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Title: Disease Investigations into Bovine Ischaemic Teat Necrosis: a severe, emerging disease of economic importance

Bovine Ischaemic Teat Necrosis in Dairy Cattle

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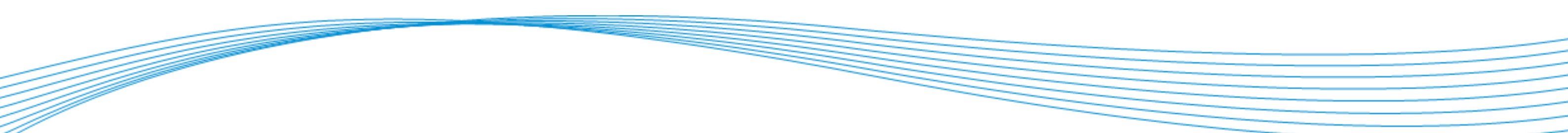

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1. Industry Summary

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** (based on 2018 prices). 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.

2. Introduction

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.

There are many diseases that affect the bovine teat and udder in dairy cattle that often have a complex aetiology. 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). 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.
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:

- Objective 1) perform an epidemiology study to report the GB incidence of disease and identify potential risk factors.
- Objective 2) documenting the pathology and attempt to look for potential aetiological agents.
- Objective 3) investigating the microbiological involvement by assessing the possibility of the involvement of digital dermatitis associated treponemes in ITN.
- Objective 4) investigating the microbiological involvement by surveying the microbiome for potential ITN aetiological agents.

3. Materials and methods

3.1. Objective 1: Perform an epidemiology study to report the GB incidence of disease and identify potential risk factors.

3.1.1. Observational farmer-based questionnaire study into potential farm-level risk factors for ITN

Sample size calculation

The study population was selected from producers designated as dairy farmers in Great Britain (GB) in a database of the AHDB. 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. 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%. 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

Questionnaire design

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. 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. 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. 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. The dataset was anonymised.

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 udder cleft dermatitis (UCD) and chapped teats.

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.

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.

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.

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.

3.2. Objective 2: Documenting the pathology and attempt to look for potential aetiological agents.

3.2.1. 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 bovine herpes mammillitis (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 ischaemic 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 ischaemic 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 (*).*

3.2.2. 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 (Objectives 3 & 4).
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.

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 *Treponema medium*, *Treponema phagedenis* and *Treponema pedis* (Evans et al., 2009) with an antigen retrieval step as previously published (Crosby-Durrani *et al.* 2016) and using a CODD grade 2 lesion as a positive control. In a similar manner, the orthopox IHC utilised 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.

3.2.3. 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. Objective 3: investigating the microbiological involvement by assessing the possibility of the involvement of digital dermatitis associated treponemes in ITN

3.3.1. Sample collection

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 Objective 4). 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 (Objective 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.

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).

3.3.2. DNA extraction

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., 2015). 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.

3.3.3. Polymerase chain reaction

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 3.2. 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 3.2. 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, DD associated *T. medium* phylogroup, *T. phagedenis* phylogroup and *T. pedis* phylogroup as previously published (Evans et al., 2009).

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 as previously published (Evans et al., 2009).

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 3.3). 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 3.4 and 3.5 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 3.3 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 3.4 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 3.5 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	

3.4. Objective 4: investigating the microbiological involvement by surveying the microbiome for potential ITN aetiological agents.

3.4.1. Sample collection

Tissue samples were obtained as described in Objectives 2 & 3. Two methods were used to assess the microbiome. The first was shotgun metagenomic study using the Illumina platform that uses short reads and a complex post-sequencing assembly to assess the microbiome. The second was using long read sequencing and real-time assembly and alignment of sequences to give a rapid result using the Oxford Nanopore technology. 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).

3.4.2. Shotgun metagenomic study using Illumina sequencing

DNA extraction

For the Illumina sequencing all tissue samples were processed as described in Objective 3. 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.

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.

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™ 6000 (Illumina Inc., San Diego, CA, USA). Bioformatic analysis was performed by Dr. Matthew Gemmell, CGR.

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.

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.

