Paper

Bovine ischaemic teat necrosis: a further potential role for digital dermatitis treponemes

S. R. Clegg, S. D. Carter, J. P. Stewart, D. M. Amin, R. W. Blowey, N. J. Evans

A recent outbreak of ischaemic teat necrosis (ITN) on mainland UK has resulted in large economic losses for dairy farmers. Typical cases start as an area of dry, thickened and encrusted skin on the medial aspect of the base of the teat, where the teat joins the udder, often with a fetid odour. The erosion spreads down the teat, often causing intense irritation, which in turn leads to more severely affected animals removing the entire teat. Due to the severity of ITN and the substantial economic costs to the industry, analyses were undertaken to ascertain if an infectious agent might be involved in the pathology. The study has considered a role for digital dermatitis (DD) treponemes in the aetiopathogenesis of ITN because, as well as being the prime bacteria associated with infectious lameness, they have been associated with a number of emerging skin diseases of cattle, including udder lesions. A high association between presence of DD-associated treponemes and incidence of ITN (19/22), compared with absence in the control population is reported. Furthermore, sequencing of the 16S rRNA gene of treponeme isolates supports the hypothesis that the identified treponemes are similar or identical to those isolated from classical foot DD lesions in cattle (and sheep). Further studies are required to allow effective targeted prevention measures and/or treatments to be developed.

Introduction

Mastitis, udder and teat lesions are of major economic importance to the dairy industry and are significant animal welfare issues. Mastitis causes economic losses of around £170 million per annum to the UK dairy industry (DEFRA 2002). However, there are many other infective issues of mammary tissues in cattle. These include bovine herpesvirus (BHV-2), which is known as bovine ulcerative mammillitis and causes an acute ulcerative condition of the teat and udder (Syring and others 2010). Pseudocowpox can cause a mild infection of the teats in cattle and several strains of papillomavirus can lead to the formation of papillomas (warts) on the teats and udders, (Ogawa and others 2004).

Although first reported some 10 years ago (Blowey 2004), during 2014/2015 there has been an anecdotal increase in reported cases of ischaemic teat necrosis (ITN) on farms across mainland UK. This disease leads to severe teat pathology, economic losses and, in a significant number of cases, culling of the affected cow(s).

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Most recently, a number of emerging skin diseases in farm animals have been observed, all linked to infections with specific treponemal bacterial linked originally to digital dermatitis (DD) in dairy cattle. These treponemes are commonly isolated from DD skin lesions in beef and dairy cattle and the association has been reported worldwide (Walker and others 1995, Dawson 1998, Klitgaard and others 2008, Nordhoff and others 2008, Evans and others 2009, Yano and others 2010, Sullivan and others 2014a). These bacteria are now also viewed as the primary infections in contagious ovine digital dermatitis (lesions in sheep in the UK and Ireland (Demirkan and others 2001, Sayers and others 2009), analogous foot lesions in goats (Sullivan and others 2014a, b), severe non-treatable foot lesions in dairy cattle (Evans and others 2011), skin lesions in pigs (Svartstrom and others 2013, Karlsson and others 2013, 2014) and a newly reported 'hoof disease' in wild North American elk (Clegg and others 2014). DD associated treponemes have also been detected in horse canker lesions (Sykora and others 2014).

In all of these clinical skin manifestations, lesions from the affected animals generally contain spirochaetes from several *Treponema* phylogroups. Three commonly isolated and characterised treponemes from the various animal skin lesions belong to the *Treponema medium, Treponema phagedenis* and *Treponema pedis* phylogroups (Evans and others 2008, 2009).

No microbiological studies have previously been carried out on ITN lesions. Given that the teat necrosis lesions had a similar pungent fetid odour to DD lesions, a moist, stippled appearance resembling other treponeme skin infections, and the recent report of treponemes isolated from ulcerative mammary dermatitis (Evans and others 2010), this study aimed to determine if DD treponemes were associated with ITN.

