Do use ACR	1	97 (43.7%)	81 (36.5%)		
what teat liner material is used				218	10
Have rubber teat liners	0	106	99 (45.4%)		
Have silicon teat liners	1	6	7 (3.2%)		
How often is the milking machine serviced				226	2
service milking machine every 1-5 months	1	5 (2.2%)	5 (2.2%)		
every 6-9 months	2	31 (13.7%)	16 (7.1%)		
every 10-12 months	3	61 (27.0%)	64 (28.3%)		
every 12-18 months	4	17 (7.5%)	22 (9.7%)		
when needed	5	3 (1.3%)	2 (0.88%)		
Has there been a dynamic parlour test				222	6
Haven't had a dynamic parlour test	0	34 (15.3%)	41 (18.5%)		
Have had a dynamic parlour test	1	81 (36.5%)	66 (29.7%)		
Who milks the cows				224	4
Family members milk cows	1	50 (22.3%)	39 (17.4%)		
Staff milk the cows	2	15 (6.7%)	14 (6.3%)		
Family members and staff milk cows	3	46 (20.5%)	53 (23.7%)		
Cows are milked by robots	4	5 (2.2%)	2 (0.89%)		
Do cows leak before/after milking				223	5
No cows leak milk	0	6 (2.7%)	8 (3.6%)		
cows leak before milking	1	93 (41.7%)	83 (37.2%)		
cows leak before and after milking	2	16 (7.2%)	17 (7.6%)		
Other animals on the farm				224	4
Farms without beef cows	0	64 (28.6%)	56 (25.0%)		
Farms with beef cows	1	53 (23.7%)	51 (22.8%)		
Other animals on the farm				224	4
Farms without cats	0	72 (32.1%)	68 (30.4%)		
Farms with cats	1	45 (20.1%)	39 (17.4%)		
Other animals on the farm				224	4
Farms without dogs	0	56 (25.0%)	54 (24.1%)		
farms with dogs	1	61 (27.2%)	53 (23.7%)		
Other animals on the farm				224	4
Farms without horses	0	94 (42.0%)	85 (37.9%)		
Farms with horses	1	23 (10.3%)	22 (9.8%)		
Other animals on the farm				224	4
Farms without pigs	0	113 (50.4%)	101 (45.1%)		
Farms with pigs	1	4 (1.8%)	6 (2.7%)		
Other animals on the farm				224	4
Farms without poultry	0	112 (50.0%)	101 (45.1%)		
Farms with poultry	1	5 (2.2%)	6 (2.7%)		
Other animals on the farm				224	4
Farms without sheep	0	69 (30.8%)	66 (29.5%)		
Farms with sheep	1	48 (21.4%)	41 (18.3%)		
Are clusters disinfected between milking cows				208	20
Don't disinfect clusters	0	25 (12.0%)	47 (22.6%)		
Disinfect clusters between every cow	1	38 (18.3%)	29 (13.9%)		
Disinfect cluster if mastitis/high SCC	2	41 (19.7%)	28 (13.5%)		
Where are replacements obtained from				221	7
All replacements bred on farm	0	77 (34.8%)	74 (33.5%)		
Buy direct from the same farms	1	6 (2.7%)	6 (2.7%)		
Buy from auction	2	25 (11.3%)	22 (10.0%)		
Buy from the European Union (EU)	3	4 (1.8%)	3 (1.4%)		

Other not specified	4	3 (1.4%)	1 (0.45%)		
Any isolation period of new livestock coming on to the farm				222	6
Farms with an isolation period	0	78 (35.1%)	79 (35.6%)		
Farms without an isolation period	1	38 (17.1%)	27 (12.2%)		
Are heifers reared on the farm				213	15
Heifers are reared on the same site	1	82 (37.3%)	62 (28.2%)		
Heifers reared on the same farm different site	2	21 (9.5%)	31 (14.1%)		
Reared on different farm	3	7 (3.2%)	10 (4.5%)		
Are calving pens shared with sick/lame animals				220	8
Calving pens not shared with sick/lame animals/ no calving pens/ calve outside	0	73 (33.2%)	74 (33.6%)		
Calving pens are shared or sometimes shared	1	41 (18.6%)	32 (14.5%)		
How are lactating cows managed				221	7
lactating cows housed year round	1	14 (6.3%)	9 (4.1%)		
lactating cows at pasture and housed at night	2	19 (8.6%)	11 (5.0%)		
lactating cows at pasture housed in winter	3	52 (23.5%)	68 (30.8%)		
lactating cows at pasture housed at night and in winter	4	12 (5.4%)	8 (3.6%)		
lactating cows at pasture	5	15 (6.8%)	8 (3.6%)		
lactating cows housed year round but have pasture access	6	4 (1.8%)	1 (0.45%)		
How are lactating cows housed				221	7
lactating cows housed in cubicles	1	81 (38.4%)	85 (40.3%)		
lactating cows loose housed	2	11 (5.2%)	9 (4.3%)		
lactating cows with cubicles and loose housing	3	20 (9.5%)	11 (5.2%)		
lactating cows have no housing	4	3 (1.4%)	1 (0.47%)		
what bedding is used for lactating cows				217	11
Lactating cows without straw	0	78 (35.9%)	63 (29.0%)		
lactating cows with straw	1	35 (16.1%)	41 (18.9%)		
what bedding is used for lactating cows				217	11
lactating cows without shavings	0	112 (51.6%)	102 (47.0%)		
lactating cows with shavings	1	1 (0.46%)	2 (0.92%)		
what bedding is used for lactating cows				217	11
lactating cows without sawdust	0	46 (21.2%)	55 (25.3%)		
lactating cows with sawdust	1	67 (30.9%)	49 (22.6%)		
what bedding is used for lactating cows				217	11
lactating cows without sand	0	95 (43.8%)	90 (41.5%)		
lactating cows with sand	1	18 (8.3%)	14 (6.5%)		
what bedding is used for lactating cows				217	11
lactating cows without lime	0	100 (46.1%)	96 (44.2%)		
lactating cows with lime	1	13 (6.0%)	8 (3.7%)		
what bedding is used for lactating cows				217	11
lactating cows without husk	0	112 (51.6%)	103 (47.5%)		
lactating cows with husk	1	1 (0.46%)	1 (0.46%)		
what bedding is used for lactating cows				217	11
lactating cows without paper	0	108 (49.8%)	102 (47.0%)		
lactating cows with paper	1	5 (2.3%)	2 (0.92%)		
How are dry cows managed				214	14
dry cows housed year round	1	12 (5.6%)	11 (5.1%)		
dry cows at pasture housed at night	2	6 (2.8%)	1 (0.47%)		
dry cows at pasture housed in winter	3	59 (27.6%)	66 (30.8%)		
dry cows at pasture housed at night and in winter	4	7 (3.3%)	5 (2.3%)		

Dry cows at pasture	5	25 (11.7%)	18 (8.4%)		
Dry cows housed year round with pasture access	6	2 (0.93%)	2 (0.9%)		
How are dry cows housed				218	10
Dry cow cubicles	1	44 (20.2%)	45 (20.6%)		
dry cow loose housing	2	36 (16.5%)	32 (14.7%)		
dry cow cubicles and loose	3	26 (11.9%)	24 (11.0%)		
dry cow no housing	4	7 (3.2%)	4 (1.8%)		
Dry cow bedding				208	20
Dry cows without straw	0	42 (20.2%)	38 (18.3%)		
Dry cows with straw	1	66 (31.7%)	62 (29.8%)		
Dry cow bedding				208	20
Dry cows without shavings	0	106 (51.0%)	99 (47.6%)		
Dry cows with shavings	1	2 (0.96%)	1 (0.48%)		
Dry cow bedding				208	20
Dry cows without sawdust	0	65 (31.3%)	65 (31.3%)		
Dry cows with sawdust	1	43 (20.7%)	35 (16.8%)		
Dry cow bedding				208	20
Dry cows without paper	0	105 (50.5%)	100 (48.1%)		
Dry cows with paper	1	3 (1.4%)	0 (0.0%)		
Dry cow bedding				208	20
Dry cows without lime	0	102 (49.0%)	96 (46.2%)		
Dry cows with lime	1	6 (2.9%)	4 (1.9%)		
Dry cow bedding				208	20
Dry cows without sand	0	97 (46.6%)	89 (42.8%)		
Dry cows with sand	1	11 (5.3%)	11 (5.3%)		
How are freshly calved cows managed				216	12
Fresh cows housed year round	1	25 (11.6%)	12 (5.6%)		
Fresh cows at pasture housed at night	2	17 (7.9%)	12 (5.6%)		
Fresh cows at pasture housed in winter	3	52 (24.1%)	59 (27.3%)		
Fresh cows at pasture housed at night and in winter	4	10 (4.6%)	9 (4.2%)		
Fresh cows at pasture	5	9 (4.2%)	11 (5.1%)		
How are freshly calved cows housed				216	12
Fresh cows cubicle housing	1	44 (20.4%)	58 (26.9%)		
Fresh cows loose housing	2	50 (23.1%)	30 (13.9%)		
Fresh cows cubicles and loose housing	3	16 (7.4%)	13 (6.0%)		
Fresh cows no housing	4	2 (0.93%)	3 (1.4%)		
Fresh cow bedding				210	18
Fresh cows not on straw	0	34 (16.2%)	47 (22.4%)		
Fresh cows on straw	1	75 (35.7%)	54 (25.7%)		
Fresh cow bedding				210	18
Fresh cows not on shavings	0	107 (51.0%)	100 (47.6%)		
Fresh cows on shavings	1	2 (0.95%)	1 (0.48%)		
Fresh cow bedding				210	18
Fresh cows not on sawdust	0	70 (33.3%)	68 (32.4%)		
Fresh cows on sawdust	1	39 (18.6%)	33 (15.7%)		
Fresh cow bedding				210	18
Fresh cows not on paper	0	107 (51.0%)	99 (47.1%)		
Fresh cows on paper	1	2 (0.95%)	2 (0.95%)		
Fresh cow bedding				210	18
Fresh cows not on lime	0	102 (48.6%)	97 (46.2%)		
Fresh cows on lime	1	7 (3.3%)	4 (1.9%)		
Fresh cow bedding				210	18
Fresh cows not on sand	0	97 (46.2%)	91 (43.3%)		
Fresh cows on sand	1	12 (5.7%)	10 (4.8%)		
How are heifers managed				210	18

Heifers housed year round	1	3 (1.4%)	0 (0.0%)		
Heifers at pasture and housed at night	2	6 (2.8%)	1 (0.47%)		
Heifers at pasture and house in winter	3	63 (29.9%)	81 (38.4%)		
heifers at pasture and housed at night and winter	4	7 (3.3%)	2 (0.95%)		
heifers at pasture	5	26 (12.3%)	15 (7.1%)		
Heifers housed year round with pasture access	6	5 (2.4%)	1 (0.47%)		
Heifer housing				205	23
Heifers in cubicles	1	49 (23.7%)	35 (16.9%)		
Heifers in loose housing	2	25 (12.1%)	39 (18.8%)		
Heifers in cubicles and loose	3	27 (13.0%)	20 (9.7%)		
No housing	4	7 (3.4%)	3 (1.4%)		
Heifer bedding				198	30
Heifers without straw	0	38 (19.2%)	32 (16.2%)		
Heifers with straw	1	64 (32.3%)	64 (32.3%)		
Heifer bedding				198	30
Heifers without shavings	0	99 (50.0%)	95 (48.0%)		
Heifers with shavings	1	3 (1.5%)	1 (0.51%)		
Heifer bedding				198	30
Heifers without sawdust	0	68 (34.3%)	69 (34.8%)		
Heifers with sawdust	1	34 (17.2%)	27 (13.6%)		
Heifer bedding				198	30
Heifers without sand	0	93 (47.0%)	91 (46.0%)		
Heifers with sand	1	9 (4.5%)	5 (2.5%)		
Heifer bedding				198	30
Heifers without lime	0	95 (48.0%)	94 (47.5%)		
Heifers with lime	1	7 (3.5%)	2 (1.0%)		
Breeds of dairy cows on farm				217	11
Multiple breeds/crossbreeds	1	13 (6.0%)	13 (6.0%)		
Multiple breeds excluding holstein/HF	2	6 (2.8%)	3 (1.4%)		
Multiple breeds excluding Jerseys	3	23 (10.6%)	23 (10.6%)		
Multiple breeds excluding holstein/HF & jerseys	4	2 (0.92%)	6 (2.8%)		
Pure Holstein	5	32 (14.7%)	13 (6.0%)		
Pure Friesians	6	2 (0.92%)	4 (1.8%)		
Pure Channel Isles	7	3 (1.4%)	3 (1.4%)		
Pure British Friesians	13	2 (0.92%)	6 (2.8%)		
Pure Holstein Friesians	14	30 (13.8%)	28 (12.9%)		
Other pure breed	15	2 (0.92%)	3 (1.4%)		
Type of insemination used				222	6
Artificial insemination (AI) only	1	49 (22.1%)	54 (24.3%)		
Bull only	2	5 (2.3%)	8 (3.6%)		
AI & Bull	4	54 (24.3%)	41 (18.5%)		
AI & Embryo transfer (ET)	5	2 (0.9%)	1 (0.45%)		
AI, Bull & ET	7	5 (2.3%)	3 (1.4%)		
Calving pattern				223	5
Calve year round	1	94 (42.2%)	77 (34.5%)		
Seasonal calving pattern	2	20 (9.0%)	27 (12.1%)		
Combination of year round and seasonal	5	2 (0.90%)	3 (1.3%)		
Length of dry cow period				218	10
30-39 days	1	2 (0.92%)	5 (2.3%)		
40-49 days	2	26 (11.9%)	23 (10.6%)		
50-59 days	3	38 (17.4%)	30 (13.8%)		
60+ days	4	45 (20.6%)	49 (22.5%)		
Length of time fresh cows are separate from milking herd				221	7
No time	0	42 (19.0%)	45 (20.4%)		

1-2 days		1	20 (9.0%)	18 (8.1%)		
3-6 days		2	33 (14.9%)	31 (14.0%)		
7+ days		4	20 (9.0%)	12 (5.4%)		
Time calves with dams					221	7
0-1 hours		1	3 (1.4%)	11 (5.0%)		
1<12 hours		2	47 (21.3%)	29 (13.1%)		
12<24 hours		3	27 (12.2%)	26 (11.8%)		
24<48 hours		4	20 (9.0%)	18 (8.1%)		
>48 hours		5	19 (8.6%)	21 (9.5%)		
Number of cows in milk					173	55
1-50 cows in milk	1		4 (1.8%)	8 (3.6%)		
51-100 cows in milk	2		23 (10.5%)	25 (11.4%)		
101-150 cows in milk	3		17 (7.7%)	20 (9.1%)		
151-200 cows in milk	4		23 (10.5%)	12 (5.5%)		
201-250 cows in milk	5		8 (3.6%)	10 (4.5%)		
251-300 cows in milk	6		9 (4.1%)	4 (1.8%)		
301+ cows in milk	7		9 (4.1%)	1 (0.45%)		
Total cattle herd size					205	23
Small total herd (60-179 animals)		1	12 (5.4%)	29 (13.0%)		
small to medium total herd (180-250)		2	25 (11.2%)	21 (9.4%)		
medium total herd (251-399)		3	24 (10.8%)	20 (9.0%)		
medium to large total herd (400-500)		4	21 (9.4%)	18 (8.1%)		
large total herd (501+)		5	33 (14.8%)	20 (9.0%)		
Total milking herd size					223	5
small milking herd (5-100 cows)		1	15 (6.7%)	30 (13.5%)		
small to medium milking herd (101-140)		2	22 (9.9%)	23 (10.3%)		
medium milking herd (141-200)		3	29 (13.0%)	22 (9.9%)		
medium to large milking herd (201-300)		4	28 (12.6%)	24 (10.8%)		
large milking herd (300+)		5	21 (9.4%)	9 (4.0%)		
Length of lactation					198	30
270-300 days		1	10 (5.1%)	11 (5.6%)		
301-350 days		2	45 (22.7%)	48 (24.2%)		
351-400 days		3	30 (15.2%)	31 (15.7%)		
401+ days		4	15 (7.6%)	8 (4.0%)		
Average milk yield per lactation					216	12
<6000 litres		1	17 (7.9%)	22 (10.2%)		
6001-8000 litres		2	28 (13.0%)	44 (20.4%)		
8001-10000 litres		3	53 (24.5%)	30 (13.9%)		
>10001 litres		4	14 (6.5%)	8 (3.7%)		

A.2.1.2 Comparison of study population characteristics with similar published national data for Great Britain (GB). The mean with the standard deviation (sd) in parenthesis. The median has the interquartile range (IQR) in parenthesis.

