Identifying infection reservoirs of digital dermatitis in dairy cattle

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CONTENTS

1.1 Farmer recommendations

• Digital dermatitis (DD) is an infectious ulcerative dermatitis typically affecting the skin of the hind feet of dairy cattle worldwide with substantial welfare and economic implications making this disease an important issue for the dairy industry. The DD treponemes are considered the causal microbes of DD and across the last decade these difficult to grow bacteria have been reported to also cause skin lesions in beef cattle, sheep, goats and pigs. This report focused on understanding relationships between the bacteria, dairy cattle and the farm environment to underpin implementation of effective treatment/preventative measures.

• When considering within host infection reservoirs other than within the foot lesion, the DD treponemes were identified in the bovine gastro-intestinal (GI) tract, primarily the gingiva and recto-anal junction (RAJ) and they do not appear to cause damage to the host in these locations. Furthermore for the first time the ability to culture/isolate DD treponemes from faeces was demonstrated adding to evidence for faecal shedding as one route of transmission of DD. Survival studies in the laboratory demonstrated that DD treponemes have the ability to survive in sterile faeces for a median of 1 day and a maximum of 6 days which would enable transmission of viable bacteria to another animal's foot. Given involvement with the GI tract and faeces, increasing hygiene on farm should help to reduce DD on farm.

• The DD treponemes were detected in dairy cattle fomites (surfaces touched by affected feet/lesions) including hoof trimming knives, trimming equipment and gloves as well as surface footprints made on crush and parlour floor surfaces. These fomites should be considered important infection reservoirs in addition to DD lesions themselves and the GI tract. Improving biosecurity and foot trimming practices should help reduce disease spread. Further research is needed into protocols for disinfecting hoof trimming equipment and to mitigate on farm infection sources.

• In the laboratory, the best farm beddings to use to reduce the presence of DD treponemes were 1) straw or 2) sand containing 5% (w/w) lime as the DD bacteria were not viable in these beddings. The straw bedding data corresponds with several epidemiological studies suggesting straw is lower risk for DD. Field trials are now needed to gage how this knowledge may be applied to control DD and to verify the effectiveness of these bedding types on actual farms. In contrast, DD treponemes can remain viable in recycled manure solids (RMS) for five days, in sawdust for six days and in sand for at least seven days.

• From a broader perspective, in order to effectively tackle DD in the future, further research should investigate (but not be limited too) the development of novel antimicrobials and/or disinfectants as well as alternative novel methods to reduce transmission of the disease. Further research into effective vaccines is also needed.

1.2 Executive Summary – max 2 pages.

Introduction: Digital dermatitis (DD) is an infectious ulcerative dermatitis typically affecting the skin of the hind feet of dairy cattle worldwide with substantial welfare and economic implications making this disease an important issue for the dairy industry. A polytreponemal aetiology has been described with three distinct cultivable treponeme phylogroups (*Treponema medium, Treponema phagedenis* and *Treponema pedis*) consistently detected within DD lesions. Current control strategies fail to eliminate DD on farm and little is known about transmission. Identifying the infection reservoirs of DD treponemes should inform new prevention strategies for DD.

Results:

Presence of digital dermatitis treponemes in animal tissues and faeces:

Dairy cattle gingiva, recto-anal junction (RAJ) and DD-unaffected foot tissue along with samples from the dairy farm environment were surveyed for presence of DD treponemes by molecular and cultivation techniques to determine their role as infection reservoirs. DD treponemes were detected in 6.3% (14/122) gingiva sampled, 1.6% (2/121) RAJ sampled and 18.9% (41/217) DD-unaffected feet. No temporal association with presence of DD treponemes in these tissue types was identified. Detection of DD treponemes in dairy cattle faeces (*n*=62*),* mucin casts (*n*=31), water (*n*= 19) and feed samples (*n*=36) failed by direct PCR, despite use of optimised detection techniques for faecal material. However, for the first time a treponeme belonging to the *T. phagedenis* DD treponeme phylogroup was isolated from a dairy cattle faecal culture and a second faecal culture was also positive by PCR for this DD treponeme phylogroup resulting in a culture detection rate in faeces of 6.5% (2/31 faecal cultures).

Presence of digital dermatitis in fomites:

DD treponemes were readily detected on dairy cattle fomites by PCR. For hoof trimming blades, 79% (19/24) cattle blades were positive for DD treponemes by PCR, with 100% (17/17) of cattle with DD lesions resulting in a DD treponeme positive blade. From just two culture attempts a DD trepponeme was grown from a foot trimming knife used on a foot exhibiting DD. Furthermore 9/16 foot trimmer gloves were positive for DD treponemes following DD-affected foot handling. DD treponemes were also detected on a small number of foot trimming tools other than the foot trimming knife blades. Additionally, for the first time, DD treponemes were detected in 13% (22/169) dairy cattle footprints, with the largest proportions detected in footprints on concrete and rubber floors.

Role of digital dermatitis treponemes in asymptomatic feet and carriage sites:

Investigation into carriage of DD treponemes in host tissues using histopathology and immunohistochemistry demonstrated that gingiva and RAJ tissue had no signs of disease.

Contrastingly, clinically healthy foot tissues which were PCR positive for DD treponemes had microscopic tissue changes consistent with infection. Multi-locus sequence typing revealed that the same sequence types of DD treponeme phylogroups found in DD lesions of various host species could also be found in gingiva, RAJ and DD-unaffected foot tissue of dairy cattle.

Survival of digital dermatitis treponemes in relevant environments:

Survival of DD treponemes was assessed in a range of different conditions, with DD treponemes remaining viable when cultured between the pH values of 5.5 and 9 and in temperatures of 4-37 °C under anaerobic conditions. DD treponemes remained viable in faecal microcosms incubated aerobically for a median of 1 day (range of 0-6 days). In five different bedding microcosms under aerobic conditions, DD treponemes were viable for the full 7 days of the study in sand bedding, for 6 days in sawdust and for 5 days in recycled manure solids (RMS). However, DD treponemes were not viable at any time point when inoculated into bedding microcosms of straw or sand containing 5% (w/w) lime.

Conclusions: These studies have demonstrated that DD treponemes have a diverse range of infection reservoir sites including the gastrointestinal (GI) tract and fomites, and along with survival information, this knowledge can be applied to the development of preventative measures to mitigate DD transmission.

Review of Digital Dermatitis Research:

A review of recent bovine DD research including DD treponemes, risk factors and control strategies was completed and accepted for publication in the Veterinary Journal. The conclusions of the review were:

• DD infection reservoirs are DD lesions, hoof trimming tools and bovine GI tract.

• Improving on farm hygiene and foot trimming practice could help prevent disease spread.

• Currently used antibiotics and their application methods are not the most effective against DD treponemes.

• Novel antibiotics, additional transmission prevention and/or effective vaccines are needed.

Publications from project thus far:

Sullivan LE, Blowey RW, Carter SD, Duncan JS, Grove-White DH, Page P, Iveson T, Angell JW, Evans NJ. Presence of digital dermatitis treponemes on cattle and sheep hoof trimming equipment. Vet Rec. 2014, 175: 201.