Methods

Ethical approval

This study was carried out with ethics approval obtained from the University of Liverpool Veterinary Ethics Committee (VREC157).

Questionnaire

A short questionnaire was developed to obtain information about cases of ITN on a small number of farms. The questionnaire was distributed by a veterinarian (RWB), following consultation with the farm veterinarian, and after having viewed photographs and circulated a description of the disease. Questions were asked including which cows were affected, age, date of calving, date of disease onset, type of milking cups used, clinical outcome, treatments, clinical appearance and animal identification. When postpartum ITN was seen, the treatments used and their effectiveness and recovery rates were also recorded.

Additionally, the questionnaire asked questions about herd size, milk sales, DD status and prevalence on the farm, milking methodology and bedding.

Samples

Samples were taken from animals on farms that reported cases to the veterinarian (RWB). These farms had a large geographical range across mainland UK, and were chosen to give as great a geographical spread as possible. A photograph of the case was taken by a field vet, and reviewed by the veterinarian associated with this study (RWB), and was confirmed to be the same condition as that under investigation. When this was confirmed, a swab, and if possible, a tissue sample were taken from the animal. This usually consisted of a scab that was falling off the lesion or, in some cases, such as when the animal was culled, deeper tissue samples, which were placed in oral treponeme enrichment broth (OTEB: Anaerobe Systems, Morgan Hill, California, USA) containing 5 µg/ml rifampicin and 5 µg/ml enrofloxacin and posted to the laboratory, where they were inoculated immediately for culture and isolation of DD treponemes.

In cases where it was not possible to obtain a tissue sample, a dry swab was taken, with care being taken to ensure sufficient lesion material was on the swab. Swabs were posted to the laboratory and stored at 4°C until subjected to DNA extraction for PCR detection of DD treponemes.

Isolation of spirochaetes

Spirochaete isolation attempts were carried out on all tissues taken from affected ITN samples, as described previously for other cattle samples, in OTEB (Evans and others 2008) including 5 µg/ml rifampicin and 5 µg/ml enrofloxacin. Samples were inoculated into OTEB containing fetal calf serum (Gibco, Paisley, UK), to maximise growth of *T. phagedenis* and *T. pedis* phylogroups, and rabbit serum (RS, GE Healthcare Life Sciences, Buckinghamshire, UK) was added to maximise growth of *T. medium* phylogroup treponemes. All isolation attempts were carried out in an anaerobic cabinet (85 per cent N_2, 10 per cent H_2 and 5 per cent CO_2, 36°C). Cultures were screened by phase contrast microscopy and analysed by specific nested PCR assays to identify any specific treponeme phylogroups present.

Passage was continued on fastidious anaerobe agar plates, supplemented with 5 per cent defibrinated sheep blood and antibiotics as above, and single colonies from the plates were inoculated into further OTEB tubes as described above to allow pure cultures to be obtained.

DNA extraction

For isolation of bacterial genomic DNA from OTEB cultures, 2 ml of the culture was centrifuged (5000× *g*, 10 min, 4°C) in a bench-top centrifuge. DNA was then extracted from the cell pellet using Chelex-100, as previously described (Chua and others 2005) and stored at −20°C.

For extraction of DNA from tissues and swabs, a QIAquick DNeasy blood and tissue kit (Qiagen, Manchester, UK) was used following manufacturer's instructions.

PCR

Tissues taken from ITN cases and culture samples were subjected to nested PCR assays, specific for the three cultivable DD-associated treponeme phylogroups, *T. medium, T. phagedenis* and *T. pedis*, as described previously (Evans and others 2008, 2009) with resulting PCR products encompassing 300 to 500 base pairs of the 16S rRNA gene. For gene sequencing, the large 16S rRNA gene was subjected to PCR and the resulting amplicon sequenced (Evans and others 2008).

To validate the PCR assays, each experiment included positive controls (genomic DNA from each of the three bovine DD treponeme phylogroups) and a no template control (water) (Evans and others 2009), with all assays carried out in triplicate.