Variable	Mean	Median	Reference
Milking herd size			
Study dataset average (mean)	202 (sd: 82)	155 (IQR: 111-240)	dataset
AHDB all year round calving herd size, upper 25% of farms based on performance	311		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
AHDB all year round calving herd size, middle 50% based on performance	219		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
AHDB autumn calving herd top, upper 25% of farms based on performance	251		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
AHDB autumn calving herd middle performing herds 50%	217		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf

Average milk yield per cow (litres/cow/year)			
Study dataset average	8093 (sd:1773)	8000 (IQR: 6838-9055)	dataset
AHDB all year round calving herd top 25%	8,749		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
AHDB all year round calving herd middle 50%	8,396		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
AHDB autumn calving herd top 25%	7,550		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
AHDB autumn calving herd middle 50%	7,808		https://projectblue.blob.core.windows.net/media/Default/Dairy/Publications/DairyPerformResults3265_200317_WEB.pdf
John Nix Pocketbook all year round calving	8,000		Redman G. The John Nix Farm Management Pocketbook 2021 (51st Edition) ~ Contents. 2020.
John Nix Pocketbook autumn calving herd	6,000		Redman G. The John Nix Farm Management Pocketbook 2021 (51st Edition) ~ Contents. 2020.

Clinical mastitis rate (number of cases per 100 cows per year)			
Study dataset average	19 (SD: 15)	16 (IQR: 10-24)	dataset
AHDB Sentinel herd median 2018	26		https://ahdb.org.uk/knowledge-library/mastitis-in-dairy-cows-what-do-records-tell-us
National milk records 500 herd study median 2019	30		https://ahdb.org.uk/knowledge-library/mastitis-in-dairy-cows-what-do-records-tell-us
National milk records 500 herd study top 25% 2019	<18		https://ahdb.org.uk/knowledge-library/mastitis-in-dairy-cows-what-do-records-tell-us

Somatic cell count data (calculated bulk milk SCC, cells/ml)			
Study dataset average	145000 (sd:45,000)	140,000 (IQR: 114,000-180,000)	dataset
AHDB Sentinel herd median 2018	159,000		https://ahdb.org.uk/knowledge-library/mastitis-in-dairy-cows-what-do-records-tell-us
National milk records 500 herd study median 2019	171,000		https://ahdb.org.uk/knowledge-library/mastitis-in-dairy-cows-what-do-records-tell-us
National milk records 500 herd study top 25% 2019	<136,000		https://ahdb.org.uk/knowledge-library/mastitis-in-dairy-cows-what-do-records-tell-us

Calving patterns		
Study dataset year round calving pattern	77%	dataset
Study dataset seasonal calving pattern	21%	dataset
John Nix Pocketbook approximated year round calving pattern	85%	Redman G. The John Nix Farm Management Pocketbook 2021 (51st Edition) ~ Contents. 2020.

A.2.1.3 The univariable data using the presence or absence of ischaemic teat necrosis (ITN) on the farm as outcome variable. The proportion of each farm depending on the outcome is presented along with the number of farmers responding to the question and the odds ratio (OR). Lci-uci lower confidence interval-upper confidence interval. + positive, - negative

Variables	ITN + farms	ITN - farms	Number of responders	OR	lci	uci	p-value	variable name
Country			225					
England	86 (38.2%)	75 (33.3%)		1.15	0.84	1.56	*	47_country
Wales	18 (8.0%)	23 (10.2%)		0.68	0.34	1.36	0.28	47_country
Scotland	13 (5.8%)	10 (4.4%)		1.13	0.47	2.74	0.78	47_country
Disease factors								
The presence of teat licking on the farm			224					
No teat licking	28 (12.5%)	100 (44.6%)		0.28	0.18	0.43	*	q1
Teat licking	88 (39.3%)	8(3.57%)		39.29	17.02	90.67	0.00	q1
Presence of bovine papillomas on farm			217					
No cases of bovine warts	49 (22.6%)	66 (30.4%)		0.74	0.51	1.07	*	7_bwart
Cases of bovine warts	61 (28.1%)	41 (18.9%)		2.00	1.17	3.44	0.01	7_bwart
Presence of bovine herpes mammillitis (BHM)			217					
No cases of BHM	104 (47.9%)	107 (49.3%)		0.97	0.74	1.27	*	7_bum
Cases of BHM	6 (2.8%)	0 (0.0%)		######	0.00	Inf	0.99	7_bum
Presence of Udder Cleft Dermatitis (UCD)			217					
No cases of UCD	59 (27.2%)	81 (37.3%)		0.73	0.52	1.02	*	7_ucd
Cases of UCD	51 (23.5%)	26 (12.0%)		2.69	1.51	4.81	0.00	7_ucd
Presence of udder acne on farm			217					
No cases of udder acne	103 (47.5%)	105 (49.5%)		0.98	0.75	1.29	*	7_acne
Cases of udder acne	7 (3.2%)	2 (0.92%)		3.57	0.72	17.58	0.12	7_acne
Presence of pseudocowpox on farm			217					
No cases of pseudocowpox	109 (50.2%)	106 (48.8%)		1.03	0.79	1.34	*	7_pcpox
cases of pseudocowpox	1 (0.46%)	1 (0.46%)		0.97	0.06	15.75	0.98	7_pcpox
Presence of chapped teats on farm			217					
no cases of chapped teats	90 (41.5%)	103 (47.5%)		0.87	0.66	1.16	*	7_chatea
cases of chapped teats	20 (9.2%)	4 (1.8%)		5.72	1.89	17.37	0.00	7_chatea
Presence of digital dermatitis (DD) on the farm			225					
No DD on farm	8 (3.6%)	10 (4.4%)		0.80	0.32	2.03	*	q12 DD y/n
Farms with DD	109 (48.4%)	98 (43.6%)		1.39	0.53	3.66	0.51	q12 DD y/n
Presence of DD in spring farm level			212					
Farms never had DD in spring	37 (17.5%)	42 (19.8%)		0.88	0.57	1.37	*	q12c_spri_dd
Farms with DD in spring	72 (34.0%)	61 (28.8%)		1.34	0.77	2.34	0.30	q12c_spri_dd
Presence of DD summer farm level			212					
Farms never had DD in summer	50 (23.6%)	64(30.2%)		0.78	0.54	1.13	*	q12c_sum_dd
Farms with DD in summer	59 (27.8%)	39 (18.4%)		1.94	1.12	3.35	0.02	q12c_sum_dd
Presence of DD in autumn farm level			212					

Farms never had DD in autumn	21 (9.9%)	34 (16.0%)		0.62	0.36	1.06	*	q12c_aut_dd
Farms with DD in autumn	88 (41.5%)	69 (32.5%)		2.06	1.10	3.87	0.02	q12c_aut_dd
Presence of DD in winter farm level			212					
Farms never had DD in winter	15 (7.1%)	15 (7.1%)		1.00	0.49	2.05	*	q12c_win_dd
Farms with DD in winter	94 (44.3%)	88 (41.5%)		1.07	0.49	2.31	0.87	q12c_win_dd
DD worst in spring farm level			197					
DD not worst in spring	77 (39.1%)	77 (39.1%)		1.00	0.73	1.37	*	12d spring
Farms with DD worst in spring	25 (12.7%)	18 (9.1%)		1.39	0.70	2.75	0.35	12d spring
DD worst in summer farm level			197					
DD not worse in summer	96 (48.7%)	89 (45.2%)		1.08	0.81	1.44	*	12d summer
Farm with DD worst in summer	6 (3.0%)	6 (3.0%)		0.93	0.29	2.98	0.90	12d summer
DD worst in the autumn farm level			197					
DD not worst in autumn	67 (34.0%)	76 (38.6%)		0.88	0.63	1.22	*	12d autumn
farms with DD worst in autumn	35 (17.8%)	19 (9.6%)		2.09	1.09	3.99	0.03	12d autumn
DD worst in the winter farm level			197					
DD not worst in winter	28 (14.2%)	21 (10.7%)		1.33	0.76	2.35	*	12d winter
Farms with DD worst in winter	74 (37.6%)	74 (37.6%)		0.75	0.39	1.44	0.39	12d winter
Any bovine viral diarrhoea virus (BVD) persistently infected (PI) animals removed from farm			212					
No BVD PI removed from farm in last year	97 (45.8%)	94 (44.3%)		1.03	0.78	1.37	*	q13
BVD PI removed from farm in last year	9 (4.2%)	12 (5.7%)		0.73	0.29	1.80	0.49	q13
Have you had any cases of tuberculosis (TB) on farm			225					
No cases of TB in last year	87 (37.0%)	85 (36.2%)		1.02	0.76	1.38	*	q15 tb
Confirmed cases of TB	23 (9.8%)	13 (5.5%)		1.73	0.82	3.63	0.15	q15 tb
Reactor, but no lesions on pm	6 (2.4%)	11 (4.7%)		0.53	0.19	1.51	0.24	q15 tb
Any contagious ovine digital dermatitis (CODD) in sheep?			192					
Farms without CODD	83 (43.2%)	79 (41.1%)		1.05	0.77	1.43	*	34a_CODD
Farms with CODD	15 (7.8%)	15 (7.8%)		0.95	0.44	2.07	0.90	34a_CODD
Average somatic cell count (SCC) in last year			214					
< 100,000 cells	15 (7.0%)	20 (9.3%)		0.75	0.38	1.46	*	6_scc
101-150,000 cells	55 (25.7%)	43 (20.1%)		1.71	0.78	3.72	0.18	6_scc
151-200,000 cells	29 (13.6%)	37 (17.3%)		1.05	0.46	2.39	0.92	6_scc
> 200,000 cells	8 (3.7%)	7 (3.3%)		1.52	0.45	5.14	0.50	6_scc
Type of mastitis on the farm	7 (4.6%) not interpretable		152					
No testing for mastitis	22 (14.5%)	38 (25.0%)		0.58	0.34	0.98	*	q4
Environmental mastitis	26 (17.1%)	25 (16.4%)		1.66	0.78	3.55	0.19	q4
Contagious mastitis	4 (2.6%)	6 (3.9%)		2.59	0.66	10.19	0.17	q4
Mixed environmental & contagious	9 (5.9%)	11 (7.2%)		2.11	0.76	5.89	0.15	q4
Test but don't specify	1 (0.66%)	3 (2.0%)		5.18	0.51	52.90	0.17	q4
Chemical factors								

Any pre milking teat product used			224					
No pre milking teat product used	42 (18.8%)	42 (18.8%)		1.00	0.65	1.53	*	q17
Pre milking teat product used	73 (32.6%)	67 (29.9%)		1.09	0.63	1.87	0.76	q17
Which disinfectant used in pre milking preparation	70 (37.6%)	don't disinfect	186					
Clean water	1 (0.54%)	2 (1.1%)		0.50	0.05	5.51	*	27awdisc
hypochlorite	5 (2.7%)	7 (3.8%)		1.43	0.10	20.44	0.79	27awdisc
Iodine	2 (1.1%)	2 (1.1%)		2.00	0.09	44.35	0.66	27awdisc
Peracetic acid	52 (28.0%)	39 (21.0%)		2.67	0.23	30.48	0.43	27awdisc
Others	5 (2.7%)	1 (0.54%)		10.00	0.40	250.42	0.16	27awdisc
Iodine in pre-dip?			111					
No iodine in pre-dip	38 (34.2%)	38 (34.2%)		1.00	0.64	1.57	*	17_iod
Iodine in pre dip	20 (18.0%)	15 (13.5%)		1.33	0.60	2.99	0.48	17_iod
Chlorhexidine in pre-dip?			111					
No chlorhexidine in pre-dip	49 (44.1%)	38 (34.2%)		1.29	0.84	1.97	*	17_chlorhex
Chlorhexidine in pre-dip	9 (8.1%)	15 (13.5%)		0.47	0.18	1.18	0.11	17_chlorhex
Peracetic acid in pre-dip?			111					
No peracetic acid in pre dip	48 (43.2%)	49 (44.1%)		0.98	0.66	1.46	*	17_perac
Peracetic acid in pre-dip	10 (9.0%)	4 (3.6%)		2.55	0.75	8.70	0.13	17_perac
Chloride dioxide in pre-dip?			111					
No chloride dioxide in pre dip	52 (46.8%)	47 (42.3%)		1.11	0.75	1.64	*	17_chldio
Chloride dioxide in pre-dip	6 (5.4%)	6 (5.4%)		0.90	0.27	3.00	0.87	17_chldio
Lactic acid in pre-dip?			111					
No lactic acid in pre-dip	40 (36.0%)	35 (31.5%)		1.14	0.73	1.80	*	17_lacaci
Lactic acid in pre-dip	18 (16.2%)	18 (16.2%)		0.88	0.40	1.94	0.74	17_lacaci
Other active ingredients in pre-dip?			111					
No other things in teat pre-dip	53 (47.7%)	49 (44.1%)		1.08	0.73	1.60	*	17_other
Other things in teat pre-dip	5 (4.5%)	4 (3.6%)		1.16	0.29	4.55	0.84	17_other
How are the teats cleaned	86 (38.4%)	no pre dip	224					
Spray the product	24 (10.7%)	21 (9.4%)		1.14	0.64	2.05	*	q17b
dip the product	14 (6.3%)	8 (3.6%)		1.53	0.54	4.37	0.43	q17b
Use a foam cup	20 (8.9%)	30 (13.4%)		0.58	0.26	1.32	0.20	q17b
Use a cloth/wipe	9 (4.0%)	3 (1.3%)		2.63	0.63	10.99	0.19	q17b
Use a teat scrubber	6 (2.7%)	3 (1.3%)		1.75	0.39	7.88	0.47	q17b
Use of an automatic dipping and flushing (ADF) system			213					
Don't use ADF	74 (34.7%)	82 (38.5%)		0.90	0.66	1.24	*	q26
Do use ADF	37 (17.4%)	20 (9.4%)		2.05	1.09	3.84	0.03	q26
what is used in the flushing part of ADF	157 (75.1%)	no ADF system	209					
Flush with water	6 (2.9%)	5 (2.4%)		1.20	0.37	3.93	*	q26a_flushing_fluid
Flush with peracetic acid	30 (14.4%)	11 (5.3%)		2.27	0.58	8.97	0.24	q26a_flushing_fluid
Are clusters disinfected/flushed between cows			208					
Don't disinfect clusters	25 (12.0%)	47 (22.6%)		0.53	0.33	0.86	*	q27
Disinfect clusters between every cow	38 (18.3%)	29 (13.9%)		2.46	1.24	4.89	0.01	q27