Evans NJ, Murray RD, Carter SD. Bovine digital dermatitis: Current concepts from laboratory to farm. Vet J. 2016, 211: 3-13.

2. INTRODUCTION

Bovine digital dermatitis (BDD) is an infectious disease causing serious lameness in dairy cattle worldwide. This important disease raises substantial issues for the UK dairy industry. BDD is very painful, making it an important animal welfare issue. The disease is endemic on the majority of UK dairy farms, providing a real challenge for the dairy industry as a whole. Considerable economic implications result from treatment costs and reduction in milk yield and reproductive ability [1-3]. BDD is estimated to cost a dairy holding of 65 cows $1,517/year [4] which equates to costing the UK dairy industry (1.85 million dairy cows) a substantial £26.4 million/year.

Spirochetes (bacteria) belonging to the genus *Treponema* have been identified by several research groups as the primary causal agents of BDD [5-7]. However, these organisms are very difficult to isolate and grow and there has been limited success worldwide in obtaining relevant isolates, even though they are found in all BDD lesions. The research team at the University of Liverpool (UoL) has been responsible for isolation and characterisation of a large number (90+) of BDD treponemes [8, 9] and for designating one phylotype as a new species; *Treponema pedis* [10].

Identifying the infection reservoirs and routes of BDD treponeme transmission are central to preventing BDD spreading on and between dairy farms and are a major challenge for scientists to enable real impacts at the farm level.

Whilst treponemes have been found throughout the bovine gastrointestinal (GI) tract these organisms are typically very different to the DD treponemes [11]. However, recent to the start of this study we had provided preliminary evidence that BDD treponemes may well be present in the bovine GI tract, specifically in the oral and rectal tissues [12]. Further studies into these specific tissues and identifying additional infection reservoirs of DD treponemes should inform new prevention strategies for DD. New approaches are required because current control strategies fail to eliminate DD on farms and little is known about transmission.

Thus, this project sets out to further characterise the relationships between BDD treponemes, the bovine host and the dairy farm environment so that BDD transmission routes can be clarified to enable implementation of effective treatment/preventative measures.

2.1. Objectives

The overall objective of this study was to try and identify and characterise specific associations between BDD treponeme carriage and the bovine host and environment so that farmers might be informed of better intervention strategies for BDD.

The project best suited a PhD study programme, with specific objectives as follows:

1. Improve techniques for the molecular detection of DD treponemes in bovine faeces

2. Identify potential infection reservoirs of DD treponemes in the dairy cow and dairy farm environment:

- Build upon the previous work [12] by surveying a larger number of dairy cattle for the detection of DD treponemes in the gingiva, RAJ and healthy pedal tissue and to determine if there are any temporal associations of DD treponemes with these tissues

- To survey the dairy farm environment for the presence of DD treponemes in faeces, feed, water, gloves, foot trimming tools and footprints

3. Further characterise the carriage of DD treponemes in dairy cattle tissues other than DD lesions:

- Determine if carriage in tissues can occur without disease and determine the localisation of treponemes in these various tissues using histopathology and immunohistochemistry

- Investigate whether it is actually the same DD treponeme strains (sequence types) found in DD lesions [13] that are present in the different anatomical sites

4. To determine the growth and survival of DD treponemes under different conditions relating to the host and dairy farm environment:

- To conduct survival studies to investigate DD treponeme survival at different temperatures, different pH values, in bovine faeces and in different types of bedding typically used in dairy systems

3. METHODS

3.1 Identification of most appropriate method for molecular detection of DD treponemes from bovine faeces

Serial two-fold dilutions of a digital dermatitis treponeme (*T. phagedenis* phylogroup strain T320A) in culture media (1/2 – 1/1048576) were prepared using pooled cultures containing 1.14 x 10^8 cells/ml. Faecal samples from four different dairy cows (low milk yield and high milk yield cows) were pooled and mixed. Five hundred milligrams of faeces was spiked with 500µl of each dilution from the dilutions series and were stored at -20°C till DNA extraction. Spiked faeces underwent DNA extraction using the various DNA extraction techniques (Table 1) and using different relevant methods for each kit, resulting in 4 kits and 8 different methods being tested using PCR.

Table 1: DNA extraction kits and protocols used for DNA extraction protocols experiment

Abbreviation	Kit/ method name	Manufacturer	Material amount (mg)	Extraction type	Elution volume (µl)
QS	QIAamp® Fast DNA Stool Mini Kit	Qiagen, Manchester, UK	200 mg	chemical	200
QS.1	QIAamp® Fast DNA Stool Mini Kit – alternative lysis method	Qiagen, Manchester, UK	200	chemical	200
QS.2	QIAamp® Fast DNA Stool Mini Kit – pretreatment protocol (Klitgaard et al., 2014)	Qiagen, Manchester, UK	200	chemical	200
MP	PowerSoil® DNA Isolation Kit	MO BIO laboratories Inc, Carslbad, CA, USA	250 mg	physical/ chemical	100
MP.1	PowerSoil® DNA Isolation Kit – wet soil sample protocol	MO BIO laboratories Inc, Carslbad, CA, USA	250	Physical/ chemical	100
MP.2	PowerSoil® DNA Isolation Kit- alternative lysis protocol	MO BIO laboratories Inc, Carslbad, CA, USA	250	Physical/ chemical	100
PF	PowerFecal® DNA Isolation Kit	MO BIO laboratories Inc, Carslbad, CA, USA	250	Physical / chemical	100
BN	Stool DNA isolation Kit	Norgen Biotek Corp, Thorold, Canada	100 (BN.1), 150 (BN.2), 200 (BN.3)	Physical/ chemical	50

3.2 Survey of the dairy host and farm environment for digital dermatitis treponeme infection reservoirs

3.2.1 Sample Information

Details of farms from which environment samples including faeces, mucin casts, feed, water, foot trimming equipment and footprints were collected are listed in Table 2. All farms were endemic for DD in dairy cattle.

Table 2: Farm information [a]

Farm	County	Herd size	Daily milking frequency	Summer grazing access?	Parlour floor surface	Crush floor surface	DD footbath prevention
A	Cheshire	220	3	No	NT	Metal	Either formalin once weekly or copper sulphate twice weekly
B	Glouc	300	2	Yes	Rubber	Rubber	2x Daily at parlour exit
C	Glouc	450	3	No	Concrete	Rubber	2x Daily at parlour entrance
D	Glouc	220	2	Yes	Rubber	NT	2x Daily at parlour exit
E	Worcs	450	3	No	NT	Rubber	2x Daily at parlour exit
F	Glouc	-	-	-	NT	NT	-
G	Cheshire	-	-	-	NT	NT	-

[a] *Abbreviations: Glouc, Gloucestershire; Worcs, Worcestershire; NT, not tested. (-) denotes not known.*

Tissue samples were collected post mortem from dairy cows sent to slaughter with 123 cows surveyed. Cows were classified as DD-affected if a lesion corresponding to one of the stages of DD (M1-M4.1) was present on one or more of the feet.