A PCR assay for herpesviruses was performed using degenerate primers that detect all herpesviruses (Ehlers and others 1999, Hughes and others 2010). Ovine herpesvirus-2 was used as a positive control for the pan herpesvirus PCR. Water was used as a no template control.

Sequencing and sequence analysis

Amplified PCR products were sequenced commercially (Macrogen, Amsterdam, The Netherlands) and gene sequences assembled using the Chromas Pro sequence analysis package (Technelysium). Gene sequences were aligned using CLUSTALW as implemented in MEGA 5.0 (Tamura and others 2011). The DNA alignment was subjected to Modeltest, as implemented in Topali (Milne and others 2009), which revealed that the best fit model was General Time Reversible. This was used to produce maximum likelihood phylogenetic trees (bootstrap values based on 10,000 iterations).

Sequences of the 16S rRNA gene were compared with available analogous sequences from treponemes in foot lesions using BlastN, and visually using Mega 5.0 (Tamura and others 2011).

Results

Clinical description

The lesion was seen primarily in first lactation cattle in the first two months to three months of lactation. In order to be classified as a case of ITN for this study, all cases had an area of dry, thickened and encrusted skin on the medial aspect of the base of the teat. A few cases spontaneously healed from this stage. In the other cases, the area of epidermal erosion extended down the medial aspect of the teat towards the orifice. The latter appeared to be the result of intense irritation, and some of the more severely affected animals had removed the whole teat, or in occasional cases, all four teats, by progressive licking (Fig 1).

Questionnaire

Seven completed questionnaires were received from affected dairy farms (housing 2290 cows), and four from unaffected farms (housing 1155 cows). All affected and unaffected farms reported current cases of DD. There was no identifiable ITN association with the presence of DD lesions because all farms had current cases of DD.

The six farms with ITN reported 43 cases (range 1–15 cases per farm). Cases were reported throughout the year.

The disease was generally seen in first lactation heifers, 1–12 weeks after calving (n=36), but sometimes (n=7) older cows (up to fourth lactation) were affected.

Complete recovery rates from ITN were low, with only 8/43 animals recovering. The remainder (n=35) were removed from the herd and culled.

As additional data was collected, including milking cups and time of lactation, it was possible to assess if there were any similarities between cases. Although case numbers were too small to analyse statistically, there was no apparent trend towards

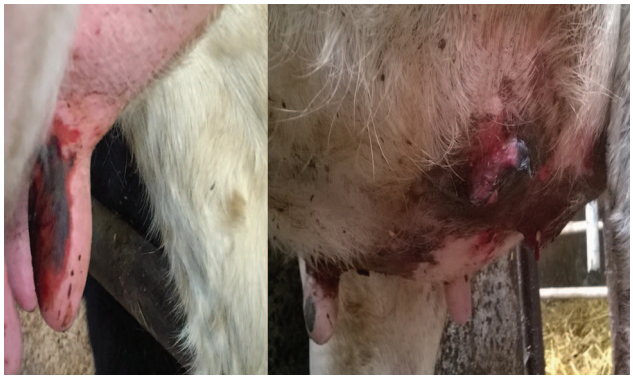

FIG 1: Photographs representative of stages of ischaemic teat necrosis (ITN) lesions. The left hand image shows an early stage lesion with a raw and intensely irritant area, spreading down the teat. The right hand photo shows severe lesions which have been licked and chewed off by the cow due to intense irritation and pain. Regeneration of the teat in the right photo is impossible and in the left photo highly unlikely (Photographs courtesy of Roger Blowey (Left) and Andrew Cooke (Right))

particular cattle or management variables which defined the survivor cattle.

A range of treatment approaches were used on farms with ITN, with proprietary teat dip and udder salve being commonly used without response. Other treatments attempted, again with little success, included topical iodine, copper sulfate solution, hypochlorite, fusidic acid, injectable antibiotics (including ceftiofur and standard penicillin), injectable steroids, topical antibiotic sprays (including oxytetracycline and lincospectin) and Stockholm tar and anti-sucking nose clips were also used with little or no improvement in the clinical condition. The in vitro susceptibility of these isolated treponemes to antibiotics was not tested as part of this study.