Disinfect cluster if mastitis/high SCC	41 (19.7%)	28 (13.5%)		2.75	1.39	5.45	0.00	q27
If clusters are flushed, what are they flushed with?			116					
Don't use water to flush	64 (55.2%)	49 (42.2%)		1.31	0.90	1.89	*	27_water
Use water to flush	1 (0.86%)	2 (1.7%)		0.38	0.03	4.34	0.44	27_water
If clusters are flushed, what are they flushed with?			116					
Don't use hypochlorite to flush	60 (51.7%)	44 (37.9%)		1.36	0.92	2.01	*	27_hypoc
Use hypochlorite to flush	5 (4.3%)	7 (6.0%)		0.52	0.16	1.76	0.30	27_hypoc
If clusters are flushed, what are they flushed with?			116					
Don't use iodine to flush	63 (54.3%)	49 (42.2%)		1.29	0.89	1.87	*	27_iod
Use iodine to flush	2 (1.7%)	2 (1.7%)		0.78	0.11	5.72	0.81	27_iod
If clusters are flushed, what are they flushed with?			116					
Don't peracetic acid to flush	12 (10.3%)	11 (9.5%)		1.09	0.48	2.47	*	27_peraci
Peracetic acid to flush	53 (45.7%)	40 (34.5%)		1.21	0.49	3.03	0.68	27_peraci
If clusters are flushed, what are they flushed with?			116					
Don't use hydrogen peroxide to flush	64 (55.2%)	50 (43.1%)		1.28	0.88	1.85	*	27_hydroper
Use hydrogen peroxide to flush	1 (0.86%)	1 (0.86%)		0.78	0.05	12.80	0.86	27_hydroper
If clusters are flushed, what are they flushed with?			116					
Don't use other ingredient to flush	61 (52.6%)	51 (44.0%)		1.20	0.82	1.73	*	27_other
Use other ingredients to flush	4 (3.4%)	0 (0.0%)		######	0.00	Inf	0.99	27_other
Management and milking machine factors								
Teat discolouration after milking farm level			168					
no teat discolouration	59 (35.1%)	66 (39.3%)		0.89	0.63	1.27	*	8teat discolouration
cases of teat discolouration	26 (15.5%)	17 (10.1%)		1.71	0.85	3.46	0.14	8teat discolouration
Teat ringing after milking farm level			169					
no teat ringing	53 (31.4%)	65 (38.5%)		0.82	0.57	1.17	*	8 teat ringing
cases of teat ringing	32 (18.9%)	19 (11.2%)		2.07	1.05	4.05	0.03	8 teat ringing
Presence of teat end keratosis farm level			169					
no teat end keratosis	36 (21.3%)	56 (33.1%)		0.64	0.42	0.98	*	8 teat end keratosis
cases of teat end keratosis	49 (29.0%)	28 (16.6%)		2.72	1.46	5.09	0.00	8 teat end keratosis
Presence of teat end eversion			167					
no teat end eversion	70 (41.9%)	76 (45.5%)		0.92	0.67	1.27	*	8 teat evertion
cases of teat end eversion	14 (8.4%)	7 (4.2%)		2.17	0.83	5.69	0.12	8 teat evertion
Dry cow therapy practice			218					
no dry cow therapy	4 (1.8%)	8 (3.7%)		0.50	0.15	1.66	*	9 SDCT
Selective dry cow therapy (SDCT)	56 (25.7%)	52 (23.9%)		2.15	0.61	7.58	0.23	9 SDCT
Blanket dry cow therapy (DCT)	51 (23.4%)	43 (19.7%)		2.37	0.67	8.42	0.18	9 SDCT
Other DCT	2 (0.9%)	2 (0.9%)		2.00	0.20	19.91	0.55	9 SDCT
Do you vaccinate against bovine viral diarrhoea virus (BVD)			212					
No vaccination against BVD	39 (18.4%)	49 (23.1%)		0.80	0.52	1.21	*	q14 BVD vaccinated?
Vaccinate against BVD	69 (32.5%)	55 (25.9%)		1.58	0.91	2.73	0.11	q14 BVD vaccinated?

Which vaccine do you use against BVD	90 (44.6%)	no vaccination	202				
Use Bovillis	35 (17.3%)	25 (12.4%		1.40	0.84	2.34	*	q14 BVD vacine used
Use Bovella	13 (6.4%)	10 (5.0%)		0.93	0.35	2.45	0.88	q14 BVD vacine used
Use Bovidec	16 (7.9%)	13 (6.4%)		0.88	0.36	2.15	0.78	q14 BVD vacine used
How many times a day the cows are milked			225				
Milked voluntarily (robot)	6 (2.7%)	3 (1.3%)		2.00	0.50	8.00	*	q16
Milked twice daily	99 (44%)	102 (45.3%)		0.49	0.12	1.99	0.32	q16
Milked three times daily	8 (3.6%)	3 (1.3%)		1.33	0.20	9.08	0.77	q16
Milked other	3 (1.3%)	1 (0.44%)		1.50	0.11	21.31	0.77	q16
Foremilking on the farm			224				
Don't foremilk	9 (4.0%)	22 (9.8%)		0.41	0.19	0.89	*	q18
Yes, always foremilk	42 (18.8%)	29 (12.9%)		3.54	1.43	8.78	0.01	q18
Foremilk most of the time	12 (5.4%)	14 (6.3%)		2.10	0.70	6.25	0.19	q18
Foremilk occasionally	14 (6.3%)	17 (7.6%)		2.01	0.70	5.75	0.19	q18
Foremilk if suspect mastitis	38 (17.0%)	27 (12.1%)		3.44	1.37	8.63	0.01	q18
Time between preparing the teats and attaching the cluster			221				
No time between attaching the cluster	8 (3.6%)	8 (3.6%)		1.00	0.38	2.66	*	q19
<30 seconds	19 (8.6%)	22 (10.0%)		0.86	0.27	2.74	0.80	q19
30s-1 mins	50 (22.6%)	47 (21.3%)		1.06	0.37	3.06	0.91	q19
1-2 mins	30 (13.6%)	23 (10.4%)		1.30	0.43	4.00	0.64	q19
>2 mins	7 (3.2%)	7 (3.2%)		1.00	0.24	4.20	1.00	q19
Glove use when milking			220				
Don't use gloves	14 (6.4%)	16 (7.3%)		0.88	0.43	1.79	*	20_combined
Use gloves	99 (45%)	91 (41.4%		1.24	0.57	2.69	0.58	20_combined
Paper towel use to dry teats	2 (0.9%)	others excluded	221				
no paper towel	15 (6.8%)	12 (5.4%)		1.25	0.59	2.67	*	q21
yes, new paper towels each cow	54 (24.4%)	49 (22.1%)		0.88	0.38	2.07	0.77	q21
same towel multiple cows	23 (10.4%)	32 (14.5%)		0.58	0.23	1.46	0.24	q21
wet wipes	6 (2.7%)	8 (3.6%)		0.60	0.16	2.21	0.44	q21
washable cloths	10 (4.5%)	5 (2.3%)		1.60	0.43	5.96	0.48	q21
robots	3 (4.5%)	2 (0.9%)		1.20	0.17	8.38	0.85	q21
When are freshly calved cows milked in relation to rest of the herd			222				
fresh cows milked before the herd	18 (8.1%)	12 (5.4%)		1.50	0.72	3.11	*	q22
fresh cows milked after the herd, before mastitis	18 (8.1%)	12 (5.4%)		1.00	0.36	2.81	1.00	q22
fresh cows milked after the herd	28(12.6%)	29 (13.1%)		0.64	0.26	1.58	0.34	q22
fresh cows milked with the herd	50(22.5%)	53 (23.9%)		0.63	0.28	1.44	0.27	q22
other	1 (0.45%)	1 (0.45%)		0.67	0.04	11.72	0.78	q22
When are mastitis cows milked in relation to the rest of the herd			225				
mastitis cows milked before herd	1 (0.44%)	1 (0.44%)		1.00	0.06	15.99	*	q23
mastitis cows milked after the herd	32 (14.2%)	27 (12%)		1.19	0.07	19.86	0.91	q23
milked with the herd, same cluster	48 (21.3%)	46 (20.4%)		1.04	0.06	17.18	0.98	q23
milked with the herd separate cluster	33 (14.7%)	33(14.7%)		1.00	0.06	16.67	1.00	q23

other	3 (1.3%)	1 (0.44%)		3.00	0.08	107.45	0.55	q23
Use of an automatic cluster release (ACR)			222					
Don't use ACR	17 (7.7%)	27 (12.2%)		0.63	0.34	1.16	*	q25
Do use ACR	97 (43.7%)	81 (36.5%)		1.90	0.97	3.73	0.06	q25
what teat liner material is used			218					
Have rubber teat liners	106	99 (45.4%)		1.07	0.81	1.41	*	q28
Have silicon teat liners	6	7 (3.2%)		0.80	0.26	2.46	0.70	q28
How often is the milking machine serviced			226					
service milking machine every 1-5 months	5 (2.2%)	5 (2.2%)		1.00	0.29	3.45	*	q30
every 6-9 months	31 (13.7%)	16 (7.1%)		1.94	0.49	7.69	0.35	q30
every 10-12 months	61 (27.0%)	64 (28.3%)		0.95	0.26	3.46	0.94	q30
every 12-18 months	17 (7.5%)	22 (9.7%)		0.77	0.19	3.11	0.72	q30
when needed	3 (1.3%)	2 (0.88%)		1.50	0.17	13.23	0.72	q30
Has there been a dynamic parlour test			222					
Haven't had a dynamic parlour test	34 (15.3%)	41 (18.5%)		0.83	0.53	1.31	*	q31
Have had a dynamic parlour test	81 (36.5%)	66 (29.7%)		1.48	0.85	2.59	0.17	q31
Who milks the cows			224					
Family members milk cows	50 (22.3%)	39 (17.4%)		1.28	0.84	1.95	*	32_wmc
Staff milk the cows	15 (6.7%)	14 (6.3%)		0.84	0.36	1.94	0.68	32_wmc
Family members and staff milk cows	46 (20.5%)	53 (23.7%)		0.68	0.38	1.20	0.18	32_wmc
Cows are milked by robots	5 (2.2%)	2 (0.89%)		1.95	0.36	10.59	0.44	32_wmc
Do cows leak before/after milking			223					
No cows leak milk	6 (2.7%)	8 (3.6%)		0.75	0.26	2.16	*	q33
cows leak before milking	93 (41.7%)	83 (37.2%)		1.49	0.50	4.48	0.47	q33
cows leak before and after milking	16 (7.2%)	17 (7.6%)		1.25	0.36	4.42	0.72	q33
Other animals on the farm			224					
Farms without beef cows	64 (28.6%)	56 (25.0%)		1.14	0.80	1.64	*	34_beef
Farms with beef cows	53 (23.7%)	51 (22.8%)		0.91	0.54	1.54	0.72	34_beef
Other animals on the farm			224					
Farms without cats	72 (32.1%)	68 (30.4%)		1.06	0.76	1.47	*	34_cats
Farms with cats	45 (20.1%)	39 (17.4%)		1.09	0.63	1.87	0.76	34_cats
Other animals on the farm			224					
Farms without dogs	56 (25.0%)	54 (24.1%)		1.04	0.71	1.51	*	34_dogs
farms with dogs	61 (27.2%)	53 (23.7%)		1.11	0.66	1.88	0.70	34_dogs
Other animals on the farm			224					
Farms without horses	94 (42.0%)	85 (37.9%)		1.11	0.82	1.48	*	34_horse
Farms with horses	23 (10.3%)	22 (9.8%)		0.95	0.49	1.82	0.87	34_horse
Other animals on the farm			224					
Farms without pigs	113 (50.4%)	101 (45.1%)		1.12	0.86	1.46	*	34_pigs
Farms with pigs	4 (1.8%)	6 (2.7%)		0.60	0.16	2.17	0.43	34_pigs
Other animals on the farm			224					
Farms without poultry	112 (50.0%)	101 (45.1%)		1.11	0.85	1.45	*	34_poultry