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, which also included all available Fusobacterium, Treponeme, Pseudocowpox, Bovine popular stomatitis virus, Cowpox, Vaccinia virus, Orf virus, Buffalopox virus, Parapox virus, Bovine Herpesvirus 4, and Bovine Herpesvirus 2 genomes from the National Center for Biotechnology Information (NCBI) Refernce Sequence Database (RefSeq) (at 21st November 2019). 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).

Functional profiling of microbial communities

The presence/absence and abundance of microbial pathways was profiled for each sample using HUMAnN2 (Franzosa et al., 2018).

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.

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.

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

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 with DNA extracted using the DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) following manufacturer guidelines.

Assessing quality of the extracted DNA
DNA quantity and quality were assessed the same as in Objective 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. 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™ software (ONT, Oxford, UK) built into the MinION Mk1c (ONT, Oxford, UK).

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.

4. Results

4.1. Objective 1: Perform an epidemiology study to report the GB incidence of disease and identify potential risk factors.

4.1.1. 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 4.1 shows the reported reasons for not completing the questionnaire.

Table 4.1 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

4.1.2. 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. There was a reported increase in farmers witnessing cases for the first time within the last decade.

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. 4.1). 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.

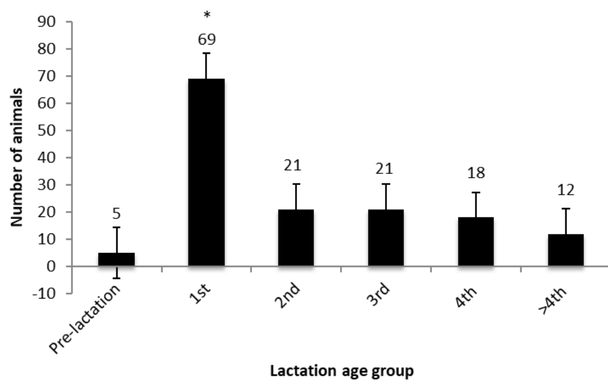


Figure 4.1 ***The production age of animals depending on the lactation the cow presented with an ischaemic teat necrosis (ITN) lesion on the teat****. First lactation heifers are significantly over-reported as developing ITN lesions on their teats. * Represents a significant difference (p-value <0.001).*

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). Seventeen farmers (14.8%; 95% CI: -0.9 – 30.5%) of 115 that responded reported the lesions appearing in more than one DIM category.

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. 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.

4.1.3. Univariable associations with the presence of ITN on the farm (primary outcome 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; chemical factors; management and milking machine factors.

4.1.4. 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 4.2).

Table 4.2 ***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.

4.1.5. 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 4.3, 4.4, 4.5 and 4.6).

Table 4.3. ***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 4.4, 4.5, 4.6.*

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 4.4 ***The estimated cost for an uncomplicated case of ITN that recovers.*** *The calculations utilise the assumptions displayed in Table 4.3. 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 4.5 ***The estimated cost for a complicated case of ITN.*** *The calculations utilise the assumptions displayed in Table 4.3. 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 4.6 ***The estimated cost for a case of ITN that required culling before the end of lactation****. The calculations utilise the assumptions displayed in Table 4.3. 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.

4.1.6. 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). 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 4.7).

Table 4.7 ***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.

4.1.7. 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. The final multivariable model contained two variables (Table 4.8).

Table 4.8 ***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.

4.2. Objective 2: Documenting the pathology and attempt to look for potential aetiology agents.

4.2.1. 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. 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 4.9) 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 4.9. 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 4.9).
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.