Sterile scalpels were used to extract single tissue biopsies from the recto anal junction (RAJ) (*n*=121), gingiva (gum between the upper first and second premolars) (*n*=122), the skin above the coronet between the bulbs of the heel on both hind limbs for healthy foot tissue (*n*=217), DD lesional tissue (*n*=12) and foot tissue classed as 'other' (*n*=16). Approximately 3mm^3 of tissue for each sample was transferred into 10% neutral buffered formalin (NBF) for immunohistochemistry (IHC). Another small piece (~5mm$_3$) of each tissue was placed in transport medium for subsequent culturing and the remainder stored at -20˚C.

A total of 62 faecal samples were collected from two dairy farms (A and B, Table 3) where DD was endemic. Fresh faecal samples (30-50g) were collected immediately after defecation.

A total of 31 mucin casts were collected from one farm (Farm A Table 3). To obtain mucin casts, each faeces sample was sieved with water until mucin casts were visible as clay-like clumps. Twenty samples of mixed ration feed from the same farm were taken over several visits. A further 16 different components of feed were also surveyed.

Water samples (*n*=19) were taken using sterile universals from troughs in the housing areas from two farms (A and F, Table 3).

Hoof trimming blades were swabbed from cattle blades as described in an already published study [14], briefly samples were taken by the attending vet during routine hoof trimming of cattle (n=24).

Hoof trimming equipment (hoof grinder disc and handle, gloves before and after use and clippers) were swabbed during routine visitation on three different farms (B, C and E Table 3). Swabs of the hoof grinder disc (*n*= 24), hoof grinder handle (*n*=19) and clippers (*n*=20) were taken before the foot trimming session began and after use on each cow's hind foot and after the clippers were washed in cold water (*n*=18). Gloves worn by the foot trimmer were swabbed when first put on before each cow (*n*=11) and then again after use on each cow (*n*=16). For all these samples the DD status of the animals were recorded.

Footprints left behind on three types of material used on floor surfaces (rubber *n*=72, concrete *n*=53 and metal *n*=52) were sampled through swabbing footprints on rubber (Farms B, C and E, Table 3) and metal crush floors (Farm A, Table3) as well as footprints on rubber (Farms B and D, Table 3) and concrete parlour floors (Farm C, Table 3). Floor surfaces were regularly washed (between each cow) and swabs of the floor surface taken where the hind feet would have stood.

All sample types were kept on ice for transportation and stored at -20°C for subsequent PCR analysis.

3.2.2 Inoculation of samples into liquid media and subsequent isolation of spirochaetes

Inoculation of tissue and environmental samples for the isolation of spirochaetes, specifically treponemes, was carried out in accordance with culture techniques developed by Evans et al., (2008) for the isolation of treponemes. Samples included gingival tissue (*n*=113), RAJ tissue (*n*=115), lesional tissue (*n*=11), healthy foot tissue (*n*=204), 'other' foot tissue (*n*=16), feed samples (n=16) and hoof trimming blades (n=2). For each faecal (*n*=31) and mucin cast sample (*n*=16), treponeme culture was attempted as per Evans et al (2012). Inoculated cultures were checked every 2-3 days during incubation, by phase contrast microscopy, for the presence of spirochaetes. If spirochaetes were present the culture was sub-cultured onto plates and single colonies reinoculated and phase contrast microscopy used to ensure a pure

treponeme culture was obtained. Cultures containing isolated spirochaetes were stored at -80°C in 10% glycerol. For some samples, complete isolation was not possible due to other contaminating bacteria in the cultures. These samples were also stored in glycerol at -80˚C.

3.2.3 DNA extraction from different sample types

Tissue and swab samples (foot trimming equipment and foot prints) were thawed and underwent DNA extraction with the DNeasy Blood and Tissue kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. Faeces and mucin casts underwent DNA extraction with the QIAamp Fast DNA Stool Mini kit (Qiagen, Manchester, UK) with the alternative lysis method (QS.1). DNA was extracted from feed samples using the PowerSoil® DNA Isolation Kit (MP; MO BIO laboratories Inc, Carslbad, CA, USA) according to the manufacturer's instructions. In order to extract genomic DNA from water collected from water troughs, 1.5 ml of each sample was initially centrifuged at 13 500 rpm for 5 minutes. The supernatant was discarded and the remaining pellet of material underwent DNA extraction with the DNeasy blood and tissue kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. DNA was extracted from isolated cultures using a chelex resin method [8]. Extracted genomic DNA was aliquoted and stored at -20°C for subsequent PCR analysis.

3.2.4 PCR assays for the detection of DD treponemes

PCR assays specific for each of the three DD treponeme phylogroups (*T. medium*, *T. phagedenis* and *T. pedis*) and a *Treponema* genus specific 16S rRNA gene targeted PCR assay were carried out on extracted genomic DNA from all tissues, trimming equipment swabs and footprint swabs as previously described [9] as well as for faeces, mucin casts, feed and water samples.

3.2.5 16S rRNA gene sequencing and phylogenetic analysis

Extracted cultures of isolated spirochaetes were subjected to a universal bacterial 16S rRNA gene PCR assay and submitted for Sanger sequencing as previously described [8]. Phylogenetic analysis of the 16S rRNA gene consensus sequences obtained were carried out in order to determine the relatedness of isolated spirochaetes to pathogenic and commensal treponemes.

3.3 Tissue pathology in DD carriage niches and typing of carriage bacteria

3.3.2 Immunohistochemistry and histopathology of PCR positive healthy tissues

Immunohistochemistry (IHC) was carried out by the Veterinary Pathology Services, School of Veterinary Science at the University of Liverpool (Leahurst Field Station, Wirral, UK). Gingiva, RAJ and healthy foot tissue that were PCR positive for DD treponemes were submitted for IHC and haematoxylin and eosin (HE) staining to investigate whether DD treponemes could be visualised in these tissues and whether they could be present without associated tissue damage. IHC was carried out with rabbit anti-treponemal polyclonal antibodies using an automated protocol optimised for the labelling of treponemes [9].

3.3.3 Multi locus sequence typing of DD treponemes in host tissue samples

Multi locus sequence typing (MLST) of DD treponemes was carried out on either extracted genomic DNA from cultured gingiva (n=6), RAJ (n=1) and healthy foot tissue (n=14) samples containing DD treponemes or directly from extracted genomic DNA from tissue samples that were PCR positive for DD treponemes in the gingiva (n=9) and RAJ (n=1), DD lesions (n=2) and healthy foot tissue (n=5). Extracted genomic DNA from the rumen reticular pillar, rumen dorsal sac, gingiva and lesion of a single dairy cow from a previous study were also kindly donated [12]. Overall, this resulted in 8 cows having extracted genomic DNA from more than one tissue location analysed by MLST and a further 17 cows with only one sample location analysed. The MLST protocol used in this study followed a protocol previously developed for a study investigating the population structure and diversity of DD treponemes isolated from cloven hoofed animals [13].

3.4 DD treponeme survival under different host and farm environment conditions

3.4.1 Faeces and bedding sample collection

Faecal samples were collected from DD-unaffected low milk yield and high milk yield cows from farm A (Table 2). Following collection, faecal samples were stored at -20°C and were later pooled before microcosm set up. A total of five different types of unused bedding were collected: sand and sand containing 5% (w/w) lime (calcium oxide) were collected from Farm F, wheat straw was collected from Farm B, recycled manure solids (RMS) was collected from Farm C and sawdust was collected from Farm G (Table 2). Straw was chopped into approximately 5 mm^2 pieces for ease of handling. Samples were stored at 4°C prior to sterilisation via autoclaving and microcosm preparation.