Farms with poultry	5 (2.2%)	6 (2.7%)		0.75	0.22	2.54	0.65	34_poultry
Other animals on the farm			224					
Farms without sheep	69 (30.8%)	66 (29.5%)		1.05	0.75	1.47	*	34_sheep
Farms with sheep	48 (21.4%)	41 (18.3%)		1.12	0.66	1.91	0.68	34_sheep
Where are replacements obtained from			221					
All replacements bred on farm	77 (34.8%)	74 (33.5%)		1.04	0.76	1.43	*	35_buyin
Buy direct from the same farms	6 (2.7%)	6 (2.7%)		0.96	0.30	3.11	0.95	35_buyin
Buy from auction	25 (11.3%)	22 (10.0%)		1.09	0.57	2.10	0.79	35_buyin
Buy from the European Union (EU) outside Great Britain (GB)	4 (1.8%)	3 (1.4%)		1.28	0.28	5.92	0.75	35_buyin
Other not specified	3 (1.4%)	1 (0.45%)		2.88	0.29	28.34	0.36	35_buyin
Any isolation period of new livestock coming on to the farm			222					
Farms with an isolation period	78 (35.1%)	79 (35.6%)		0.99	0.72	1.35	*	35a_ison
Farms without an isolation period	38 (17.1%)	27 (12.2%)		1.43	0.79	2.56	0.23	35a_ison
Are heifers reared on the farm	7 (3.2%) don't rear heifers		220					
Heifers are reared on the same site	82 (37.3%)	62 (28.2%)		1.32	0.95	1.84	*	q36
Heifers reared on the same farm different site	21 (9.5%)	31 (14.1%)		0.51	0.27	0.98	0.04	q36
Reared on different farm	7 (3.2%)	10 (4.5%)		0.53	0.19	1.47	0.22	q36
Are calving pens shared with sick/lame animals			220					
Calving pens not shared with sick/lame animals/ no calving pens/ calve outside	73 (33.2%)	74 (33.6%)		0.99	0.71	1.36	*	37combined
Calving pens are shared or sometimes shared	41 (18.6%)	32 (14.5%)		1.30	0.74	2.28	0.36	37combined
How are lactating cows managed			221					
lactating cows housed year round	14 (6.3%)	9 (4.1%)		1.56	0.67	3.59	*	39lacc
lactating cows at pasture and housed at night	19 (8.6%)	11 (5.0%)		1.11	0.36	3.40	0.86	39lacc
lactating cows at pasture housed in winter	52 (23.5%)	68 (30.8%)		0.49	0.20	1.22	0.13	39lacc
lactating cows at pasture housed at night and in winter	12 (5.4%)	8 (3.6%)		0.96	0.28	3.28	0.95	39lacc
lactating cows at pasture	15 (6.8%)	8 (3.6%)		1.21	0.36	4.00	0.76	39lacc
lactating cows housed year round but have pasture access	4 (1.8%)	1 (0.45%)		2.57	0.25	26.85	0.43	39lacc
How are lactating cows housed			211					
lactating cows housed in cubicles	81 (38.4%)	85 (40.3%)		0.95	0.70	1.29	*	39_lacch
lactating cows loose housed	11 (5.2%)	9 (4.3%)		1.28	0.51	3.26	0.60	39_lacch
lactating cows with cubicles and loose housing	20 (9.5%)	11 (5.2%)		1.91	0.86	4.23	0.11	39_lacch
lactating cows have no housing	3 (1.4%)	1 (0.47%)		3.15	0.32	30.89	0.33	39_lacch
what bedding is used for lactating cows			217					
Lactating cows without straw	78 (35.9%)	63 (29.0%)		1.24	0.89	1.73	*	laccstraw
lactating cows with straw	35 (16.1%)	41 (18.9%)		0.69	0.39	1.21	0.19	laccstraw
what bedding is used for lactating cows			217					
lactating cows without shavings	112 (51.6%)	102 (47.0%)		1.10	0.84	1.44	*	laccsha

lactating cows with shavings	1 (0.46%)	2 (0.92%)		0.46	0.04	5.10	0.52	laccsha
what bedding is used for lactating cows			217					
lactating cows without sawdust	46 (21.2%)	55 (25.3%)		0.84	0.57	1.24	*	lacsaw
lactating cows with sawdust	67 (30.9%)	49 (22.6%)		1.63	0.95	2.80	0.07	lacsaw
what bedding is used for lactating cows			217					
lactating cows without sand	95 (43.8%)	90 (41.5%)		1.06	0.79	1.41	*	laccsand
lactating cows with sand	18 (8.3%)	14 (6.5%)		1.22	0.57	2.59	0.61	laccsand
what bedding is used for lactating cows			217					
lactating cows without lime	100 (46.1%)	96 (44.2%)		1.04	0.79	1.38	*	lacclim
lactating cows with lime	13 (6.0%)	8 (3.7%)		1.56	0.62	3.93	0.35	lacclim
what bedding is used for lactating cows			217					
lactating cows without husk	112 (51.6%)	103 (47.5%)		1.09	0.83	1.42	*	lacchusk
lactating cows with husk	1 (0.46%)	1 (0.46%)		0.92	0.06	14.89	0.95	lacchusk
what bedding is used for lactating cows			217					
lactating cows without paper	108 (49.8%)	102 (47.0%)		1.06	0.81	1.39	*	laccpap
lactating cows with paper	5 (2.3%)	2 (0.92%)		2.36	0.45	12.44	0.31	laccpap
How are dry cows managed			214					
dry cows housed year round	12 (5.6%)	11 (5.1%)		1.09	0.48	2.47	*	39_dryc
dry cows at pasture housed at night	6 (2.8%)	1 (0.47%)		5.50	0.57	53.22	0.14	39_dryc
dry cows at pasture housed in winter	59 (27.6%)	66 (30.8%)		0.82	0.34	2.00	0.66	39_dryc
dry cows at pasture housed at night and in winter	7 (3.3%)	5 (2.3%)		1.28	0.31	5.25	0.73	39_dryc
Dry cows at pasture	25 (11.7%)	18 (8.4%)		1.27	0.46	3.52	0.64	39_dryc
Dry cows housed year round with pasture access	2 (0.93%)	2 (0.9%)		0.92	0.11	7.67	0.94	39_dryc
How are dry cows housed			218					
Dry cow cubicles	44 (20.2%)	45 (20.6%)		0.98	0.65	1.48	*	39_drych
dry cow loose housing	36 (16.5%)	32 (14.7%)		1.15	0.61	2.16	0.66	39_drych
dry cow cubicles and loose	26 (11.9%)	24 (11.0%)		1.11	0.55	2.22	0.77	39_drych
dry cow no housing	7 (3.2%)	4 (1.8%)		1.79	0.49	6.55	0.38	39_drych
Dry cow bedding			208					
Dry cows without straw	42 (20.2%)	38 (18.3%)		1.11	0.71	1.71	*	drycstraw
Dry cows with straw	66 (31.7%)	62 (29.8%)		0.96	0.55	1.68	0.90	drycstraw
Dry cow bedding			208					
Dry cows without shavings	106 (51.0%)	99 (47.6%)		1.07	0.81	1.41	*	drycshav
Dry cows with shavings	2 (0.96%)	1 (0.48%)		1.87	0.17	20.92	0.61	drycshav
Dry cow bedding			208					
Dry cows without sawdust	65 (31.3%)	65 (31.3%)		1.00	0.71	1.41	*	drycsaw
Dry cows with sawdust	43 (20.7%)	35 (16.8%)		1.23	0.70	2.16	0.47	drycsaw
Dry cow bedding			208					
Dry cows without paper	105 (50.5%)	100 (48.1%)		1.05	0.80	1.38	*	drycpap
Dry cows with paper	3 (1.4%)	0 (0.0%)		######	0.00	Inf	0.99	drycpap
Dry cow bedding			208					

Dry cows without lime	102 (49.0%)	96 (46.2%)		1.06	0.80	1.40	*	dryclim
Dry cows with lime	6 (2.9%)	4 (1.9%)		1.41	0.39	5.16	0.60	dryclim
Dry cow bedding			208					
Dry cows without sand	97 (46.6%)	89 (42.8%)		1.09	0.82	1.45	*	drycsand
Dry cows with sand	11 (5.3%)	11 (5.3%)		0.92	0.38	2.22	0.85	drycsand
How are freshly calved cows managed			216					
Fresh cows housed year round	25 (11.6%)	12 (5.6%)		2.08	1.05	4.15	*	39_fresc
Fresh cows at pasture housed at night	17 (7.9%)	12 (5.6%)		0.68	0.25	1.87	0.45	39_fresc
Fresh cows at pasture housed in winter	52 (24.1%)	59 (27.3%)		0.42	0.19	0.93	0.03	39_fresc
Fresh cows at pasture housed at night and in winter	10 (4.6%)	9 (4.2%)		0.53	0.17	1.66	0.28	39_fresc
Fresh cows at pasture	9 (4.2%)	11 (5.1%)		0.39	0.13	1.20	0.10	39_fresc
How are freshly calved cows housed			216					
Fresh cows cubicle housing	44 (20.4%)	58 (26.9%)		0.76	0.51	1.12	*	39_fresch
Fresh cows loose housing	50 (23.1%)	30 (13.9%)		2.20	1.21	4.00	0.01	39_fresch
Fresh cows cubicles and loose housing	16 (7.4%)	13 (6.0%)		1.62	0.71	3.72	0.25	39_fresch
Fresh cows no housing	2 (0.93%)	3 (1.4%)		0.88	0.14	5.49	0.89	39_fresch
Fresh cow bedding			210					
Fresh cows not on straw	34 (16.2%)	47 (22.4%)		0.72	0.47	1.12	*	fresstra
Fresh cows on straw	75 (35.7%)	54 (25.7%)		1.92	1.09	3.37	0.02	fresstra
Fresh cow bedding			210					
Fresh cows not on shavings	107 (51.0%)	100 (47.6%)		1.07	0.81	1.41	*	fresshav
Fresh cows on shavings	2 (0.95%)	1 (0.48%)		1.87	0.17	20.93	0.61	fresshav
Fresh cow bedding			210					
Fresh cows not on sawdust	70 (33.3%)	68 (32.4%)		1.03	0.74	1.44	*	fressaw
Fresh cows on sawdust	39 (18.6%)	33 (15.7%)		1.15	0.65	2.03	0.64	fressaw
Fresh cow bedding			210					
Fresh cows not on paper	107 (51.0%)	99 (47.1%)		1.08	0.82	1.42	*	frespap
Fresh cows on paper	2 (0.95%)	2 (0.95%)		0.93	0.13	6.69	0.94	frespap
Fresh cow bedding			210					
Fresh cows not on lime	102 (48.6%)	97 (46.2%)		1.05	0.80	1.39	*	freslim
Fresh cows on lime	7 (3.3%)	4 (1.9%)		1.66	0.47	5.86	0.43	freslim
Fresh cow bedding			210					
Fresh cows not on sand	97 (46.2%)	91 (43.3%)		1.07	0.80	1.42	*	fressand
Fresh cows on sand	12 (5.7%)	10 (4.8%)		1.13	0.46	2.73	0.79	fressand
How are heifers managed	1 (0.47%)	no heifers	211					
Heifers housed year round	3 (1.4%)	0 (0.0%)		######	0.00	Inf	*	39_heif
Heifers at pasture and housed at night	6 (2.8%)	1 (0.47%)		0.00	0.00	Inf	0.99	39_heif
Heifers at pasture and house in winter	63 (29.9%)	81 (38.4%)		0.00	0.00	Inf	0.99	39_heif
heifers at pasture and housed at night and winter	7 (3.3%)	2 (0.95%)		0.00	0.00	Inf	0.99	39_heif
heifers at pasture	26 (12.3%)	15 (7.1%)		0.00	0.00	Inf	0.99	39_heif
Heifers housed year round with pasture access	5 (2.4%)	1 (0.47%)		0.00	0.00	Inf	0.99	39_heif

Heifer housing	2 (0.96%) no heifers		207					
Heifers in cubicles	49 (23.7%)	35 (16.9%)		1.40	0.91	2.16	*	39_heifh
Heifers in loose housing	25 (12.1%)	39 (18.8%)		0.46	0.24	0.89	0.02	39_heifh
Heifers in cubicles and loose	27 (13.0%)	20 (9.7%)		0.96	0.47	1.99	0.92	39_heifh
No housing	7 (3.4%)	3 (1.4%)		1.67	0.40	6.90	0.48	39_heifh
Heifer bedding			198					
Heifers without straw	38 (19.2%)	32 (16.2%)		1.19	0.74	1.90	*	heifstraw
Heifers with straw	64 (32.3%)	64 (32.3%)		0.84	0.47	1.51	0.56	heifstraw
Heifer bedding			198					
Heifers without shavings	99 (50.0%)	95 (48.0%)		1.04	0.79	1.38	*	heisha
Heifers with shavings	3 (1.5%)	1 (0.51%)		2.88	0.29	28.16	0.36	heisha
Heifer bedding			198					
Heifers without sawdust	68 (34.3%)	69 (34.8%)		0.99	0.71	1.38	*	heifsaw
Heifers with sawdust	34 (17.2%)	27 (13.6%)		1.28	0.70	2.34	0.43	heifsaw
Heifer bedding			198					
Heifers without sand	93 (47.0%)	91 (46.0%)		1.02	0.77	1.36	*	heifsand
Heifers with sand	9 (4.5%)	5 (2.5%)		1.76	0.57	5.46	0.33	heifsand
Heifer bedding			198					
Heifers without lime	95 (48.0%)	94 (47.5%)		1.01	0.76	1.34	*	heiflim
Heifers with lime	7 (3.5%)	2 (1.0%)		3.46	0.70	17.10	0.13	heiflim
Breeds of dairy cows on farm			217					
Multiple breeds/crossbreeds	13 (6.0%)	13 (6.0%)		1.00	0.46	2.16	*	40_catbre2
Multiple breeds excluding holstein/HF	6 (2.8%)	3 (1.4%)		2.00	0.41	9.76	0.39	40_catbre2
Multiple breeds excluding Jerseys	23 (10.6%)	23 (10.6%)		1.00	0.38	2.62	1.00	40_catbre2
Multiple breeds excluding holstein/HF & jerseys	2 (0.92%)	6 (2.8%)		0.33	0.06	1.97	0.23	40_catbre2
Pure Holstein	32 (14.7%)	13 (6.0%)		2.46	0.90	6.71	0.08	40_catbre2
Pure Friesians	2 (0.92%)	4 (1.8%)		0.50	0.08	3.22	0.47	40_catbre2
Pure Channel Isles	3 (1.4%)	3 (1.4%)		1.00	0.17	5.90	1.00	40_catbre2
Pure British Friesians	2 (0.92%)	6 (2.8%)		0.33	0.06	1.97	0.23	40_catbre2
Pure Holstein Friesians (HF)	30 (13.8%)	28 (12.9%)		1.07	0.42	2.70	0.88	40_catbre2
Other pure breed	2 (0.92%)	3 (1.4%)		0.67	0.10	4.67	0.68	40_catbre2
Type of insemination used			222					
Artificial insemination (AI) only	49 (22.1%)	54 (24.3%)		0.91	0.62	1.34	*	q42
Bull only	5 (2.3%)	8 (3.6%)		0.69	0.21	2.25	0.54	q42
AI & Bull	54 (24.3%)	41 (18.5%)		1.45	0.83	2.54	0.19	q42
AI & embryo transfer (ET)	2 (0.9%)	1 (0.45%)		2.20	0.19	25.07	0.52	q42
AI, Bull & ET	5 (2.3%)	3 (1.4%)		1.84	0.42	8.09	0.42	q42
Length of the dry cow period			218					
30-39 days	2 (0.92%)	5 (2.3%)		0.40	0.08	2.06	*	10_ldryp
40-49 days	26 (11.9%)	23 (10.6%)		2.83	0.50	15.99	0.24	10_ldryp
50-59 days	38 (17.4%)	30 (13.8%)		3.17	0.57	17.48	0.19	10_ldryp
60+ days	45 (20.6%)	49 (22.5%)		2.30	0.42	12.43	0.33	10_ldryp