4.2.2. Microscopic examination

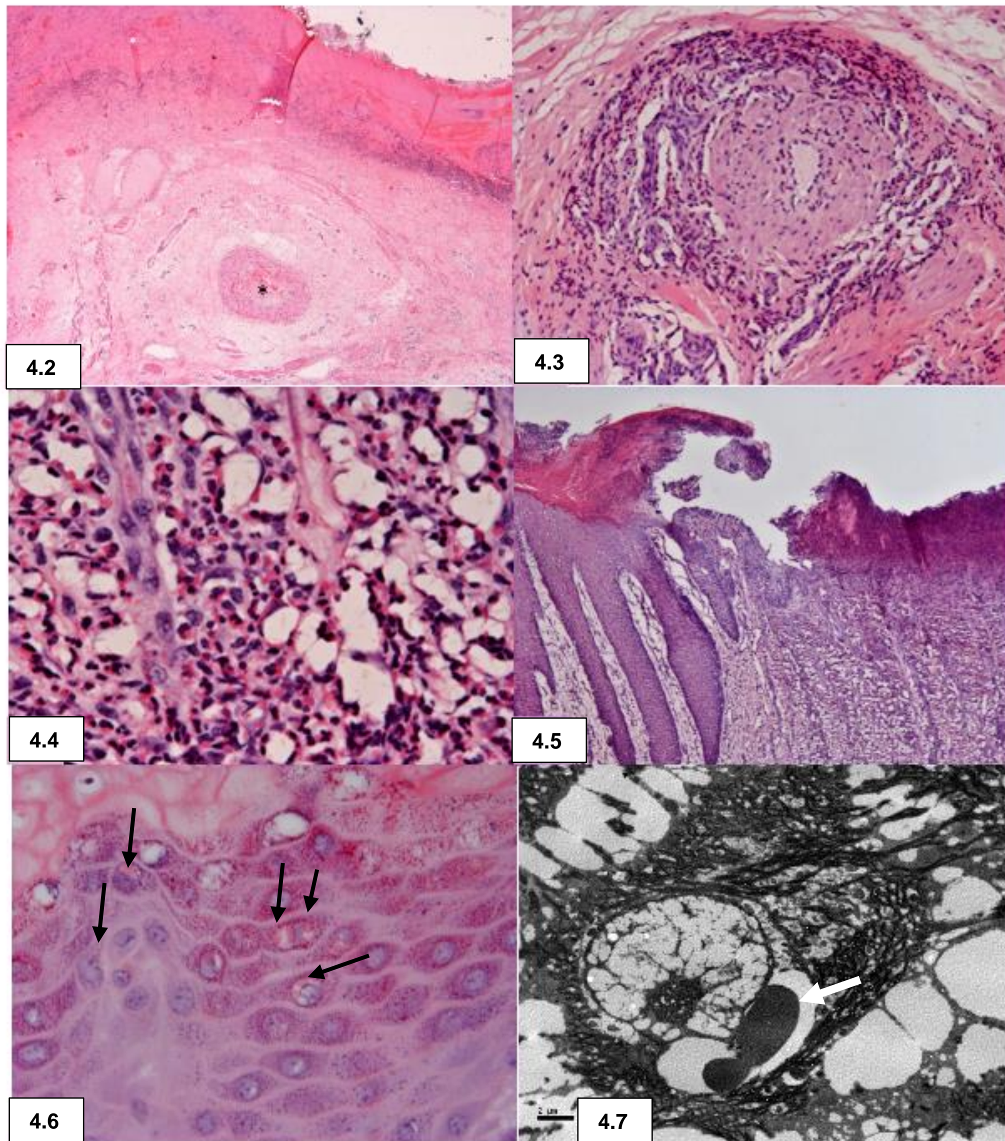
Haematoxylin and eosin stained sections of teats were viewed by light microscope. A transection through normal microanatomy of the bovine teat, in a superficial to deep direction, consists of a thick keratinised stratified squamous epidermis and dermis comprising the teat skin; and deep to this the lamina 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 4.10. 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 4.10.

Table 4.10 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. 4.2). 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. 4.3). 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. 4.4) were observed in the highest proportion in Type 3 lesions. Serocellular crusting was observed most frequently in Type 2 lesions. Ulceration (Fig. 4.5) 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. 4.6). The teat sinus epithelium, when present, was within normal limits.



Figures 4.2-4.7 ***Bovine ischaemic teat necrosis****, teat, cow.* ***Figure 4.2*** *The lamina propria of the teat sinus with a thrombosed vessel (*) surrounded by coagulative necrosis extending to the associated dermis. (HE, 20x magnification).* ***Figure 4.3*** *The lamina propria of the teat cistern with eosinophilic and lymphoplasmacytic vasculitis. (HE, 100x magnification).* ***Figure 4.4*** *The dermis subjacent to the ulceration showing marked infiltration of eosinophils. (HE, 400x magnification).* ***Figure 4.5*** *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 4.6*** *The thickened stratum granulosum of the epidermis with variable intracytoplasmic globules of eosinophilic keratin (arrows). (HE, 400x magnification).* ***Figure 4.7*** *Transmission electron micrograph of a keratinocyte with an intracytoplasmic keratin in a vacuole (arrow).*

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 mixed 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.