3.4.2 DD treponeme survival in faeces and bedding microcosms

Faecal and bedding microcosm experiments were carried out in triplicate on separate days and within each experiment there were three replicates of each microcosm. Figure 1 depicts a summary of the microcosm methodology. Briefly following inoculation each microcosm was mixed well and allowed to incubate aerobically at 12°C. Samples were collected from microcosms on day 0 and then each day for 7 days total. Each day microcosms inoculated into media were allowed to incubate in an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) and scored for growth and motility via phase contrast microscopy after 7 and 28 days incubation.

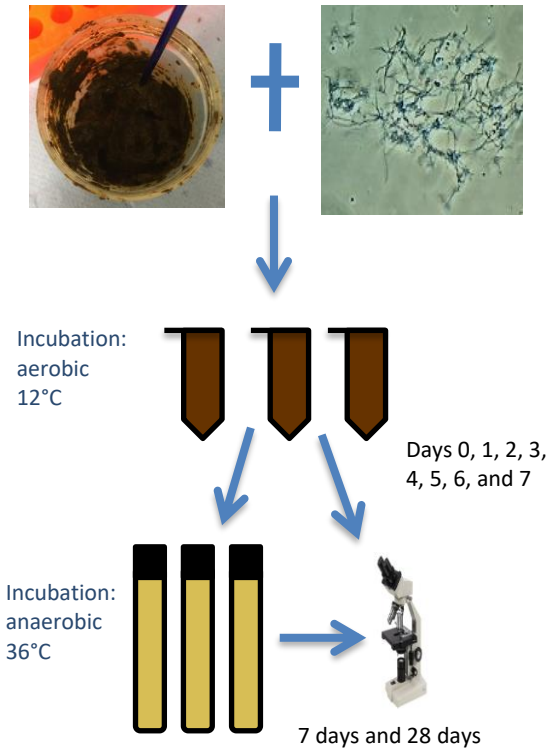

Figure 1: Pictogram of DD treponeme survival experiment procedure

Pictogram describing experimental procedure for investigation into the survival of DD treponemes in faecal microcosms (which can also be applied to study of bedding microcosms). Faecal microcosms were spiked with DD treponemes either T. medium phylogroup strain T19, T. phagedenis phylogroup strain T320A or T. pedis phylogroup strain T3552B in triplicate. Faecal microcosms were aerobically incubated at 12 °C for 7 days. On each of the 7 days the microcosms were inoculated into culture medium enriched for treponemes and growth and motility scored by phase contrasts microscopy. Microcosms inoculated into media were then growth and motility scored by phase contrast microscopy after 7 days and 28 days anaerobic incubation at 36 °C.

3.4.3 DD treponeme cultivation under different temperatures

DD treponeme cultures were incubated under anaerobic conditions at either 4°C, 12°C, 20°C, 37°C, 45°C or 60°C. Cultures were sampled on day 0, 2, 4, 7, 10, 15 and 21, except for cultures kept at 60 °C which were only sampled until day 7.

On sampling days, each of the cultures were growth and motility scored by phase contrast microscopy and sub-cultured. The secondary cultures were transferred into an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) for incubation. Growth and motility scores of secondary cultures were carried out using phase contrast microscopy after 7 days incubation and again at 14 days incubation if growth was poor after the initial 7 days.

3.4.4 DD treponeme cultivation under different pHs

A microplate methodology was adapted from a study investigating the minimum bactericidal concentrations (MBCs) of antibiotics against DD treponemes [15] in order to investigate DD treponeme survival in pHs 4.5-9 (0.5 increments). For each DD treponeme phylogroup, the experiment was carried out in triplicate on three separate occasions and each experiment had three technical replicates of each strain.

4. RESULTS

4.1 Identification of most appropriate DNA extraction method for DD treponemes in faeces

Extracted genomic DNA underwent PCR specific for DD *T. phagedenis* phylogroup in order to detect the DD treponeme dilutions spiked into the bovine faeces. The results are depicted in Table 3 with band strength of PCR products scored. Kit BN, which was tested using three different starting amounts of material (BN.1-3), failed to detect the bacteria in any of the spiked faecal samples. Kits QS, MP and PF were all able to detect the DD treponeme to varying degrees in the spiked faeces; with the alternative protocols for QS and MP (QS.1, QS.2, MP.1 and MP.2) enabling the best detection via PCR.

Table 3: PCR detection of DD T. phagedenis strain T320A from extracted genomic DNA from four different extraction kits including alternative methods

T. phagedenis	PCR Detection of T320A[a]									
T320A dilution	**QS**	**QS.1**	**QS.2**	**MP**	**MP.1**	**MP.2**	**PF**	**BN.1**	**BN.2**	**BN.3**
2X	++	++	++	+	+	++	+++	-	-	-
1	+	++	++	+	+	+	+	-	-	-
½	+	++	+	+	+	+	+	-	-	-
¼	+	++	+	-	+	+	+	-	-	-
1/8	+	++	+	-	+	+	NT	-	-	-
Faeces[b]	-	-	-	-	-	-	-	-	-	-

[a] *QS, QS.1, QS.2, MP, MP.1, MP.2, PF, BN, BN.1, BN.2 and BN.3 refer to the commercial DNA extraction kits detailed in Table 1. T. phagedenis phylogroup specific single step PCR assay. (+) denotes a faint positive PCR product band, (++) denotes a medium strength PCR product band, (+++) denotes a strong PCR product band and (-) denotes no PCR product band present. NT denotes not tested.*

[b] *Faeces refers to faeces not spiked with T. phagedenis phylogroup strain T320A.*

Overall, kit QS.1, which included an alternate 95°C incubation step during the lysis stage of the manufacturer's protocol, was the only DNA extraction kit to produce medium strength bands across all DD treponeme dilutions (2X-1/8); the other kits either weakly detected the DD treponeme in faeces with the production of faint bands or bands were initially strong or medium strength and became faint as T320A became more dilute. DNA extractions were repeated for kit QS.1 but with a larger range of two-fold serial dilutions of the DD treponeme (1-1/1048576) spiked into an equal volume of bovine faeces to ascertain the largest dilution to which kit QS.1 would enable downstream detection. Extracted genomic DNA underwent PCR specific for *T. phagedenis*-like phylogroup to detect the DD treponeme within the faeces.

The DD treponeme was detectable in bovine faeces down to a final dilution of 1/1024 in spiked faeces.

4.2 Identification of digital dermatitis infection reservoirs

In this study, dairy cattle gingiva, recto-anal junction (RAJ) and DD-unaffected foot tissue (previously identified as potential infection reservoirs) together with samples from the dairy farm environment were surveyed for the presence of DD treponemes by molecular and cultivation techniques to determine their role as infection reservoirs.

DD treponemes were detected in 14/122 (11.5%) gingiva sampled, 2/121 (1.7%) RAJ sampled and 41/217 (18.9%) DD-unaffected feet when using DD treponeme specific PCR. No temporal association with presence of DD treponemes in the gingiva or RAJ tissue types were identified.