Length of time fresh cows are separate from milking herd			221					
No time	42 (19.0%)	45 (20.4%)		0.93	0.61	1.42	*	q38
1-2 days	20 (9.0%)	18 (8.1%)		1.19	0.56	2.55	0.65	
3-6 days	33 (14.9%)	31 (14.0%)		1.14	0.60	2.18	0.69	
7+ days	20 (9.0%)	12 (5.4%)		1.79	0.78	4.10	0.17	
Time calves with dams			221					
0-1 hours	3 (1.4%)	11 (5.0%)		0.27	0.08	0.98	*	41_tcwd
1<12 hours	47 (21.3%)	29 (13.1%)		5.94	1.53	23.10	0.01	41_tcwd
12<24 hours	27 (12.2%)	26 (11.8%)		3.81	0.95	15.22	0.06	41_tcwd
24<48 hours	20 (9.0%)	18 (8.1%)		4.07	0.98	16.97	0.05	41_tcwd
>48 hours	19 (8.6%)	21 (9.5%)		3.32	0.80	13.72	0.10	41_tcwd
Number of cows in milk (year round calving system)	47 (21.4%) seasonal calvers		220					
1-50 cows in milk	4 (1.8%)	8 (3.6%)		0.50	0.15	1.66	*	43aimc
51-100 cows in milk	23 (10.5%)	25 (11.4%)		1.84	0.49	6.94	0.37	
101-150 cows in milk	17 (7.7%)	20 (9.1%)		1.70	0.43	6.65	0.45	
151-200 cows in milk	23 (10.5%)	12 (5.5%)		3.83	0.96	15.37	0.06	
201-250 cows in milk	8 (3.6%)	10 (4.5%)		1.60	0.35	7.30	0.54	
251-300 cows in milk	9 (4.1%)	4 (1.8%)		4.50	0.84	24.18	0.08	
301+ cows in milk	9 (4.1%)	1 (0.45%)		18.00	1.65	196.28	0.02	
Number of cows dry (year round calving system)	47 (21.5%) seasonal calvers		219					
1-20 dry cows	47 (21.5%)	48 (21.9%)		0.98	0.65	1.46	*	43adry
21-40 dry cows	32 (14.6%)	28 (12.8%)		1.17	0.61	2.23	0.64	
41-65 dry cows	10 (4.6%)	3 (1.4%)		3.40	0.88	13.15	0.08	
65+ dry cows	3 (1.4%)	1 (0.46%)		3.06	0.31	30.52	0.34	
Total cattle herd size			223					
Small total herd (60-179 animals)	12 (5.4%)	29 (13.0%)		0.41	0.21	0.81	*	44_totcat
small to medium total herd (180-250)	25 (11.2%)	21 (9.4%)		2.88	1.18	6.99	0.02	44_totcat
medium total herd (251-399)	24 (10.8%)	20 (9.0%)		2.90	1.18	7.11	0.02	44_totcat
medium to large total herd (400-500)	21 (9.4%)	18 (8.1%)		2.82	1.12	7.08	0.03	44_totcat
large total herd (500+)	33 (14.8%)	20 (9.0%)		3.99	1.67	9.54	0.00	44_totcat
Total milking herd size			223					
small milking herd (5-100 cows)	15 (6.7%)	30 (13.5%)		0.50	0.27	0.93	*	44_totmilk
small to medium milking herd (101-140)	22 (9.9%)	23 (10.3%)		1.91	0.82	4.48	0.14	44_totmilk
medium milking herd (141-200)	29 (13.0%)	22 (9.9%)		2.64	1.15	6.05	0.02	44_totmilk
medium to large milking herd (201-300)	28 (12.6%)	24 (10.8%)		2.33	1.02	5.33	0.04	44_totmilk
large milking herd (300+)	21 (9.4%)	9 (4.0%)		4.67	1.72	12.65	0.00	44_totmilk
Length of lactation			198					
270-300 days	10 (5.1%)	11 (5.6%)		0.91	0.39	2.14	*	45_length_of_lactation
301-350 days	45 (22.7%)	48 (24.2%)		1.03	0.40	2.66	0.95	45_length_of_lactation
351-400 days	30 (15.2%)	31 (15.7%)		1.06	0.39	2.87	0.90	45_length_of_lactation

401+ days	15 (7.6%)	8 (4.0%)	2.06	0.61	6.93	0.24	45_length_of_lactation
Average milk yield per lactation		216					
<6000 litres	17 (7.9%)	22 (10.2%)	0.77	0.41	1.46	*	46_milkyield
6001-8000 litres	28 (13.0%)	44 (20.4%)	0.82	0.37	1.82	0.63	46_milkyield
8001-10000 litres	53 (24.5%)	30 (13.9%)	2.29	1.05	4.96	0.04	46_milkyield
>10001 litres	14 (6.5%)	8 (3.7%)	2.26	0.77	6.63	0.14	46_milkyield

* reference

A.2.2 ***Probability of having ischaemic teat necrosis (ITN) in relation to the presence of udder cleft dermatitis (UCD) or chapped teats on the farm****: Predicted percentage probabilities from the final multivariable model.*

UCD on Farm	Chapped teats on Farm	Predicted Percentage probability of having ITN from the model	Observed percentage of farms with ITN
No	No	37.8% (29.8-46.5%)	37.6%
Yes	No	63.0% (55.2-91.7%)	63.2 %
No	Yes	78.6% (51.1-73.4%)	80.0%
Yes	Yes	91.2% (76.0-97.1%)	88.9%

A.3.1 The univariable data using the presence of udder cleft dermatitis (UCD) on the farm as the outcome variable.

Variables	OR	lci	uci	p-value	variable
Country					
England	0.53	0.38	0.74	*	47_country
Wales	0.91	0.43	1.91	0.80	47_country
Scotland	1.57	0.64	3.88	0.33	47_country
Disease factors					
Presence of ischaemic teat necrosis (ITN) on the farm					
No ITN	0.32	0.21	0.50	*	q2
Have ITN	2.69	1.51	4.81	<0.001	q2
Presence of bovine papillomas on farm					
No bovine papilloma	0.40	0.27	0.60	*	7_bwart
Have bovine papilloma	1.89	1.07	3.31	0.03	7_bwart
Presence of bovine herpes mammillitis (BHM)					
No BHM	0.53	0.40	0.70	*	7_bum
Have BHM	3.78	0.68	21.13	0.13	7_bum
Presence of chapped teats on farm					
No chapped teats	0.54	0.40	0.73	*	7_chapped teats
Chapped teats	1.10	0.46	2.65	0.83	7_chapped teats
Presence of udder acne on farm					
No udder acne	0.52	0.39	0.69	*	7_acne
Udder acne	3.86	0.94	15.89	0.06	7_acne
Presence of pseudocowpox on the farm					
No pseudocowpox	0.55	0.41	0.72	*	7_pcpox
Pseudocowpox	1.83	0.11	29.65	0.67	7_pcpox
The presence of teat licking on the farm					
no teat licking	0.36	0.24	0.53	*	q1
has teat licking	2.66	1.50	4.74	0.00	q1
Presence of digital dermatitis (DD) on the farm					
no DD on the farm	0.07	0.01	0.54	*	q12 DD y/n

DD on the farm	8.29	1.07	64.32	0.04	q12 DD y/n
Presence of DD in spring farm level					
Farms never had DD in spring	0.31	0.18	0.52	*	q12c_spri_dd
Farms with DD in spring	2.41	1.28	4.56	0.01	q12c_spri_dd
Presence of DD summer farm level					
Farms never had DD in summer	0.36	0.24	0.55	*	q12c_sum_dd
Farms with DD in summer	2.27	1.26	4.10	0.01	q12c_sum_dd
Presence of DD in autumn farm level					
Farms never had DD in autumn	0.31	0.16	0.59	*	q12c_aut_dd
Farms with DD in autumn	2.08	1.01	4.30	0.05	q12c_aut_dd
Presence of DD in winter farm level					
Farms never had DD in winter	0.23	0.09	0.60	*	q12c_win_dd
Farms with DD in winter	2.66	0.96	7.37	0.06	q12c_win_dd
DD worst in spring farm level					
DD not worst in spring	0.41	0.29	0.59	*	12d spring
Farms with DD worst in spring	2.80	1.38	5.69	0.00	12d spring
DD worst in summer farm level					
DD not worse in summer	0.50	0.37	0.69	*	12d summer
Farm with DD worst in summer	1.98	0.61	6.42	0.25	12d summer
DD worst in the autumn farm level					
DD not worst in autumn	0.48	0.34	0.69	*	12d autumn
farms with DD worst in autumn	1.36	0.70	2.62	0.36	12d autumn
DD worst in the winter farm level					
DD not worst in winter	0.24	0.12	0.50	*	12d winter
Farms with DD worst in winter	2.68	1.20	5.97	0.02	12d winter
Any bovine viral diarrhoea (BVD) persistently infected (PI) animals removed from farm					
No BVD PI removed from farm in last year	0.51	0.38	0.70	*	q13
BVD PI removed from farm in last year	1.20	0.47	3.05	0.70	q13
Have you had any cases of tuberculosis (TB) on farm					
No TB	0.59	0.43	0.80	*	q15 tb
Confirmed case	1.11	0.51	2.39	0.79	q15 tb
Reactors no disease	0.11	0.01	0.82	0.03	q15 tb
Any contagious ovine digital dermatitis (CODD) in sheep					
Farms without CODD	0.46	0.32	0.64	*	34a_CODD
Farms with CODD	1.46	0.65	3.27	0.36	34a_CODD
Average somatic cell count (SCC) in last year					
< 100,000 cells	0.83	0.42	1.65	*	6_scc
101-150,000 cells	0.77	0.35	1.72	0.53	6_scc
151-200,000 cells	0.43	0.18	1.03	0.06	6_scc
> 200,000 cells	0.33	0.08	1.39	0.13	6_scc
Type of mastitis on the farm					
No testing for mastitis	0.40	0.23	0.71	*	q4
Environmental mastitis	1.43	0.64	3.22	0.38	q4
Contagious mastitis	0.62	0.12	3.21	0.57	q4
Mixed environmental & contagious	2.47	0.87	7.00	0.09	q4
Test but don't specify	2.47	0.32	18.99	0.38	q4
Chemical factors					
Any pre milking teat product (pre-dip) used					
Don't use premilking teat dip	0.52	0.33	0.82	*	q17
Use premilking teat dip	1.03	0.58	1.84	0.92	q17
Iodine in pre-dip?					
No iodine in pre-dip	0.62	0.39	1.00	*	17_iod
Iodine in pre dip	0.45	0.17	1.18	0.10	17_iod
Chlorhexidine in pre-dip?					
No chlorhexidine in pre-dip	0.49	0.31	0.78	*	17_chlorhex

Chlorhexidine in pre-dip	1.09	0.41	2.88	0.87	17_chlorhex
Peracetic acid in pre-dip?					
No peracetic acid in pre dip	0.47	0.30	0.73	*	17_perac
Peracetic acid in pre-dip	1.60	0.51	5.05	0.42	17_perac
Chloride dioxide in pre-dip?					
No chloride dioxide in pre dip	0.45	0.29	0.70	*	17_chldio
Chloride dioxide in pre-dip	2.21	0.66	7.43	0.20	17_chldio
Lactic acid in pre-dip?					
No lactic acid in pre-dip	0.48	0.29	0.79	*	17_lacaci
Lactic acid in pre-dip	1.14	0.48	2.69	0.77	17_lacaci
Other active ingredients in pre-dip?					
No other things in teat pre-dip	0.52	0.34	0.80	*	17_other
Other things in teat pre-dip	0.55	0.11	2.78	0.47	17_other
How are the teats cleaned					
Spray teat dip	0.40	0.20	0.78	*	q17b
Dip teat	0.44	0.11	1.79	0.25	q17b
Foam cup	1.94	0.81	4.69	0.14	q17b
Cloth/wipe	2.08	0.53	8.14	0.29	q17b
teat scrubber/ brush	2.00	0.46	8.75	0.36	q17b
Use of an automatic dipping and flushing (ADF) system					
Don't use ADF	0.49	0.35	0.70	*	q26
Do use ADF	1.56	0.83	2.95	0.17	q26
what is used in the flushing part of ADF					
Flush with water	0.38	0.10	1.41	*	q26a_flushing_fluid
Flush with peracetic acid	2.29	0.53	9.93	0.27	q26a_flushing_fluid
Are clusters disinfected between cows					
Don't disinfect clusters	0.37	0.22	0.63	*	q27
Disinfect clusters between every cow	1.47	0.71	3.06	0.30	q27
Disinfect cluster if mastitis/high SCC	2.03	0.99	4.17	0.05	q27
If clusters are flushed, what are they flushed with?					
Don't use water to flush	0.66	0.45	0.97	*	27_water
Use water to flush	0.00	0.00	Inf	0.99	27_water
If clusters are flushed, what are they flushed with?					
Don't use hypochlorite to flush	0.64	0.43	0.96	*	27_hypoc
Use hypochlorite to flush	0.89	0.25	3.26	0.86	27_hypoc
If clusters are flushed, what are they flushed with?					
Don't use iodine to flush	0.62	0.42	0.92	*	27_iod
Use iodine to flush	1.61	0.22	11.87	0.64	27_iod
If clusters are flushed, what are they flushed with?					
Don't peracetic acid to flush	0.75	0.32	1.78	*	27_peraci
Peracetic acid to flush	0.81	0.31	2.12	0.67	27_peraci
If clusters are flushed, what are they flushed with?					
Don't use hydrogen peroxide to flush	0.62	0.42	0.91	*	27_hydroper
Use hydrogen peroxide to flush	9322173.20	0.00	Inf	0.99	27_hydroper
If clusters are flushed, what are they flushed with?					
Don't use other ingredient to flush	0.62	0.42	0.92	*	27_other
Use other ingredients to flush	1.61	0.22	11.87	0.64	27_other
Which disinfectant used in pre milking preparation					
Clean water	0.00	0.00	Inf	*	27awdisc
hypochlorite	8943635.02	0.00	Inf	0.99	27awdisc
Iodine	15651361.28	0.00	Inf	0.99	27awdisc
Peracetic acid	9854560.81	0.00	Inf	0.99	27awdisc
Others	23477041.92	0.00	Inf	0.99	27awdisc
Management and milking machine factors					
Teat discolouration after milking farm level					