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.

4.2.3. Ultrastructure examination

Areas where there were intracytoplasmic inclusions as determined by light microscopy (Fig. 4.7) 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. 4.7) and/or abnormally large keratohyalin granules.

4.3. Objective 3: investigating the microbiological involvement by assessing the possibility of the involvement of digital dermatitis associated treponemes in ITN

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 4.11). No swabs from the age and production stage matched animals were positive for DD associated treponemes using the PCR assays.

Table 4.11 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) of swabs from teat liners after milking two animals with an ITN affected teat were 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 4.12).

Table 4.12 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.

4.4. Objective 4: investigating the microbiological involvement by surveying the microbiome for potential ITN aetiological agents.

4.4.1. Results of the Shotgun Metagenomics from the Illumina sequencing

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 4.13). Prior to submitting to CGR for Illumina sequencing, all extracted DNA from samples M1-M20 were loaded on to the NanoDrop^{TM} (Thermo Scientific^{TM}, Waltham, MA, USA) for assessment and were deemed adequate in terms of concentration and quality.

Table 4.13 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

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 Table 4.14.

Table 4.14 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)

The read length distributions after adapter and quality trimming showed 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.

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.

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.

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 fewer 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 4.8).

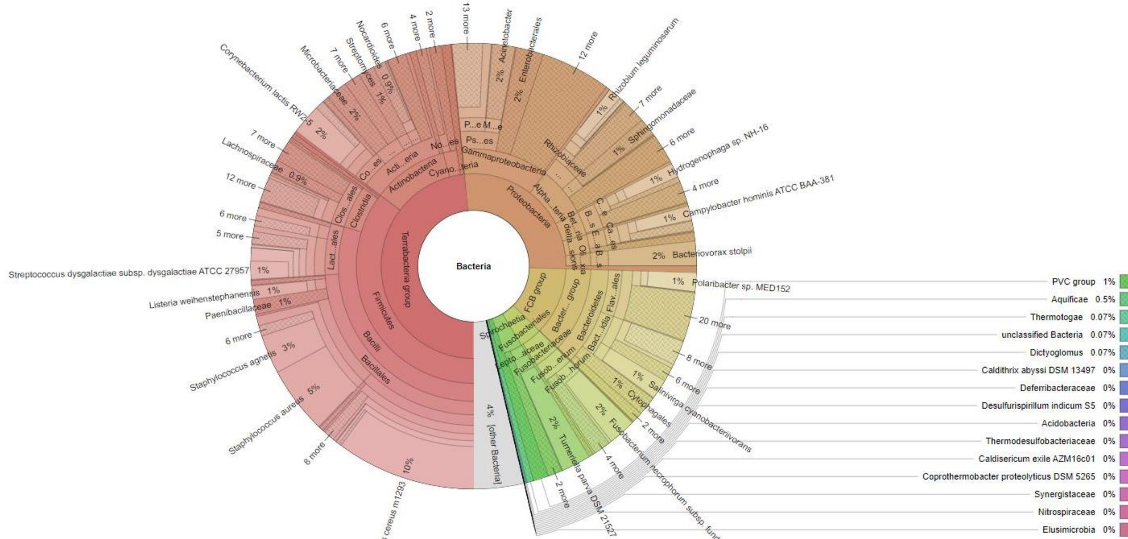


Figure 4.8 Krona output created via Kraken 2 with the bacteria identified in the sample M10 comprising of 4% of the root.

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. 4.9). 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.