The DD treponemes could not be detected in dairy cattle faeces (0%) (*n*=62*),* despite the use of optimised detection techniques for DD treponemes in faecal material. However, when cultured material was analysed, 13 of 31 faecal cultures contained spirochetes. Two treponemes were isolated and whilst one was considered commensal, for the first time a DD treponeme was isolated from a dairy cattle faecal culture. Furthermore when the spirochete containing cultures were subjected to the diagnostic PCR assays, a second faecal culture was also positive by PCR for a DD treponeme phylogroup identifying a total of 2 of the 31 cultures (6.5%) as containing DD treponemes.

Mucin casts (*n*=31) were all negative for BDD treponemes although six of sixteen cultures were considered to contain spirochetes and two cultures yielded treponeme isolates that were identified as commensal. All water (*n*= 19) and feed samples (*n*=36) were negative when using PCR to test for DD treponemes

In contrast to the relatively low percentages for presence of DD treponemes in the GI tract, we were readily able to detect DD treponemes on the gloves and foot trimming blades of personnel handling dairy cattle feet. In the initial study of foot trimming blades which is already published [14], 79% (19/24) cattle blades were positive for DD treponemes by PCR, with 100% (17/17) of cattle with DD lesions resulting in a DD treponeme positive blade and 28% (2/7) of asymptomatic cattle having DD treponemes on the blade. From just two culture attempts a DD treponeme was grown from a foot trimming knife used on a foot exhibiting DD.

Gloves swabbed before foot trimming were negative by PCR for DD treponemes. However, after foot trimming DD treponemes were detected on 9/16 (56.3%) gloves. Positive PCR results were only obtained after trimming DD-affected feet; all gloves used on DD-unaffected cows were negative for DD treponemes after foot trimming.

The hoof grinder disc and handle were swabbed following use on cattle feet during foot trimming. DD treponeme DNA was detected on 1/24 (4.2%) hoof grinder discs swabbed. The positive hoof grinder disc had been swabbed following trimming of a DD-unaffected foot. DD treponeme DNA was also detected on 1/19 (5.3%) swabs of the hoof grinder handle swabbed following use on a DD-unaffected foot (different cow to the positive hoof grinder disc).

Clippers were swabbed following foot trimming of each foot per cow, washed briefly in cold water and swabbed again. Following foot trimming, 2/20 (10%) clippers were positive for DD treponemes. Clippers positive for DD treponemes were only obtained following the foot trimming of DD-affected feet. Following washing in cold water DD treponemes could no longer be detected by PCR on the clippers in which they were detected before washing (*n*=2).

Fresh foot prints were positive by PCR for DD treponemes suggesting that this also may be an important infection reservoir. Furthermore, footprints on three different types of floor material: rubber, metal and concrete typically used in dairy farms settings were compared for the presence of DD treponemes by PCR. A total of 169 footprints were surveyed across four farms for the presence of DD treponemes. Overall, 22/169 (13%) footprints were positive by PCR for DD treponemes. Footprints from DD-affected feet accounted for 18/22 of the DD treponeme PCR positive results. The remaining 4 positive results were from DD-unaffected feet. DD treponemes were detected in 11/72 (15.3%), 10/53 (18.9%), 1/44 (2.3%) footprints surveyed on rubber, concrete and metal floors respectively

4.3 Characterisation of digital dermatitis treponeme carriage

4.3.1 Histopathology and immunohistochemistry of carriage tissues

Studies to characterise the carriage of DD treponemes in tissues other than DD lesions which had previously identified as containing DD treponemes by PCR used histopathology and immunohistochemistry. These techniques were used to investigate general tissue damage or localization/presence of DD treponemes and demonstrated that the gingiva and RAJ tissue had no signs of disease.

Contrastingly nearly all of the healthy foot tissue which had been identified as PCR positive for DD treponemes did exhibit tissue changes consistent with infection.

4.3.2 Multi-locus sequence typing of DD treponemes from carriage sites

Multi-locus sequence typing allows bacterial strains to be typed and therefore can be used to investigate if the same strains are present in both lesions and other carriage tissues such as gingiva RAJ and healthy feet. The method relies on typing seven bacterial genes to help delineate strain profiles within a bacterial species (or phylogroup) rather than historically the typing of just one gene to assign a bacteria to a species. The multi-locus sequence typing of

carriage strains and comparison with lesion strains revealed that the same strains/sequence types of DD treponeme phylogroups found in DD lesions of various host species could also be found in gingiva, RAJ and DD-unaffected foot tissue of dairy cattle.

4.4 DD treponeme survival under different host and farm environment conditions

4.4.1 Faecal microcosm survival

The DD treponemes remained viable, growing in culture on inoculation of microcosm material, in faecal microcosms which were incubated aerobically for a median of 1 day and with a range of 0-6 days.

4.4.2 Bedding microcosm survival

The five different bedding microcosms were surveyed for treponeme survival under aerobic conditions by sub culturing each day. On surveying cultures using phase contrast microscopy the DD treponemes were identified as viable for the full 7 days of the study in sand bedding, for 6 days in sawdust and for 5 days in recycled manure solids (RMS).

However, DD treponemes were not viable at any time point when inoculated into bedding microcosms of straw or sand containing 5% (w/w) lime.

4.4.3 Survival of DD treponemes at different temperatures

The DD treponemes were able to remain viable when cultured in temperatures of 4-37°C under anaerobic conditions for at least seven days. One treponeme phylogroup survived to two days when incubated at 45°C whereas the other two phylogroups failed to reach the 15 minute time point at this temperature. All bacteria failed to remain viable when incubated at 60°C for 15 minutes.

4.4.4 Survival of DD treponemes at different PH values.

Survival of DD treponemes was assessed in a range of different conditions from pH5 to 9. Growth characteristics varied between phylogroups within the different pHs but overall DD treponemes remained viable when cultured between the pH values of 5.5 and 9.

5. DISCUSSION

Towards an optimal DNA extraction method from faeces

Of the commercial DNA extraction kits investigated, Kit QS.1 was the most successful for DD treponeme detection in bovine faeces and was able to detect up to 1/1024 dilution of the DD treponeme (1.14 x 10^8 cells/ ml stock culture) in bovine faeces. The inclusion of a high temperature heat lysis step in DNA extraction protocols appeared to be important for the DNA extraction of DD treponemes. Further optimisation of DD treponeme detection techniques for bovine faeces may be necessary to ensure the maximum likelihood of detection; however, for the purposes of this research project kit QS.1 was used to investigate whether DD treponemes are present in faeces.

Identification of infection reservoirs of digital dermatitis

This study aimed to further clarify the role of the cow and the dairy environment as infection reservoirs for DD by building upon previous work investigating dairy cattle gingiva, RAJ, healthy foot tissue and faeces for DD treponeme presence as well as exploring new potential infection reservoirs including mucin casts, foot trimming equipment, gloves, feed, water and footprints. Determining sites which are at risk of being a DD treponeme infection reservoir is key to understanding how transmission of the disease occurs and thus enables the development of strategies to prevent these transmission routes and progress towards elimination of DD from farms.