no teat discolouration	0.49	0.33	0.71	*	8teat discolouration
have teat discolouration	1.62	0.80	3.30	0.18	8teat discolouration
Presence of teat end keratosis farm level					
no teat end hyperkeratosis	0.39	0.25	0.62	*	8 teat end keratosis
have teat end hyperkeratosis	2.13	1.12	4.06	0.02	8 teat end keratosis
Presence of teat end eversion					
no teat end eversion	0.47	0.33	0.67	*	8 teat evertion
have teat end eversion	2.81	1.11	7.15	0.03	8 teat evertion
Dry cow therapy practice					
no dry cow therapy	0.25	0.05	1.18	*	9 SDCT
Selective Dry cow therapy	2.18	0.44	10.78	0.34	9 SDCT
Blanket treatment	2.57	0.52	12.80	0.25	9 SDCT
Do you vaccinate against BVD					
Don't vaccinate for BVD	0.38	0.24	0.62	*	q14 BVD vaccinated?
Vaccinate for BVD	1.68	0.92	3.07	0.09	q14 BVD vaccinated?
Which vaccine do you use for BVD					
Use Bovillis	0.69	0.41	1.15	*	q14 BVD vacine used
Use Bovella	0.55	0.19	1.60	0.27	q14 BVD vacine used
Use Bovidec	1.00	0.40	2.53	1.00	q14 BVD vacine used
How many times a day the cows are milked					
Milked voluntarily	0.50	0.13	2.00	*	q16
Milked twice daily	0.99	0.24	4.10	0.99	q16
Milked three times daily	4.00	0.56	28.40	0.17	q16
Milked other	2.00	0.18	22.06	0.57	q16
Foremilking on the farm					
Don't foremilk	0.32	0.14	0.74	*	q18
Always foremilk	1.95	0.73	5.19	0.18	q18
Foremilk most of the time	0.99	0.28	3.47	0.99	q18
Foremilk occasionally	1.50	0.48	4.66	0.49	q18
Foremilk if suspect mastitis	2.18	0.81	5.88	0.12	q18
Time between preparing the teats and attaching the cluster					
no time between preparing teats and attaching clusters	0.36	0.12	1.14	*	q19
<30 s	1.91	0.52	7.09	0.33	q19
30 s to 1 min	1.20	0.35	4.11	0.77	q19
1 to 2 mins	1.93	0.54	6.88	0.31	q19
more than 2 mins	1.53	0.31	7.44	0.60	q19
Glove use when milking					
No gloves used	0.43	0.20	0.94	*	20_combined
Gloves used	1.29	0.56	2.98	0.55	20_combined
Paper towel use to dry teats					
no paper towel	0.44	0.19	1.02	*	q21
yes new paper towel each cow	1.18	0.46	3.00	0.73	q21
same towel multiple cows	0.95	0.34	2.62	0.92	q21
wet wipes	5.63	1.35	23.45	0.02	q21
washable cloths	0.90	0.22	3.75	0.89	q21
robots	1.50	0.21	10.79	0.69	q21
When are freshly calved cows milked in relation to rest of the herd					
fresh cows milked before the herd	0.65	0.30	1.38	*	q22
fresh cows milked after the herd, before mastitis	0.86	0.29	2.54	0.78	q22
fresh cows milked after the herd	1.33	0.53	3.37	0.54	q22
fresh cows milked with the herd	0.57	0.24	1.37	0.21	q22
other	1.55	0.09	27.36	0.77	q22
When are mastitis cows milked in relation to the rest of the herd					
mastitis cows milked before herd	1.00	0.06	15.99	*	q23

mastitis cows milked after the herd	0.59	0.03	9.93	0.71	q23
milked with the herd, same cluster	0.57	0.03	9.40	0.69	q23
milked with the herd separate cluster	0.48	0.03	8.01	0.61	q23
other	0.50	0.01	19.56	0.71	q23
Use of an automatic cluster release (ACR)					
Don't use ACR	0.30	0.15	0.61	*	q25
Do use ACR	2.06	0.95	4.47	0.07	q25
what teat liner material is used					
Have rubber teat liners	0.59	0.44	0.78	*	q28
Have silicon teat liners	0.51	0.14	1.92	0.32	q28
How often is the milking machine serviced					
service milking machine every 1-5 months	0.43	0.11	1.66	*	q30
every 6-9 months	1.68	0.38	7.40	0.49	q30
every 10-12 months	1.43	0.35	5.81	0.62	q30
every 12-18 months	0.75	0.16	3.52	0.72	q30
when needed	0.58	0.04	7.66	0.68	q30
Has there been a dynamic parlour test					
Haven't had a dynamic parlour test	0.46	0.28	0.75	*	q31
Have had a dynamic parlour test	1.34	0.73	2.44	0.34	q31
Who milks the cows					
Family members milk cows	0.50	0.32	0.78	*	32_wmc
Staff milk the cows	1.00	0.38	2.61	1.00	32_wmc
Family members and staff milk cows	1.25	0.68	2.30	0.46	32_wmc
Cows are milked by robots	0.80	0.15	4.38	0.80	32_wmc
Do cows leak before/after milking					
No cows leak milk	0.40	0.13	1.28	*	q33
cows leak before milking	1.35	0.41	4.50	0.62	q33
cows leak before and after milking	1.38	0.35	5.43	0.65	q33
Other animals on the farm					
Farms without beef cows	0.57	0.39	0.84	*	34_beef
Farms with beef cows	0.93	0.53	1.63	0.80	34_beef
Other animals on the farm					
Farms without cats	0.53	0.37	0.76	*	34_cats
Farms with cats	1.09	0.61	1.94	0.78	34_cats
Other animals on the farm					
Farms without dogs	0.57	0.38	0.84	*	34_dogs
farms with dogs	0.95	0.54	1.66	0.85	34_dogs
Other animals on the farm					
Farms without horses	0.62	0.46	0.85	*	34_horse
Farms with horses	0.50	0.23	1.09	0.08	34_horse
Other animals on the farm					
Farms without pigs	0.58	0.43	0.77	*	34_pigs
Farms with pigs	0.22	0.03	1.77	0.15	34_pigs
Other animals on the farm					
Farms without poultry	0.59	0.44	0.78	*	34_poultry
Farms with poultry	0.17	0.02	1.36	0.09	34_poultry
Other animals on the farm					
Farms without sheep	0.47	0.32	0.68	*	34_sheep
Farms with sheep	1.49	0.84	2.62	0.17	34_sheep
Where are replacements obtained from					
All replacements bred on farm	0.48	0.34	0.69	*	35_buyin
Buy direct from the same farms	0.41	0.09	1.96	0.27	35_buyin
Buy from auction	1.65	0.83	3.27	0.15	35_buyin
Buy from the European Union (EU) outside of Great Britain (GB)	2.06	0.40	10.62	0.39	35_buyin
Other not specified	2.06	0.28	15.11	0.48	35_buyin
Any isolation period of new livestock coming on to the farm					

Farms with an isolation period	0.46	0.32	0.64	*	35a_ison
Farms without an isolation period	1.80	0.98	3.31	0.06	35a_ison
Are heifers reared on the farm					
Heifers are reared on the same site	0.55	0.38	0.78	*	q36
Heifers reared on the same farm different site	0.89	0.45	1.75	0.74	q36
Reared on different farm	1.43	0.50	4.07	0.51	q36
Are calving pens shared with sick/lame animals					
Calving pens not shared with sick/lame animals/ no calving pens/ calve outside	0.56	0.39	0.79	*	37combined
Calving pens are shared or sometimes shared	0.98	0.54	1.78	0.94	37combined
How are lactating cows managed					
lactating cows housed year round	1.20	0.52	2.78	*	39lacc
lactating cows at pasture and housed at night	1.03	0.34	3.12	0.96	39lacc
lactating cows at pasture housed in winter	0.26	0.10	0.66	0.01	39lacc
lactating cows at pasture housed at night and in winter	0.56	0.16	1.89	0.35	39lacc
lactating cows at pasture	0.63	0.19	2.08	0.44	39lacc
lactating cows housed year round but have pasture access	3.33	0.32	34.83	0.31	39lacc
How are lactating cows housed					
lactating cows housed in cubicles	0.65	0.47	0.89	*	39_lacch
lactating cows loose housed	0.33	0.09	1.19	0.09	39_lacch
lactating cows with cubicles and loose housing	0.47	0.19	1.16	0.10	39_lacch
lactating cows have no housing	1.54	0.21	11.21	0.67	39_lacch
what bedding is used for lactating cows					
Lactating cows without straw	0.75	0.53	1.05	*	laccstraw
lactating cows with straw	0.44	0.23	0.83	0.01	laccstraw
what bedding is used for lactating cows					
lactating cows without shavings	0.58	0.44	0.77	*	laccsha
lactating cows with shavings	0.86	0.08	9.65	0.90	laccsha
what bedding is used for lactating cows					
lactating cows without sawdust	0.32	0.20	0.51	*	lacsaw
lactating cows with sawdust	2.81	1.55	5.12	0.00	lacsaw
what bedding is used for lactating cows					
lactating cows without sand	0.57	0.42	0.78	*	laccsand
lactating cows with sand	1.11	0.50	2.42	0.80	laccsand
what bedding is used for lactating cows					
lactating cows without lime	0.57	0.42	0.77	*	lacclim
lactating cows with lime	1.17	0.45	3.00	0.75	lacclim
what bedding is used for lactating cows					
lactating cows without husk	0.59	0.44	0.78	*	lacchusk
lactating cows with husk	0.00	0.00	Inf	0.99	lacchusk
what bedding is used for lactating cows					
lactating cows without paper	0.60	0.45	0.80	*	laccpap
lactating cows with paper	0.28	0.03	2.35	0.24	laccpap
How are dry cows managed					
dry cows housed year round	0.62	0.26	1.48	*	39_dryc
dry cows at pasture housed at night	2.17	0.38	12.31	0.38	39_dryc
dry cows at pasture housed in winter	0.62	0.24	1.64	0.34	39_dryc
dry cows at pasture housed at night and in winter	1.35	0.31	5.94	0.69	39_dryc
Dry cows at pasture	1.22	0.42	3.56	0.72	39_dryc
Dry cows housed year round with pasture access	4.88	0.43	55.29	0.20	39_dryc
How are dry cows housed					
Dry cow cubicles	0.56	0.36	0.88	*	39_drych
dry cow loose housing	1.09	0.56	2.14	0.80	39_drych
dry cow cubicles and loose	0.81	0.38	1.71	0.58	39_drych
dry cow no housing	0.67	0.16	2.69	0.57	39_drych

Dry cow bedding					
Dry cows without straw	0.56	0.35	0.89	*	drycstraw
Dry cows with straw	1.00	0.55	1.81	0.99	drycstraw
Dry cow bedding					
Dry cows without shavings	0.56	0.42	0.75	*	drycshav
Dry cows with shavings	0.89	0.08	10.02	0.93	drycshav
Dry cow bedding					
Dry cows without sawdust	0.46	0.32	0.68	*	drycsaw
Dry cows with sawdust	1.60	0.88	2.91	0.12	drycsaw
Dry cow bedding					
Dry cows without paper	0.56	0.42	0.75	*	drycpap
Dry cows with paper	0.89	0.08	10.02	0.93	drycpap
Dry cow bedding					
Dry cows without lime	0.57	0.42	0.76	*	dryclim
Dry cows with lime	0.76	0.19	3.02	0.69	dryclim
Dry cow bedding					
Dry cows without sand	0.57	0.42	0.78	*	drycsand
Dry cows with sand	0.82	0.32	2.11	0.68	drycsand
How are freshly calved cows managed					
Fresh cows housed year round	1.43	0.72	2.83	*	39_fresc
Fresh cows at pasture housed at night	0.61	0.22	1.66	0.33	39_fresc
Fresh cows at pasture housed in winter	0.25	0.11	0.56	0.00	39_fresc
Fresh cows at pasture housed at night and in winter	0.56	0.18	1.78	0.32	39_fresc
Fresh cows at pasture	0.23	0.07	0.79	0.02	39_fresc
How are freshly calved cows housed					
Fresh cows cubicle housing	0.57	0.38	0.87	*	39_fresch
Fresh cows loose housing	1.11	0.60	2.06	0.74	39_fresch
Fresh cows cubicles and loose housing	0.70	0.28	1.75	0.44	39_fresch
Fresh cows no housing	0.44	0.05	4.05	0.47	39_fresch
Fresh cow bedding					
Fresh cows not on straw	0.66	0.42	1.04	*	fresstra
Fresh cows on straw	0.80	0.44	1.43	0.45	fresstra
Fresh cow bedding					
Fresh cows not on shavings	0.56	0.42	0.75	*	fresshav
Fresh cows on shavings	3.55	0.32	39.84	0.30	fresshav
Fresh cow bedding					
Fresh cows not on sawdust	0.47	0.32	0.67	*	fressaw
Fresh cows on sawdust	1.80	0.98	3.28	0.06	fressaw
Fresh cow bedding					
Fresh cows not on paper	0.59	0.44	0.79	*	frespap
Fresh cows on paper	0.00	0.00	Inf	0.98	frespap
Fresh cow bedding					
Fresh cows not on lime	0.56	0.41	0.75	*	freslim
Fresh cows on lime	1.79	0.50	6.42	0.37	freslim
Fresh cow bedding					
Fresh cows not on sand	0.58	0.43	0.79	*	fressand
Fresh cows on sand	0.86	0.33	2.23	0.75	fressand
How are heifers managed					
Heifers housed year round	2.00	0.18	22.06	*	39_heif
Heifers at pasture and housed at night	1.00	0.05	18.91	1.00	39_heif
Heifers at pasture and house in winter	0.20	0.02	2.31	0.20	39_heif
heifers at pasture and housed at night and winter	0.25	0.02	4.00	0.33	39_heif
heifers at pasture	0.50	0.04	5.97	0.58	39_heif
Heifers housed year round with pasture access	0.50	0.03	8.95	0.64	39_heif
Heifer housing					
Heifers in cubicles	0.69	0.44	1.07	*	39_heifh

Heifers in loose housing	0.69	0.34	1.40	0.30	39_heifh
Heifers in cubicles and loose	0.70	0.33	1.50	0.36	39_heifh
No housing	0.62	0.15	2.59	0.52	39_heifh
Heifer bedding					
Heifers without straw	0.70	0.43	1.13	*	heifstraw
Heifers with straw	0.65	0.35	1.21	0.18	heifstraw
Heifer bedding					
Heifers without shavings	0.53	0.39	0.72	*	heisha
Heifers with shavings	1.89	0.26	13.74	0.53	heisha
Heifer bedding					
Heifers without sawdust	0.44	0.31	0.65	*	heifsaw
Heifers with sawdust	1.77	0.94	3.34	0.08	heifsaw
Heifer bedding					
Heifers without sand	0.51	0.37	0.70	*	heifsand
Heifers with sand	1.97	0.66	5.87	0.23	heifsand
Heifer bedding					
Heifers without lime	0.54	0.40	0.73	*	heiflim
Heifers with lime	0.93	0.22	3.84	0.92	heiflim
Type of insemination used					
Artificial insemination (AI) only	0.63	0.42	0.95	*	q42
Bull only	0.13	0.02	1.05	0.06	q42
AI & Bull	0.94	0.52	1.70	0.84	q42
AI & embryo transfer (ET)	0.79	0.07	9.01	0.85	q42
AI, Bull & ET	0.53	0.10	2.74	0.45	q42
Length of the dry cow period					
30-39 days	0.20	0.02	1.71	*	10_ldryp
40-49 days	2.90	0.31	26.84	0.35	10_ldryp
50-59 days	2.98	0.33	26.95	0.33	10_ldryp
60+ days	2.50	0.28	22.37	0.41	10_ldryp
Length of time fresh cows are separate from milking herd					
No time	0.53	0.34	0.83	*	q38
1-2 days	1.15	0.52	2.58	0.73	q38
3-6 days	1.07	0.54	2.13	0.85	q38
7+ days	1.10	0.46	2.62	0.83	q38
Time calves with dams					
0-1 hours	1.00	0.35	2.85	*	41_tcwd
1<12 hours	0.37	0.12	1.20	0.10	41_tcwd
12<24 hours	0.89	0.27	2.90	0.85	41_tcwd
24<48 hours	0.76	0.22	2.61	0.67	41_tcwd
>48 hours	0.27	0.07	0.98	0.05	41_tcwd
Number of cows in milk (year round calving system)					
1-50 cows in milk	0.20	0.04	0.91	*	43aimc
51-100 cows in milk	1.76	0.34	9.23	0.50	43aimc
101-150 cows in milk	5.28	1.01	27.46	0.05	43aimc
151-200 cows in milk	2.50	0.47	13.44	0.29	43aimc
201-250 cows in milk	4.00	0.67	23.72	0.13	43aimc
251-300 cows in milk	5.83	0.90	37.82	0.06	43aimc
301+ cows in milk	8.33	1.03	67.14	0.05	43aimc
Total cattle herd size					
Small total herd (60-179 animals)	0.48	0.25	0.93	*	44_totcat
small to medium total herd (180-250)	0.82	0.33	2.06	0.67	44_totcat
medium total herd (251-399)	1.23	0.50	3.04	0.65	44_totcat
medium to large total herd (400-500)	1.08	0.42	2.77	0.87	44_totcat
large total herd (500+)	1.68	0.70	4.03	0.25	44_totcat
Total milking herd size					
small milking herd (5-100 cows)	0.30	0.15	0.61	*	44_totmilk
small to medium milking herd (101-140)	1.49	0.58	3.85	0.41	44_totmilk

medium milking herd (141-200)	2.02	0.81	5.02	0.13	44_totmilk
medium to large milking herd (201-300)	1.92	0.77	4.79	0.16	44_totmilk
large milking herd (300+)	4.13	1.46	11.64	0.01	44_totmilk
Length of lactation					
270-300 days	0.25	0.08	0.75	*	45_length_of_lactation
301-350 days	3.20	0.99	10.33	0.05	45_length_of_lactation
351-400 days	1.66	0.48	5.69	0.42	45_length_of_lactation
401+ days	2.77	0.69	11.08	0.15	45_length_of_lactation
Average milk yield per lactation					
<6000 litres	0.23	0.10	0.53	*	46_milkyield
6001-8000 litres	1.17	0.43	3.18	0.76	46_milkyield
8001-10000 litres	3.97	1.56	10.10	0.00	46_milkyield
>10001 litres	8.57	2.52	29.17	0.00	46_milkyield

* reference

A.3.2 Multivariable model with udder cleft dermatitis (UCD) as the outcome variable excluding disease factors and variables with large numbers of missing data.