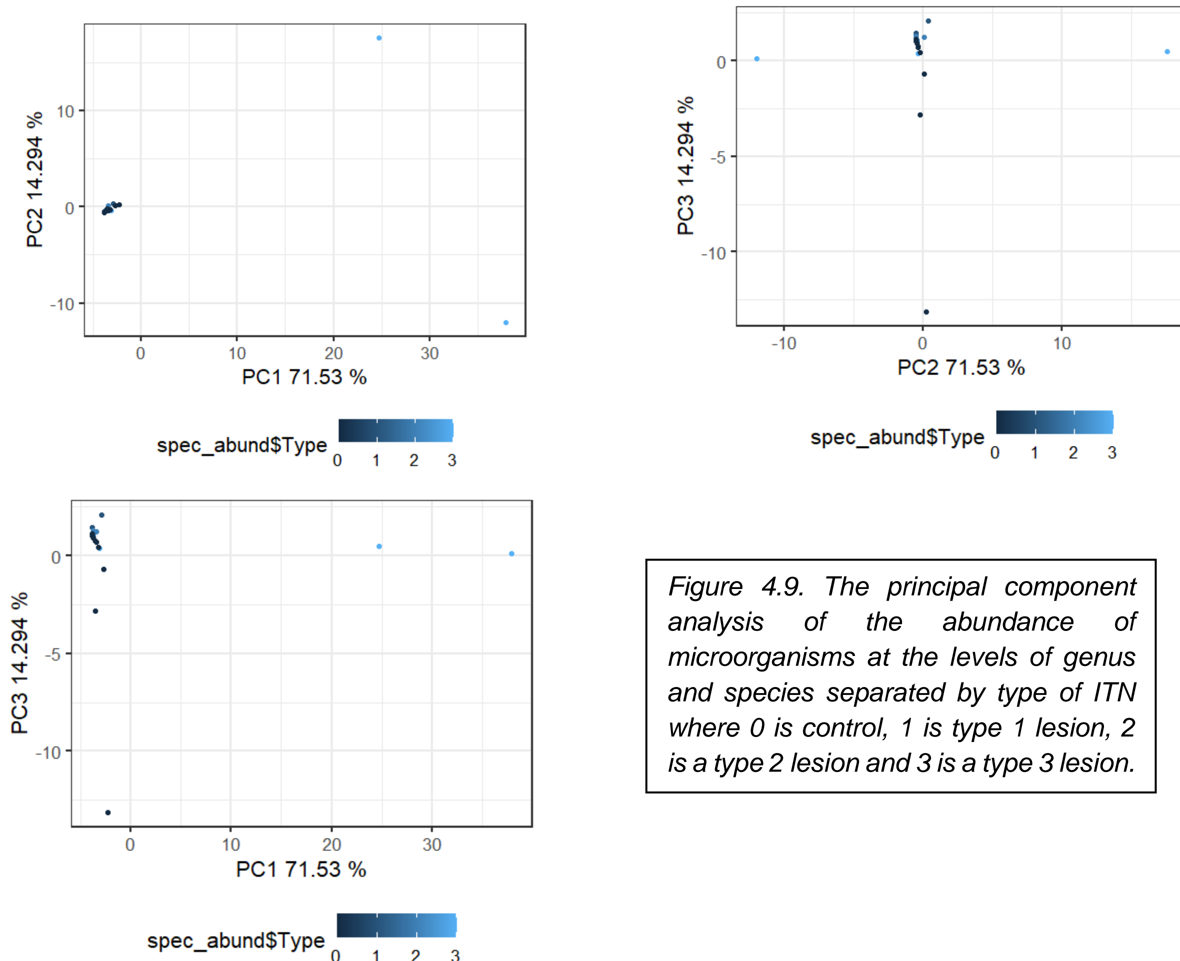


Figure 4.9. The principal component analysis of the abundance of microorganisms at the levels of genus and species separated by type of ITN where 0 is control, 1 is type 1 lesion, 2 is a type 2 lesion and 3 is a type 3 lesion.

The abundance data at a family level was inputted into a heat map (Fig. 4.10) 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.