The GI tract as an infection reservoir of DD:

The small percentage of dairy cattle surveyed positive for DD treponemes in either the gingiva or RAJ reaffirms that DD treponemes do not appear to be part of the normal microbiome found in these tissue types [16, 17], which raises the question of why carriage occurs in some animals and not others? It may be that carriage is transient as described for *Escherichia coli* O157 carriage in tissues of the lower GI tract of cattle[18, 19]. Colonisation may also be associated with cow level factors such as immune response, physiological properties of the skin and diet [20, 21]. Whilst PCR of faeces still seems problematic for detection of DD treponemes during faeces spiking, we were able to actually culture DD treponemes from faeces during analysis of field samples. This is the first time DD treponemes have been grown from faeces and is especially important as it suggests that the bacteria are viable and therefore transmissible from this infection reservoir. The culture of bacteria and PCR analysis of such cultures requires further investigation as a diagnostic technique. These data do present

substantial further evidence that the GI tract has the ability to contribute to the spread of digital dermatitis via faecal shedding.

Although DD treponemes are only present in a small number of dairy cattle GI tracts, their role as an infection reservoir should not be discounted. It may be that these GI DD treponeme carrier cattle are super shedders or super spreaders, enabling a large amount of transmission despite the small number containing these infection reservoirs. Super shedding from the RAJ occurs in cattle colonised with *E. coli* O157, in which only a small proportion of cattle carrying *E. coli* O157: H7 in the RAJ shed high amounts of the bacteria into the environment [22]. Furthermore, another spirochaete, *Brachyspira hyodysenteriae* which causes swine dysentery in pigs can be maintained in pig populations by only a small number of carrier pigs [23]. Therefore carriage of DD treponemes in the GI tract likely contributes to maintenance of DD infection on farms once the disease has been introduced or more worryingly could in fact contribute to disease introduction.

Fomites as infection reservoirs of DD:

A fomite study was completed as part of this project prior to the main PhD studentship beginning, which identified foot trimming knives as an infection reservoir of digital dermatitis treponemes and is already published (Sullivan LE et al., Vet Rec. 2014 Aug 30;175(8):201) [14]. In this collaboration with AHDB Beef and Lamb, the presence of DD treponemes in cattle and sheep foot trimming blades were investigated. For both host species the results were very interesting. When considering cattle, in contrast to the relatively low percentages for presence of DD treponemes in the GI tract we report here, we were readily able to detect DD treponemes on the foot trimming blades, with 79% cattle blades positive for DD treponemes and all blades from cattle with DD lesions positive for DD treponemes [14]. Disinfection of hoof trimming knives between feet, animals and between farms would seem an appropriate measure to limit the spread of DD. Future work is much needed to clarify this disinfection process. This infection reservoir identification changed the way in which DD treponeme infection reservoirs were thought of, placing more emphasis on the role of fomites in transmission. Subsequently here we have also detected DD treponemes on gloves and other trimming equipment used during routine foot trimming (section 4.2). As the gloves were changed between each cow and all fresh gloves tested were negative for DD treponemes, the DD treponemes must have transferred onto the gloves during the trimming of the DD-affected foot, most likely during cleaning and treatment of the lesions. These results are consistent with another study investigating the presence of DD treponemes on gloves following trimming of sheep affected by contagious ovine digital dermatitis (CODD); whereby 100% of gloves from CODD-affected sheep were positive by PCR for one or more of DD treponeme phylogroups and no DD

treponemes were detected on gloves used to trim unaffected sheep feet [24]. Changing gloves or appropriate disinfection of gloves between feet, animals and between farms would seem an appropriate measure to limit the spread of DD.

Of the other foot trimming equipment (*n*=63) examined for DD treponeme presence, 6.3% were positive for one or more of the DD treponemes. This foot trimming equipment consisted of powered hoof grinder discs and handles and clippers following dairy cow foot trimming; with one swab from a disc, one swab from a handle and two swabs from clippers positive for DD treponemes after use on different cows. Interestingly, unlike for the gloves, these samples containing DD treponeme DNA were not necessarily detected following the trimming of DD-affected feet, with the positive hoof grinder swabs obtained following trimming of DD-unaffected feet.

Here, for the first time DD treponemes have been detected in the footprints left behind by cattle standing in either the crush or the parlour. Crush and parlour floors were chosen for surveying footprints not only because it allowed ease for assessing feet for DD but cattle are confined to standing in the same spot for a period of time thus making treponeme transferral to the floor more likely. Furthermore, cattle have limited space as to where they may place their hind feet thus increasing the likelihood that they may stand on or near to the location where the last cow stood. Thus, the data presented here demonstrates DD treponeme positive footprints may provide a transmission route for DD from cow to cow.

Footprints were surveyed on three different floor types: metal, concrete and rubber for the presence of DD treponemes. Of the three floor types surveyed, footprints on metal floors had the lowest DD treponeme detection rate of 2.3% compared to 18.9% and 15.3% of concrete and rubber floors respectively. Investigations into foot-surface contact times with these different surfaces on farm, together with any changes in foot posture and *in vitro* mock contamination and decontamination of these surfaces are needed in the future to further dissect these relationships. For example, whether time standing would affect DD treponeme presence in footprints is unknown and requires future investigation.

The type of floor surface used on dairy farms has come under scrutiny in various studies for associations with lameness and claw disorders; including DD [25-28]. Concrete is the most predominant floor material used in dairy systems, which is worrying when examining the footprint data in this study as concrete has the highest detection rate when compared with metal and rubber floors for DD treponemes in footprints. The hard, slippery and abrasive nature of concrete floors as well as the ability of slurry to become trapped in certain types of

concrete floor designs such as 'grooved' have been cited as possible reasons for the increased risk of claw disorders and DD observed in these types of housing systems[26, 28-30]. The presence of DD treponemes on concrete floors from footprints further adds to this risk as not only do DD treponeme footprints potentially provide an infection reservoir for DD on this floor type but abrasion suffered by feet from the concrete could be postulated to provide an entrance route for infection. In fact, infection models of DD have demonstrated abrasion of skin on the plantar aspect of the foot between the bulbs of the heel better enables DD lesion development [31, 32]. Many farms are moving towards using rubber for floor surfacing in walkways and the parlour due to reported health benefits including reduced risk for claw disorders including DD compared to concrete flooring [33-37]. Whilst in this study DD treponemes have been detected in footprints on rubber floors, and only moderately less than concrete (15.3% and 18.9% respectively), which is still cause for concern, it could be argued that softer surfaces result in less damage to feet and thus there is less opportunity for DD treponemes to gain entry.

The increasing body of evidence for the presence of DD treponemes on fomites that readily come into contact with the foot is of great concern when considering control and prevention of DD. DD treponeme presence on foot trimming equipment, gloves and crush floors corroborates with previous studies that have cited foot trimming practices as a risk factor for DD [28, 38]. Indeed, these fomites could readily pass DD treponemes from one cow to another through direct contact if foot trimming equipment or the crush/ parlour floor is contaminated with DD treponemes from the previous cow and may also be possible in footprints made on walkways. Furthermore, evidence of DD treponeme viability as long as 3 days on the gloves [24] is worrying for between farm transmission, especially if similar viability is observed on other equipment including the footprints on the crush floor as many foot trimmers bring their own crush onto the farm.