Variable	OR	lci	uci	p-value
Intercept	*			
Lactating cows in loose housing	1.13	0.25	5.09	0.87
Lactating cows in cubicles and loose housing	0.26	0.09	0.77	0.01
Lactating cows are not housed	28.13	1.90	415.44	0.02
Lactating cows bedded on sawdust	3.13	1.45	6.76	0.00
Average milk yield per cow on the farm 6001-8000 l/year	1.75	0.54	5.70	0.35
Average milk yield per cow on the farm 8001-10000- l/year	5.72	1.85	17.68	0.00
Average milk yield per cow on the farm >10000l/year	14.74	3.23	67.29	0.00
Farm doesn't use an isolation period when introducing new animals	2.90	1.38	6.10	0.01

* reference

A.4.1 The univariable association with chapped teats as the outcome variable.

Variable name	OR	lci	uci	p-value
Country				
England	0.16	0.10	0.25	*
Wales	0.16	0.02	1.24	0.08
Scotland	0.30	0.04	2.34	0.25
Disease factors				
Presence of ischaemic teat necrosis (ITN) on the farm				
No ITN	0.04	0.01	0.11	*
Have ITN	5.72	1.89	17.37	0.00
Presence of bovine papillomas on farm				
No bovine papilloma	0.06	0.03	0.14	*
Have bovine papilloma	3.09	1.22	7.78	0.02
Presence of bovine herpes mammillitis (BHM)				
No BHM	0.11	0.07	0.17	*

Have BHM	9.05	1.72	47.71	0.01
Presence of Udder cleft dermatitis (UCD)				
No UCD on farm	0.12	0.07	0.21	*
UCD on farm	1.10	0.46	2.65	0.83
Presence of udder acne on farm				
No udder acne	0.11	0.07	0.17	*
Udder acne	7.52	1.87	30.29	0.00
Presence of pseudocowpox on the farm				
No pseudocowpox	0.12	0.08	0.18	*
Pseudocowpox	8.35	0.50	138.01	0.14
The presence of teat licking on the farm				
no teat licking	0.06	0.03	0.13	*
has teat licking	3.75	1.47	9.54	0.01
Presence of digital dermatitis (DD) on the farm				
no DD on the farm	0.07	0.01	0.54	*
DD on the farm	1.83	0.23	14.57	0.57
Presence of DD in spring farm level				
Farms never had DD in spring	0.12	0.06	0.24	*
Farms with DD in spring	1.09	0.43	2.73	0.86
Presence of DD summer farm level				
Farms never had DD in summer	0.10	0.05	0.19	*
Farms with DD in summer	1.47	0.60	3.57	0.40
Presence of DD in autumn farm level				
Farms never had DD in autumn	0.06	0.02	0.20	*
Farms with DD in autumn	2.30	0.65	8.13	0.20
Presence of DD in winter farm level				
Farms never had DD in winter	0.08	0.02	0.34	*
Farms with DD in winter	1.61	0.36	7.33	0.54
DD worst in spring farm level				
DD not worst in spring	0.14	0.09	0.23	*
Farms with DD worst in spring	0.77	0.25	2.43	0.66
DD worst in summer farm level				
DD not worse in summer	0.14	0.09	0.21	0.00
Farm with DD worst in summer	0.67	0.08	5.46	0.71
DD worst in the autumn farm level				
DD not worst in autumn	0.13	0.07	0.21	*
farms with DD worst in autumn	1.22	0.47	3.18	0.69
DD worst in the winter farm level				
DD not worst in winter	0.10	0.03	0.27	*
Farms with DD worst in winter	1.52	0.49	4.76	0.47
Any bovine viral diarrhoea virus (BVD) persistently infected (PI) animals removed from farm				
No BVD PI removed from farm in last year	0.12	0.08	0.20	*
BVD PI removed from farm in last year	1.36	0.37	5.02	0.65

Have you had any cases of tuberculosis (TB) on farm				
No TB	0.11	0.06	0.18	*
Confirmed case	2.07	0.74	5.76	0.16
Reactors no disease	1.24	0.26	5.93	0.79
Any contagious ovine digital dermatitis (CODD) in sheep				
Farms without CODD	0.13	0.08	0.21	*
Farms with CODD	0.28	0.04	2.16	0.22
Chemical factors				
Any pre milking teat product (pre-dip) used				
Don't use premilking teat dip	0.09	0.04	0.20	*
Use premilking teat dip	1.58	0.63	4.00	0.33
Iodine in pre-dip?				
No iodine in pre-dip	0.14	0.07	0.28	*
Iodine in pre dip	0.74	0.19	2.92	0.66
Chlorhexidine in pre-dip?				
No chlorhexidine in pre-dip	0.14	0.07	0.27	*
Chlorhexidine in pre-dip	0.69	0.14	3.38	0.64
Peracetic acid in pre-dip?				
No peracetic acid in pre dip	0.08	0.04	0.18	*
Peracetic acid in pre-dip	6.67	1.75	25.40	0.01
Chloride dioxide in pre-dip?				
No chloride dioxide in pre dip	0.15	0.08	0.27	*
Chloride dioxide in pre-dip	0.00	0.00	Inf	0.99
Lactic acid in pre-dip?				
No lactic acid in pre-dip	0.13	0.06	0.27	*
Lactic acid in pre-dip	1.05	0.29	3.76	0.94
Other active ingredients in pre-dip?				
No other things in teat pre-dip	0.13	0.07	0.24	*
Other things in teat pre-dip	0.97	0.11	8.47	0.98
How are the teats cleaned				
Spray teat dip	0.20	0.09	0.45	*
Dip teat	0.56	0.10	2.95	0.49
Foam cup	0.45	0.12	1.68	0.24
Cloth/wipe	1.88	0.40	8.88	0.43
Teat scrubber/ brush	0.63	0.07	5.82	0.68
Use of an automatic dipping and flushing (ADF) system				
Don't use ADF	0.09	0.05	0.16	*
Do use ADF	3.16	1.32	7.55	0.01
what is used in the flushing part of ADF				
Flush with water	0.22	0.05	1.03	*
Flush with peracetic acid	1.35	0.25	7.42	0.73

Are clusters disinfected between cows				
Don't disinfect clusters	0.04	0.01	0.14	*
Disinfect clusters between every cow	5.58	1.51	20.62	0.01
Disinfect cluster if mastitis/high somatic cell count (SCC)	2.27	0.54	9.48	0.26
If clusters are flushed, what are they flushed with?				
Don't use water to flush	0.19	0.11	0.31	*
Use water to flush	0.00	0.00	Inf	0.99
If clusters are flushed, what are they flushed with?				
Don't use hypochlorite to flush	0.20	0.12	0.35	*
Use hypochlorite to flush	0.00	0.00	Inf	0.99
If clusters are flushed, what are they flushed with?				
Don't use iodine to flush	0.19	0.11	0.32	*
Use iodine to flush	0.00	0.00	Inf	0.99
If clusters are flushed, what are they flushed with?				
Don't use hydrogen peroxide to flush	0.18	0.11	0.31	*
Use hydrogen peroxide to flush	0.00	0.00	Inf	0.99
If clusters are flushed, what are they flushed with?				
Don't use other ingredient to flush	0.19	0.11	0.32	*
Use other ingredients to flush	0.00	0.00	Inf	0.99
Management and milking machine factors				
Teat discolouration after milking farm level				
no teat discolouration	0.12	0.07	0.21	*
have teat discolouration	1.92	0.73	5.00	0.18
Teat ringing after milking farm level				
no teat ringing	0.10	0.05	0.18	*
cases of teat ringing	2.89	1.14	7.32	0.03
Presence of teat end keratosis farm level				
no teat end hyperkeratosis	0.10	0.05	0.20	*
have teat end hyperkeratosis	2.06	0.80	5.26	0.13
Presence of teat end eversion				
no teat end eversion	0.12	0.07	0.20	*
have teat end eversion	2.67	0.85	8.32	0.09
Do you vaccinate against bovine viral diarrhoea virus (BVD)				
Don't vaccinate for BVD	0.05	0.02	0.14	*
Vaccinate for BVD	3.26	1.06	10.07	0.04
Which vaccine do you use for BVD				
Use Bovillis	0.16	0.07	0.33	*
Use Bovella	1.01	0.24	4.20	0.99
Use Bovidec	1.45	0.43	4.93	0.55
Foremilking on the farm				
Don't foremilk	0.04	0.00	0.26	*
Always foremilk	6.62	0.82	53.20	0.08

Foremilk most of the time	2.43	0.21	28.58	0.48
Foremilk occasionally	3.00	0.29	30.62	0.35
Foremilk if suspect mastitis	2.50	0.28	22.44	0.41
Glove use when milking				
No gloves used	0.15	0.05	0.44	*
Gloves used	0.77	0.24	2.43	0.65
Paper towel use to dry teats				
no paper towel	0.08	0.02	0.35	*
yes new paper towel each cow	1.24	0.25	6.13	0.79
same towel multiple cows	1.22	0.22	6.78	0.82
wet wipes	2.00	0.25	15.99	0.51
washable cloths	3.27	0.48	22.46	0.23
robots	3.00	0.22	41.35	0.41
Use of an automatic cluster release (ACR)				
Don't use ACR	0.08	0.02	0.24	*
Do use ACR	1.89	0.54	6.66	0.32
what teat liner material is used				
Have rubber teat liners	0.12	0.08	0.19	*
Have silicon teat liners	0.69	0.09	5.58	0.73
Has there been a dynamic parlour test				
Haven't had a dynamic parlour test	0.11	0.05	0.23	*
Have had a dynamic parlour test	1.14	0.44	2.94	0.79
Who milks the cows				
Family members milk cows	0.07	0.03	0.17	*
Staff milk the cows	2.70	0.70	10.48	0.15
Family members and staff milk cows	1.75	0.62	4.94	0.29
Cows are milked by robots	5.40	0.86	33.92	0.07
Do cows leak before/after milking				
No cows leak milk	0.17	0.04	0.74	*
cows leak before milking	0.59	0.12	2.88	0.51
cows leak before and after milking	1.44	0.25	8.22	0.68
Other animals on the farm				
Farms without beef cows	0.13	0.07	0.23	*
Farms with beef cows	0.94	0.40	2.20	0.89
Other animals on the farm				
Farms without cats	0.11	0.06	0.19	*
Farms with cats	1.52	0.65	3.57	0.34
Other animals on the farm				
Farms without dogs	0.11	0.06	0.21	*
farms with dogs	1.23	0.53	2.89	0.63
Other animals on the farm				
Farms without horses	0.12	0.07	0.19	*
Farms with horses	1.43	0.53	3.85	0.48

Other animals on the farm				
Farms without pigs	0.12	0.08	0.19	*
Farms with pigs	2.38	0.46	12.16	0.30
Other animals on the farm				
Farms without poultry	0.13	0.09	0.21	0.00
Farms with poultry	0.00	0.00	Inf	0.99
Other animals on the farm				
Farms without sheep	0.13	0.07	0.22	*
Farms with sheep	1.03	0.43	2.43	0.95
Any isolation period of new livestock coming on to the farm				
Farms with an isolation period	0.11	0.07	0.19	*
Farms without an isolation period	1.53	0.63	3.70	0.35
Are heifers reared on the farm				
Heifers are reared on the same site	0.16	0.10	0.26	*
Heifers reared on the same farm different site	0.38	0.11	1.33	0.13
Reared on different farm	0.88	0.19	4.18	0.87
Are calving pens shared with sick/lame animals				
Calving pens not shared with sick/lame animals/ no calving pens/ calve outside	0.09	0.05	0.16	*
Calving pens are shared or sometimes shared	2.39	1.00	5.72	0.05
How are lactating cows managed				
lactating cows housed year round	0.16	0.05	0.53	*
lactating cows at pasture and housed at night	0.23	0.02	2.34	0.21
lactating cows at pasture housed in winter	0.89	0.23	3.39	0.86
lactating cows at pasture housed at night and in winter	1.12	0.20	6.30	0.90
lactating cows at pasture	0.67	0.10	4.45	0.68
lactating cows housed year round but have pasture access	1.58	0.13	19.42	0.72
what bedding is used for lactating cows				
Lactating cows without straw	0.11	0.07	0.20	*
lactating cows with straw	1.33	0.54	3.24	0.53
what bedding is used for lactating cows				
lactating cows without shavings	0.13	0.08	0.20	*
lactating cows with shavings	0.00	0.00	Inf	0.99
what bedding is used for lactating cows				
lactating cows without sawdust	0.12	0.06	0.23	*
lactating cows with sawdust	1.12	0.47	2.67	0.81
what bedding is used for lactating cows				
lactating cows without sand	0.11	0.07	0.19	*
lactating cows with sand	1.69	0.58	4.94	0.34
what bedding is used for lactating cows				
lactating cows without lime	0.13	0.08	0.20	*
lactating cows with lime	0.88	0.19	4.05	0.87
what bedding is used for lactating cows				
lactating cows without husk	0.13	0.08	0.20	*