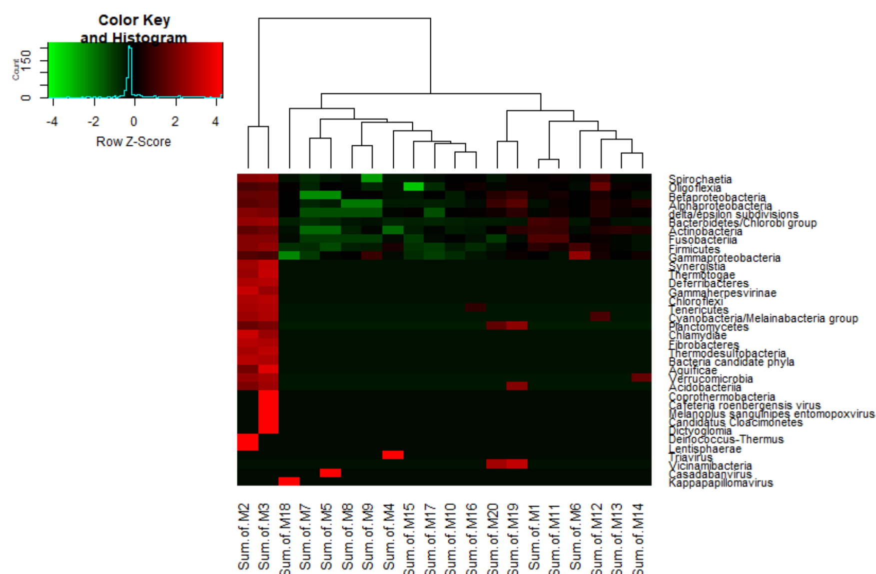


Figure 4.10 Log transformed family abundance heatmap from Bracken output data. M1-M10 are ITN diseased teats and M11-M20 are healthy control teats. Samples are clustered based on the similarity of the bacterial population present with the bacterial families present. A more intense red demonstrates an 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. 4.11). The control, non-diseased teats were far more similar using the Pearson correlation test than the ITN teats.

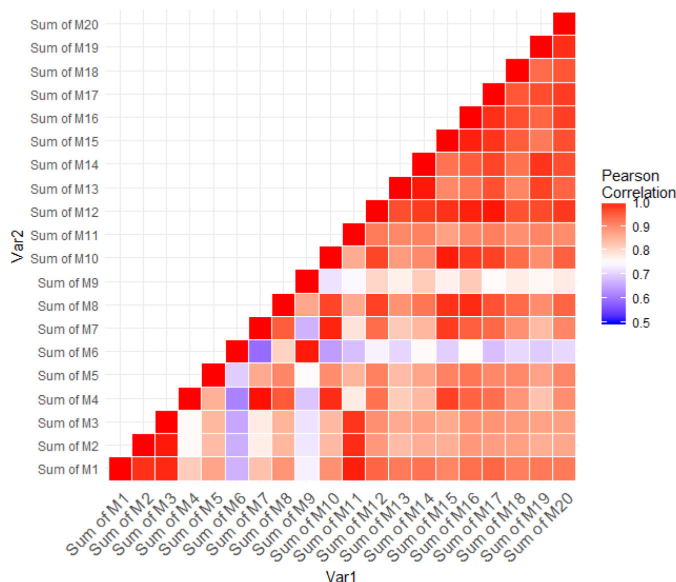


Figure 4.11 Relative abundance of family half correlation heatmap. Samples M11-M20 are closer to the Pearson correlation score 1.0 and therefore more similar than samples M1-M10, the ITN teats.

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 4.15 shows 5 diseased samples with more than 2 pathways and only 1 healthy sample with more than 2 pathways.

Table 4.15 Summary of the number of pathways discovered for samples with more than 2 pathways using HUMAnN2.

Sample	Number of Pathways
1-M1	17
2-M2	264
3-M3	311
4-M4	7
6-M6	370
11-M11	9

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 4.16). For the analysis with Bracken there were more healthy biomarkers detected than diseased.

Table 4.16 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. 4.12). *Pasteurellales* were more abundant in the diseased ITN teats than the control, non-lesion teats.