Disinfection of equipment and floors between cows and farms may be one way to control these potential infection reservoirs and prevent transmission and further work is needed to develop such protocols. Furtherher studies are also needed into the viability of DD treponemes in footprints and whether disinfectants are effective; taking into consideration contact times and concentrations of active compounds. Interestingly, cleaning gloves with disinfectants of either hand soap (with water), 1% Virkon, 1:90 dilution of FAM or 70% ethanol resulted in no growth of DD treponemes in culture and vastly reduced detection by PCR [24]. However, washing gloves with warm or cold water alone was ineffective in preventing detection of DD treponemes by PCR and culture [24]. Clearly, further study using a larger DD treponeme positive sample

population is required to determine which disinfectant, disinfectant concentration and disinfection procedure will completely disinfect equipment from DD treponemes and be practical in farming practice.

Role of digital dermatitis treponemes in asymptomatic feet and carriage sites:

The presence of DD treponemes in tissues other than DD lesions warranted further investigation into the nature of their association with these tissue types. In this study, histopathology and IHC was used to try and determine whether DD treponemes could be carried, without causing changes to the tissue associated with disease, in the GI tract and non DD lesion pedal tissue and where in these tissues the DD treponemes localised. For the GI tract tissues there was no evidence of any abnormalities in the tissue that would indicate infection or tissue damage. This suggests that unlike the human periodontal pathogen *Treponema denticola,* DD treponemes can colonise the GI tract without associated tissue abnormalities. However, interestingly DD treponemes were also not visualised in these PCR positive tissues with the polyclonal anti-treponemal rabbit antibodies. Explanations why DD treponemes were not identified by IHC in tissues may include: 1) DD treponeme colonisation was not diffuse in area and thus may be have been missed in the sections. 2) DD treponemes may not actually penetrate the tissue but be in the saliva / mucus coating the tissue and were thus lost during tissue processing or 3) there may have only been a very low concentration of DD treponemes present.

In contrast ~80% of the healthy foot tissues PCR positive for DD treponemes (*n*=36) had abnormalities present in the tissue indicative of disease pathology and in accordance with DD lesion pathology. Thus, these tissues should not be classed as 'healthy' but it is likely that these tissues may be in the very early stages of DD lesion development before normal clinical lesions can be observed by eye. Indeed, another study analysing healthy foot tissue by histopathology found that for the three samples in which DD treponemes were detected there were abnormalities in the tissues consistent with severe hyperplasia of the epidermis, hyperkeratosis and degenerated ballooning keratinocytes [39]. These changes in combination with the presence of DD treponemes lead to the author redefining these samples as subclinical DD [39]. Infection of DD treponemes within these foot tissues appears to be new as opposed to re-emerging from resolved lesions.

Additionally, a proportion of samples positive by PCR for DD treponemes, underwent MLST specific for the DD treponeme phylogroups they were positive for to determine whether the same ST types found previously in DD lesions [13] were responsible for colonisation of these

other tissue types or whether there was tissue specific tropism (preference) occurring with different strains. DD treponemes with STs that are either novel or found in DD lesions appear to be able to colonise GI tissues. Thus, it would appear that the strains that inhabit the DD lesion can go on to inhabit the GI tract or vice versa. Although further work is needed to confirm whether there are any niche limitations for some strains (STs).

Survival of digital dermatitis treponemes in relevant environments:

This study aimed to understand the ability of DD treponemes to grow and remain viable under varying conditions that may be found on farm or in the host (i.e. cattle, sheep etc) to provide further insight into the viability of identified infection reservoirs for transmission as well as providing knowledge that could help confirm or discredit newly proposed infection reservoir sites for DD. In particular, the study investigated generic conditions of pH and temperature which are important not only in terms of infection reservoirs but for culturing conditions used frequently as a method of detecting DD treponemes in reservoir environments. Furthermore, two specific niches, bovine faeces and bedding commonly used on dairy systems, were investigated to determine their role in treponemal survival and possible transmission on farm. There is a dearth of information on the effect of different pHs on DD treponeme growth and viability. This study demonstrated that DD treponemes phylogroups have slight variances in their preferred pH optimums and ranges for growth. Knowing the pH range that DD treponemes can survive in is important for assessing whether a particular site could support DD treponeme survival and thus act as a reservoir for infection. For example the skin surface of the bulb of the heel near to where DD lesions form in cattle has been cited as having a pH of 7.1 (range of 6.5-7.5); thus, any of the three DD treponeme phylogroups would be able to survive there based on pH alone [40]. The rumen, in which DD treponemes have previously been identified in the fluid [41, 42], usually has a pH between 6 and 7 which would enable survival of DD treponemes [43, 44]. However, whilst it may be expected that the lack of oxygen during fermentation of forage to produce silage would produce a good anaerobic environment for DD treponeme survival, fermentation lowers the pH, resulting in silage with a pH between 3 and 5 [45] in which DD treponemes could not survive.

Another factor which affects whether DD treponemes may be able to survive in a particular reservoir is temperature. It is known from culturing that DD treponemes are able to survive and grow between 36°C and 37°C [8]. Further investigation into temperatures in which DD treponemes survive found survival was possible at temperatures of 4°C, 12°C, 20°C and 37°C for all three DD treponeme phylogroups. Visible growth at these temperatures only occurred at 37°C. However, at the lower temperatures the treponemes remained viable and were able

to grow again once sub-cultured at 36 °C. Thus, temperatures down to 4 °C alone will not prevent DD treponeme survival in the environment and whilst refrigeration may prevent growth as with many other bacteria, it will not kill the DD treponemes when stored for 21 days.

Incubation at temperatures of 45 °C and 60 °C resulted in non-viable DD treponemes. The lack of viability of DD treponemes at 45°C and 60°C suggests that DD treponemes would not survive composting or pasteurisation techniques where temperatures can reach above 70°C and thus composting or pasteurisation may prove effective methods of removing DD treponemes from certain environments.

Faeces has come under scrutiny as a potential infection reservoir of DD, in part due to the identification of DD treponemes in the GI tract and also because it is often in close contact with the skin of the foot. Whilst previously conventional PCR and culture methods have not detected DD treponemes in bovine or ovine faeces [12, 17], deep sequencing techniques have been able to detect DD treponeme DNA although only as a very small fraction of the faecal microbiota [42, 46]. Finally here, for the first time, we have actually cultivated DD treponemes from faeces which substantially implicates this materials being able to act as an infection reservoir and makes understanding survival in this niche key to understanding transmission. Other spirochaetes have been investigated for their survival in various animal faeces and results have varied from 210 days for *Brachyspira pilosicoli* in porcine faeces to less than 4 days for the same spirochaete in avian ceacal faeces [47, 48]. Here, the DD treponemes had a median viability of 1 day in the faeces with a range of 0-6 days respectively. Although survival for one day in faeces would appear to be short, this would still allow enough time for a cow to come into contact with the faeces containing DD treponemes and enable transmission. Especially as the liquid consistency of dairy cattle faeces results in the hind feet becoming covered with this potentially infectious material and housed dairy cattle often spend a large amount of time with their hind feet in slurry. It is not feasible to remove faeces immediately after defecation and faecal slurry often builds up over a short period of time in housing systems. Although regular scraping through manual and automatic means helps to control levels of slurry, it never completely removes it, especially as floor design and quality may result in retention of the slurry. Furthermore, automatic scrapers and frequency of use have actually been linked to an increased risk of DD, which may be a result of the wave of faeces carried by the scrapers coming into contact with the feet of cows slow to move out the way [21, 49, 50]. Additionally, walkway and chute design often results in cattle walking directly behind each other and thus they are highly likely to step in other cows faeces shortly after defecation. Therefore, there are a variety of reasons for which cattle feet may come into contact with faeces within one day.