lactating cows with husk	0.00	0.00	Inf	0.99
what bedding is used for lactating cows				
lactating cows without paper	0.12	0.08	0.19	*
lactating cows with paper	1.35	0.16	11.73	0.79
How are dry cows managed				
dry cows housed year round	0.31	0.11	0.85	*
dry cows at pasture housed at night	0.53	0.05	5.55	0.60
dry cows at pasture housed in winter	0.26	0.08	0.88	0.03
dry cows at pasture housed at night and in winter	0.71	0.11	4.44	0.72
Dry cows at pasture	0.43	0.11	1.70	0.23
Dry cows housed year round with pasture access	1.07	0.09	12.69	0.96
How are dry cows housed				
Dry cow cubicles	0.10	0.05	0.21	*
dry cow loose housing	1.22	0.42	3.56	0.72
dry cow cubicles and loose	1.13	0.35	3.68	0.83
dry cow no housing	3.66	0.81	16.60	0.09
Dry cow bedding				
Dry cows without straw	0.15	0.08	0.29	*
Dry cows with straw	0.69	0.28	1.70	0.42
Dry cow bedding				
Dry cows without shavings	0.12	0.08	0.19	*
Dry cows with shavings	0.00	0.00	Inf	0.99
Dry cow bedding				
Dry cows without sawdust	0.13	0.07	0.22	*
Dry cows with sawdust	0.80	0.31	2.09	0.65
Dry cow bedding				
Dry cows without paper	0.12	0.08	0.19	*
Dry cows with paper	0.00	0.00	Inf	0.99
Dry cow bedding				
Dry cows without lime	0.12	0.07	0.19	*
Dry cows with lime	0.93	0.11	7.75	0.95
Dry cow bedding				
Dry cows without sand	0.10	0.06	0.17	*
Dry cows with sand	2.94	0.96	9.03	0.06
How are freshly calved cows managed				
Fresh cows housed year round	0.10	0.03	0.32	*
Fresh cows at pasture housed at night	0.79	0.12	5.12	0.81
Fresh cows at pasture housed in winter	1.08	0.28	4.16	0.92
Fresh cows at pasture housed at night and in winter	2.95	0.58	14.99	0.19
Fresh cows at pasture	1.82	0.33	10.04	0.49
Fresh cow bedding				
Fresh cows not on straw	0.07	0.03	0.17	*
Fresh cows on straw	2.20	0.77	6.28	0.14

Fresh cow bedding				
Fresh cows not on shavings	0.12	0.08	0.19	*
Fresh cows on shavings	0.00	0.00	Inf	0.99
Fresh cow bedding				
Fresh cows not on sawdust	0.13	0.07	0.22	*
Fresh cows on sawdust	0.75	0.28	2.04	0.58
Fresh cow bedding				
Fresh cows not on paper	0.12	0.08	0.19	*
Fresh cows on paper	0.00	0.00	Inf	0.99
Fresh cow bedding				
Fresh cows not on lime	0.12	0.07	0.19	*
Fresh cows on lime	0.94	0.11	7.85	0.96
Fresh cow bedding				
Fresh cows not on sand	0.12	0.07	0.19	*
Fresh cows on sand	0.89	0.19	4.10	0.88
How are heifers managed				
Heifers housed year round	0.50	0.05	5.51	*
Heifers at pasture and housed at night	1.00	0.05	18.91	1.00
Heifers at pasture and house in winter	0.21	0.02	2.45	0.21
heifers at pasture and housed at night and winter	0.25	0.01	5.98	0.39
heifers at pasture	0.35	0.03	4.53	0.42
Heifers housed year round with pasture access	0.40	0.02	10.02	0.58
Heifer housing				
Heifers in cubicles	0.16	0.08	0.30	*
Heifers in loose housing	0.59	0.19	1.80	0.35
Heifers in cubicles and loose	0.61	0.18	2.03	0.42
No housing	2.73	0.61	12.16	0.19
Heifer bedding				
Heifers without straw	0.15	0.08	0.31	*
Heifers with straw	0.72	0.29	1.81	0.49
Heifer bedding				
Heifers without shavings	0.13	0.08	0.20	*
Heifers with shavings	0.00	0.00	Inf	0.99
Heifer bedding				
Heifers without sawdust	0.14	0.08	0.24	0.00
Heifers with sawdust	0.66	0.23	1.89	0.44
Heifer bedding				
Heifers without sand	0.11	0.07	0.19	*
Heifers with sand	2.38	0.61	9.33	0.21
Heifer bedding				
Heifers without lime	0.13	0.08	0.21	*
Heifers with lime	0.00	0.00	Inf	0.99
Length of dry cow period				

30-39 days	0.20	0.02	1.71	*
40-49 days	0.33	0.03	3.76	0.37
50-59 days	1.09	0.12	10.21	0.94
60+ days	0.49	0.05	4.70	0.53
Length of time fresh cows are separate from milking herd				
No time	0.09	0.04	0.20	*
1-2 days	2.57	0.83	7.94	0.10
3-6 days	1.43	0.47	4.30	0.53
7+ days	1.22	0.29	5.07	0.78
Time calves with dams				
0-1 hours	0.08	0.01	0.59	*
1<12 hours	2.69	0.32	22.56	0.36
12<24 hours	1.66	0.18	15.04	0.65
24<48 hours	0.74	0.06	8.90	0.81
>48 hours	1.11	0.11	11.70	0.93
Number of cows in milk				
1-50 cows in milk	0.20	0.04	0.91	*
51-100 cows in milk	0.48	0.08	2.97	0.43
101-150 cows in milk	0.61	0.10	3.81	0.59
151-200 cows in milk	0.50	0.07	3.43	0.48
201-250 cows in milk	0.63	0.08	5.17	0.66
251-300 cows in milk	0.91	0.11	7.72	0.93
301+ cows in milk	1.67	0.18	15.13	0.65
Number of cows dry (year round calving system)				
1-20 dry cows	0.13	0.07	0.25	*
21-40 dry cows	0.72	0.24	2.18	0.56
41-65 dry cows	1.36	0.26	6.94	0.72
65+ dry cows	3.73	0.31	44.58	0.30
Total cattle herd size				
Small total herd (60-179 animals)	0.05	0.01	0.22	*
small to medium total herd (180-250)	4.62	0.94	22.84	0.06
medium total herd (251-399)	0.45	0.04	5.19	0.52
medium to large total herd (400-500)	2.24	0.38	12.98	0.37
large total herd (501+)	3.33	0.65	17.02	0.15
Total milking herd size				
small milking herd (5-100 cows)	0.08	0.02	0.24	*
small to medium milking herd (101-140)	1.67	0.37	7.45	0.50
medium milking herd (141-200)	1.16	0.24	5.49	0.85
medium to large milking herd (201-300)	1.86	0.44	7.94	0.40
large milking herd (300+)	3.03	0.66	13.90	0.15
Length of lactation				
270-300 days	0.05	0.01	0.39	*
301-350 days	1.60	0.19	13.81	0.67

351-400 days	4.45	0.54	36.87	0.17
401+ days	3.00	0.29	31.48	0.36
Average milk yield per lactation				
<6000 litres	0.19	0.08	0.46	*
6001-8000 litres	0.31	0.08	1.19	0.09
8001-10000 litres	0.75	0.25	2.24	0.61
>10001 litres	0.86	0.19	3.87	0.85

* reference

A.4.2. Predicted probability of farm developing cases of chapped teats: multivariable model with chapped teats as the outcome variable.

Peracetic acid in pre dip	**ADF system use**	**Predicted Percentage probability of having chapped teats on the farm from the model**	**Observed percentage probability of having chapped teats on the farm**
No	No	4.23% (1.38-12.3%)	5.17%
No	Yes	15.1% (6.51-31.4%)	13.33%
Yes	No	28.3% (10.0-58.3%)	22.22%
Yes	Yes	61.4% (26.6-87.5%)	75.00%

A.5.1 ***The number and proportion of missing data for farmers reporting cases of udder cleft dermatitis (UCD) for the variables****: ischaemic teat necrosis (ITN), lactating cows bedded on sawdust, teat end eversion, and the time the calves are kept with the dams. Presented are the number of missing values due to the farmer not responding to the question and also due to the farmer responding with 'don't know'. The chi squared test was used to explore the associations between proportions of missing values and the outcome.*

Variable	**Missing and farmer did not report cases of UCD (%) *n*=140**	**Missing and farmer reported cases of UCD (%) *n*=77**	**p-value**
ITN	0 (0.0%)	0 (0.0%)	
Didn't know if had ITN	0 (0.0%)	0 (0.0%)	
Lactating cows bedded on sawdust	0 (0.0%)	0 (0.0%)	
Didn't know if lactating cows were bedded on sawdust	9 (6.4%)	1 (1.3%)	0.08
Teat end eversion	0 (0.0%)	0 (0.0%)	
Didn't know if had teat end eversion	34 (24.3%)	19 (24.7%)	0.95
Time the calves were kept with the dams	0	0	
Didn't know how long the calves were kept with their dams	3 (2.1%)	2 (2.6%)	0.83

A.5.2 ***The number and proportion of missing data for farmers reporting cases of chapped teats for the variables****: peracetic acid used in the pre-milking teat preparation (pre dip), and the use of an automated dipping and flushing system (ADF). Presented are the number of missing values due to the farmer not responding to the question and also due to the farmer responding with 'don't know'. The Chi squared test or Fisher's exact test, were appropriate, were used to explore the associations between the proportion of missing values and the outcome.*

Variable	**Missing and farmer did not report cases of chapped teats *n*=193**	**Missing and farmer reported cases of chapped teats *n*=24**	**p-value**
Peracetic acid in pre dip	0 (0.0%)	0 (0.0%)	
Didn't know if had peracetic acid in pre dip	100 (51.8%)	12 (50.0%)	0.87 (Chi squared test)
Use an ADF system	0 (0.0%)	0 (0.0%)	
Didn't know if use an ADF system	14 (7.2%)	0 (0.0%)	0.37 (Fisher's exact test)

APPENDIX B

Front page of supporting paper for Chapter 2. Link to open access paper can be found here https://www.frontiersin.org/articles/10.3389/fvets.2022.748259/full

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ORIGINAL RESEARCH
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An Observational Study Investigating Potential Risk Factors and Economic Impact for Bovine Ischaemic Teat Necrosis on Dairy Farms in Great Britain

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Bovine ischaemic teat necrosis (ITN) is an emerging disease of unknown aetiology that affects the teats of dairy cattle. It causes economic and animal welfare issues with many animals being culled. No effective treatments or epidemiological data to inform control strategies are currently available. The aim of this observational study was to investigate farmer-reported experiences and identify potential farm-level risk factors. In January 2018, a questionnaire was sent to a random sample of 1,855 Great Britain (GB) dairy farmers. A usable response rate of 12.3% was obtained. Fifty-one per cent [95% confidence interval (CI): 44.4–57.8%] of farmers reported having experienced ITN on their farm between 1985 and 2018. Rising numbers of farms indicated that ITN is an emerging disease with 46.3% of farmers reporting the first case in the 3 years up to 2018. At the animal level, 47.3% (95% CI: 38.7–55.9%) of the cases occurred during the first lactation and 78.9% (95% CI: 75.2–82.6%) within the first 90 days in milk. Only 20.8% (95% CI: 15.9–26.4%) of the cases were reported to recover, whereas 22.8% (95% CI: 17.8–28.5%) of the cases required culling. The remaining cases experienced complications such as loss of a teat and/or mastitis. From these data, the cost of ITN, through production losses and expenditure, was estimated to be £1,121 per farm per year. The costs were estimated at £720, £860 and £2,133 for recovered, complicated and culled cases, respectively. Univariable and multivariable logistic regression models were used to explore the associations between the presence of ITN on farm and various risk factors. The presence of udder cleft dermatitis (UCD) (odds ratio 2.80; 95% CI: 1.54–5.07; $p < 0.01$) and chapped teats (odds ratio 6.07; 95% CI: 1.96–18.76; $p < 0.01$) in the milking herd was associated with the presence of ITN at the farm level. This is the first national questionnaire of ITN within GB and highlights the association of UCD and chapped teats with ITN at the farm level. While there are many limitations and potential bias around farmer questionnaires, these findings highlight several key areas for further disease investigation and possible intervention.

Keywords: bovine, ischaemic, necrosis, questionnaire, risk factors, dairy

APPENDIX C

Front page of supporting paper for Chapter 3. Link to open access paper can be obtained here https://www.sciencedirect.com/science/article/pii/S0021997522000913

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SPONTANEOUSLY ARISING DISEASE

Clinical and Pathological Features of Bovine Ischaemic Teat Necrosis

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Summary

Bovine ischaemic teat necrosis (ITN) is an emerging disease of unknown aetiology affecting mostly dairy cows in the early stages of first lactation and a substantial welfare concern frequently leading to premature culling and economic losses. Specific diagnostic criteria are lacking. The aims of this study were to develop an appropriate ITN grading system, describe the histopathological changes and investigate the potential aetiological role of several pathogens in 47 cows with 73 ITN lesions from 28 farms. ITN lesions were allocated to one of three broad macroscopic categories: presence of a non-proliferative lesion on the teat (type 1); proliferative teat lesion with crusting (type 2); severe purulent to eosinophilic, ulcerative and necrotising dermatitis and sloughing or total absence of the teat (type 3). Lesions were mostly observed on the medial aspect of the teat but there was no anatomical predisposition as to which teats were more frequently affected. In approximately 50% of the ITN teats reviewed, the lesions were continuous with the skin of the udder and 34.2% of cases had sloughed or partially sloughed teats. The main histological findings were: focally extensive severe purulent to eosinophilic, ulcerative and necrotizing dermatitis; serocellular crust formation; and epidermal hyperplasia with dyskeratosis. Some lesions also had leucocytoclastic to eosinophilic vasculitis and thrombosis with ischaemic necrosis. Macroscopic and histological analyses confirmed the suspected ischaemic nature of the lesions but the specific aetiopathogenesis was elusive with a wide range of bacteria present, probably as opportunistic infections. However, *Treponema* spp and *Orthopox* virus were excluded as major aetiological agents. This study establishes a foundation for further investigations of the pathogenesis of bovine ITN and a basis for consistency in diagnosis and classification of the stage of disease. The findings are also key to further understanding disease progression and prognosis.

Keywords: bovine; dairy; histology; ischaemia; necrosis; teat

Introduction

Bovine ischaemic teat necrosis (ITN) is a newly emerging skin disease causing severe udder lesions in UK dairy cattle. ITN affects the base of the teat and may extend distally towards the teat end and/or proximally to the adjacent skin of the udder (Blowey, 2004). Severe lesions can cause affected teats to slough, and in other cases teats can become firm and difficult to milk. ITN lesions may be highly irritating or pruritic with cows licking their teats until the tissue is further traumatized and eventually removed. ITN is extremely difficult to treat and many animals are culled on welfare grounds due to the severity of the lesions, with one study reporting 22.8% of cases culled within the first 100 days of lactation (Crosby-Durrani *et al*, 2022). The estimated cost of ITN in the UK has been reported to be £1,121 per farm per year (Crosby-Durrani *et al*, 2022). First

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