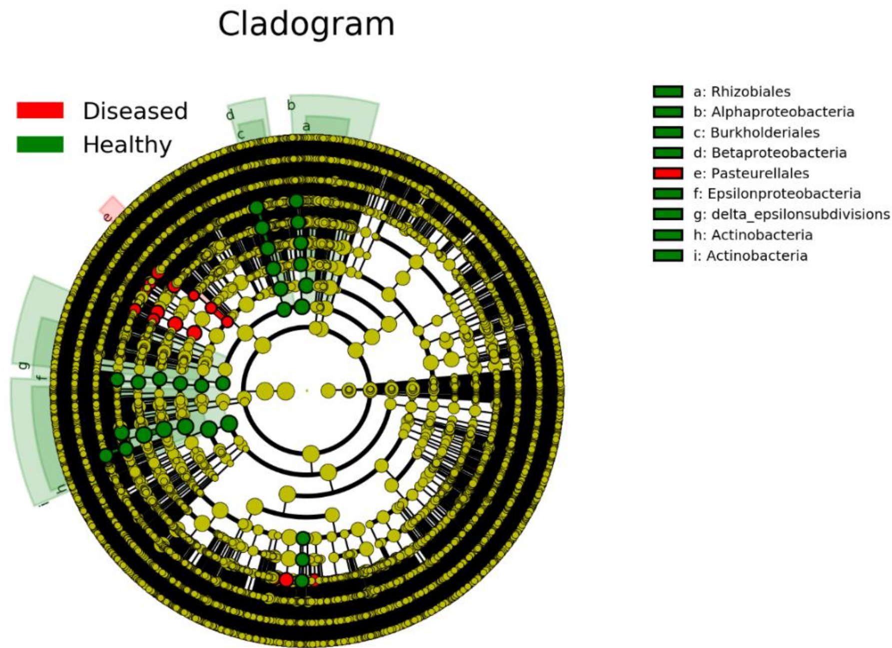


Figure 4.12 Bracken output cladogram highlighting the taxonomies detected as biomarkers.

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. 4.13.

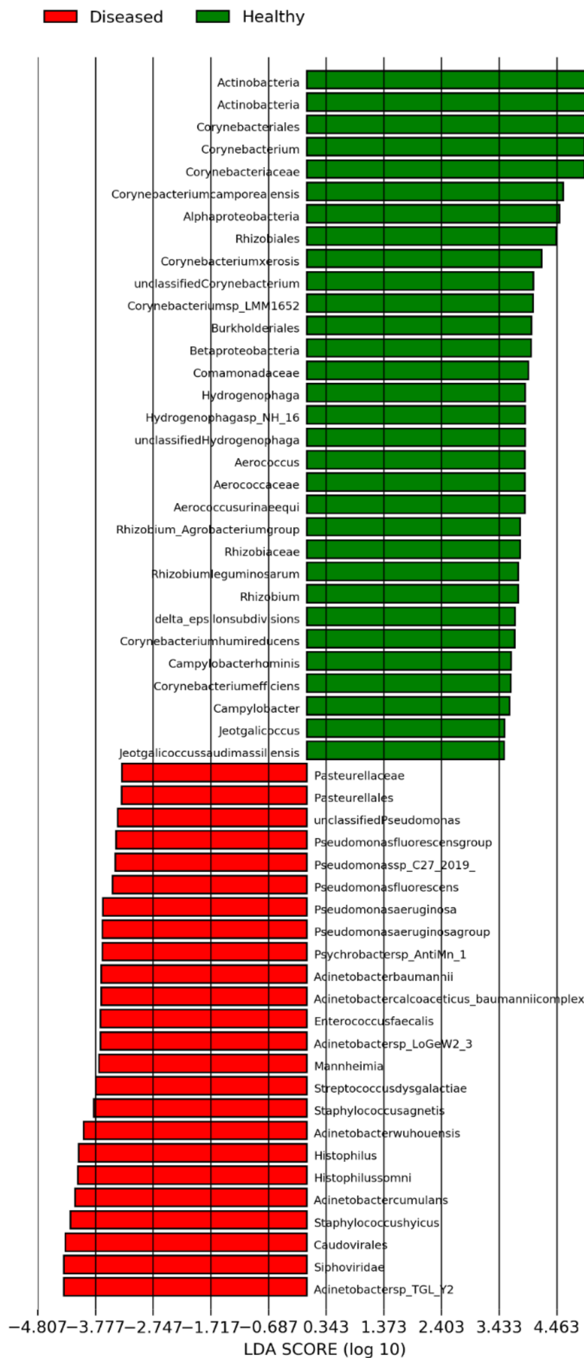


Figure 4.13 Bar chart showing the LDA values of the taxonomies detected as biomarkers from Bracken data.

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. Samples M2, M3 and M6 had perfect hits for resistance genes (Fig. 4.14). 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.