Whilst bedding was not investigated as a potential infection reservoir for DD treponemes in this study, pathogens relating to other diseases such as the mastitis causing pathogens *Escherichia coli, Klebsiella pneumonia, Mycoplasma bovis* and *Streptococcal* spp., have been found in bedding and bedding type has been cited as a risk factor for that disease. Healthy feet and DD lesion affected feet come into close contact with bedding and thus it is feasible that transference of DD treponemes may occur especially given that this thesis has identified floor surfaces as potential fomites. Thus, the survival of DD treponemes in bedding was investigated to determine the feasibility of bedding as an infection reservoir for DD.

There were clear differences in DD treponeme survivability between the five types of bedding investigated. Despite this bacteria being considered fastidious, and therefore difficult to grow, DD treponemes remained viable (as determined by secondary culture) in sawdust bedding for 6 days and in sand for the full 7 day duration of the study. Sand is a popular bedding choice in part as it is inert and provides poor support to pathogen growth, thus it usually has lower bacterial loads than other bedding types [51], resulting in its use as a method for controlling pathogens. Therefore, it is surprising that the fastidious DD treponemes survived and remained viable for 7 days within the sand microcosm given the detail above. Sawdust, on the other hand, is known to contain higher bacterial loads particularly when wet/ soiled [52]. RMS also known as 'green bedding' involves the recycling of cattle manure / slurry on farms using a separator designed to separate the solid fraction from the liquid fraction of manure/ slurry in order to produce RMS which is > 35% dry matter. RMS provides a cheaper alternative to other bedding types, is comfortable and is readily available. RMS has gained traction in many countries where it is now commonly used and is sanctioned for use in the UK, although it is subject to strict conditions of use to try and control for risks of disease spread posed by the RMS being an animal by-product [53].

Research is ongoing to determine the risks of RMS use and its effect on disease prevention and control, and of particular interest is the pathogen loads in RMS which have been shown to be comparable if not higher in RMS than in other bedding types depending upon microorganism of interest [53]. In this study, DD treponemes remained viable in RMS for 5 days following aerobic incubation at 12 °C, as determined by sub-culturing. Interestingly, this is longer than the survival we report in faeces. Hence the processing used in an attempt to reduce pathogen load in the production of RMS would appear to make this material more amenable to DD treponeme survival. Thus further investigation into RMS as a DD infection reservoir is required.

In contrast, DD treponemes were not viable (as determine by sub-culture) following incubation in wheat straw and sand 5% (w/w) lime mix bedding, with DD treponemes not present at all immediately from the initial inoculation in the sand 5% (w/w) lime mix microcosm. The poor viability in straw of DD treponemes substantially ties in with risk factor studies which cite straw yards to be low risk for DD compared to other housing systems [27, 54]. Taken together, this makes a strong case for applying straw as a control measure for DD although specific field trials for use in the control of this disease would be beneficial, to ascertain minimum bedding maintenance criteria.

Adding hydrated lime to bedding is used as a means of helping to keep the bedding dry and control bacterial load as the lime increases the pH of the bedding and acts as a desiccant [55, 56]. In this study, where sand had one of the best DD treponeme viability rates of the beddings investigated, the addition of approximately 5% (w/w) lime resulted in no survival of the DD treponemes almost immediately after inoculation. Thus, lime may be a useful measure for the control of DD treponemes both in sand as well as in other bedding types such as sawdust and RMS and further investigation is required along with investigation into concentration of hydrated lime required. However, lime must be used in moderation as it can cause skin irritation which may predispose the cow to disease.

SUMMARY

Here we have further demonstrated that the DD treponemes can be found in the bovine GI tract, and identified they do not appear to damage these host tissues. We have been able to culture/isolate DD treponemes from faeces for the first time (albeit in a small sample proportion) which further gives evidence for faecal shedding as one DD infection reservoir. Survival of DD treponemes for up to six days in faeces enables a substantial window of opportunity for subsequent transfer to another animals foot. Given involvement with the GI tract and faeces, increasing hygiene on farm should help to reduce DD on farm.

The DD treponemes were detected in a proportion of macroscopically healthy feet which we subsequently identified as diseased microscopically and represents another DD infection reservoir. Further DD infection reservoirs include dairy cattle fomites including hoof trimming equipment and gloves as well as surface footprints made on crush and parlour floor surfaces. These fomites should be considered important infection reservoirs. Improving biosecurity and foot trimming practices should help reduce disease spread. Further research is needed into protocols for disinfecting foot trimming equipment and to mitigate on farm infection sources.

The DD treponemes are able to remain viable in culture temperatures of 4, 12, 20 and 37°C as well as a pH range of 5.5 to 9. Of five bedding types assessed for DD treponeme survival, straw bedding or sand supplemented with 5% (w/w) lime appeared to prevent survival and need to be investigated further. These findings not only help to predict where DD treponeme reservoirs may be found, they point to methods for controlling DD treponemes in the environment. However, implementation of any control strategies should take into account the effect of the strategy upon other pathogenic microorganisms, for example, changing to straw bedding might increase mastitis pathogens.

From a broader perspective, in order to effectively tackle DD in the future, further research should investigate (but not be limited too) the development of novel antimicrobials and/or disinfectants, their method of application, as well as alternative novel methods to reduce transmission of the disease. Further research into effective vaccines is also needed.

6. REVIEW OF DIGITAL DERMATITIS RESEARCH

As part of this project a review of recent bovine DD research including the microbiology of DD treponemes, risk factors for the disease as well as treatment and control strategies was completed and accepted for publication in the veterinary journal:

'Evans NJ, Murray RD, Carter SD. Bovine digital dermatitis: Current concepts from laboratory to farm. Vet J. 2016 May;211:3-13.'

The conclusions of the review were:

• The DD infection reservoirs on dairy farms include the actual DD lesions themselves, hoof trimming knives and the bovine gastrointestinal tract.

• Given the identification of these reservoirs it should be considered that improvement to both on farm hygiene and foot trimming practice could help prevent this important disease.

• On comparing current *in vitro* susceptibility data, field trials and comparison with treatment of human treponematoses, it would appear currently used antibiotics and their application methods are not the most effective against DD treponemes.

• In terms of tackling this disease in the future it is of the upmost importance that novel antibimicrobials are identified for use, that additional strategies for transmission prevention are identified and implemented and that effective vaccines are much needed.

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