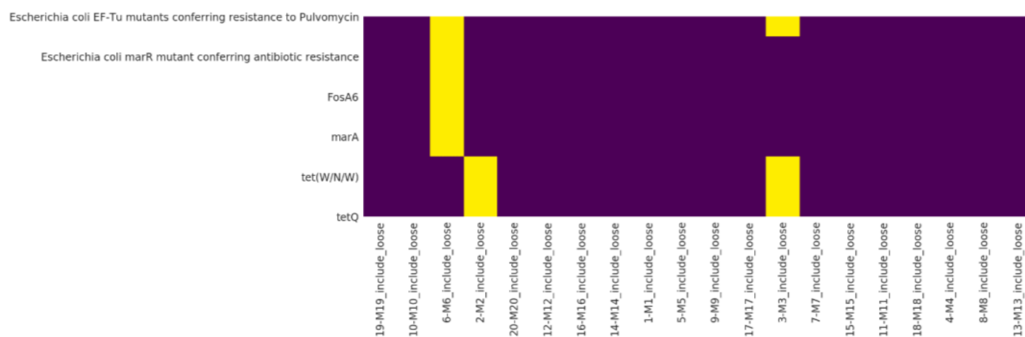


Figure 4.14 Heatmap of strict and perfect hits for resistance gene identification. Yellow on the heatmap represents a perfect hit, teal represents a strict hit and purple represents no hits.

4.4.2. 16S rRNA gene amplicons microbiome investigations using ONT minION

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 4.17). The DNA concentrations were measured using NanoDrop™ (Thermo Scientific™, Waltham, MA, USA) and deemed adequate quality and quantity.

Table 4.17 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)	

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

When reviewing the number of classified reads via EPI2ME, there were 43,202 of classified reads, 209 unclassified reads and an average accuracy of 90%. The data 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 some variations in the top 5 most abundant bacteria per sample. 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 4.15). 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 cross the samples (Table 4.18).

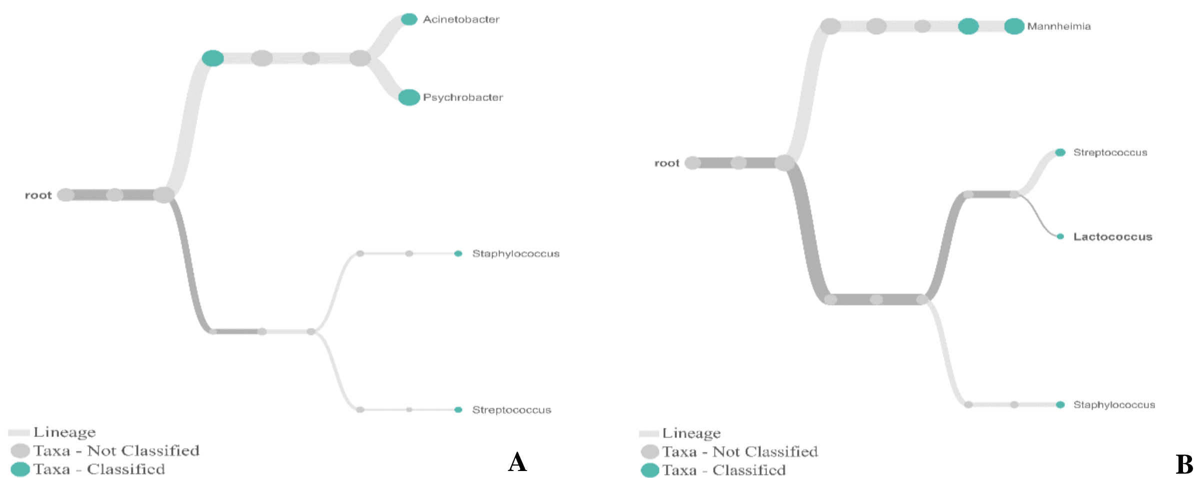


Figure 4.15 Phylogenetic tree summarising the main taxa observed form the samples of non-diseased healthy teats (A) and diseased ITN teats (B).

Table 4.18 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. 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). 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.

5.1. 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 (NGS) 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 Objective 4 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 Objective 1, 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). It is not known if there is an associated increased risk of cows with or have previously had pneumonia developing ITN. *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). 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 Objective 1 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.

5.2. 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 Objective 3 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**.

5.2.1. 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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