Spirochaete isolations

Tissue samples were taken from 12 cases of ITN from 10 farms. These were deep tissue samples (n=5), or scabs (n=7) removed from the affected teats. In addition, 20 control skin samples were taken from the udders of cattle with no teat or udder lesions at a local fallen stock centre serving most of North-West England. These were a mixture of Holstein-Friesian and Jersey cattle, the two breeds mainly affected by teat necrosis.

None of the control tissue samples taken from unaffected animals showed any signs of treponeme growth when analysed by phase contrast microscopy twice weekly during five weeks of anaerobic culture and both the tissues and the bacterial cultures were also negative when tested by *Treponema* genus PCR assay.

Of the 12 ITN lesion samples, 11 were positive for treponeme growth on examination by phase microscopy and this was confirmed by *Treponema* genus PCR assay and the nested PCR assays. The ITN lesion sample which was negative upon treponeme isolation had been delayed in the post, and consisted of a tiny dried fleck of tissue, so it may not have been suitable for isolation.

Five of the 11 treponeme-positive cultures were heavily contaminated with other (non-spirochaetal) bacterial species, rendering subsequent treponeme isolation attempts unsuccessful. In the remaining six anaerobic cultures, contamination with other bacteria was low; in these cases isolation of a single treponeme phylogroup was achieved.

For the six ITN tissues from which a pure treponeme isolate was obtained, the bacteria were further characterised by 16S rRNA gene sequencing. Five samples produced a clean 16S rRNA sequence which was then analysed using phylogenetic approaches.

Treponeme identification in teat tissue samples: PCR analysis

Of the 11 ITN tissue samples from which treponemes were cultured, at least one of the three DD-associated treponeme phylogroups was detected in each sample using direct PCR (Table 1) analysis. On culture, one or more of the *T. medium* (DD1), *T. phagedenis* (DD2) or *T. pedis* (DD3) phylogroups were present in all except one sample, which was considered of low quality. Three (3/11) of the ITN lesions contained all three DD-treponeme phylogroups, seven (7/11) contained two and one (1/11) contained only one, when tested using direct PCR on lesion tissue.

TABLE 1: Digital dermatitis (DD) associated treponeme detection in bovine ischaemic teat necrosis samples

Sample number	Microscopic identification	*Treponema* PCR	DD1	DD2	DD3
1	+	+	+ (TN4)	+	+
2	+	+	–	+	+
3	+	+	–	+	+ (TN7)
4	+	+	–	+ (TN1)	–
5	+	+	+	+	+
6	+	+	+	+	–
7	+	+	–	+	+
8	+	+	+	+	–
9	+	+	+	+	+ (TN8)
10	+	+	–	+	+
11	+	+	+	+ (TN2)	–
12	–	–	–	–	–

Results indicate presence of spirochaetes cultured from teat samples confirmed by phase contrast microscopy or detection by nested PCR. Additionally, 20 clinically normal teat control samples were tested, but were all negative by phase contrast microscopy and PCR, so are not shown. The names shown in parentheses show the isolate ID, as used in the phylogenetic tree (Fig 2)
DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is *Treponema medium* phylogroup, DD2 is *Treponema phagedenis* phylogroup and DD3 is *Treponema pedis* phylogroup
TN, teat necrosis.

Treponeme PCR detection in teat swabs

In cases where it was not possible to obtain a deep tissue sample, a swab was taken from the animal. In total, 22 swabs from ITN lesions from other cattle were subjected to DNA extraction and analysed by PCR for the presence of DD-associated treponemes. An additional 15 swabs were taken from normal teats from animals on three farms that had ITN affected cattle, and 5 swabs were taken from normal udder skin from cattle on two unaffected farms (Table 2).

When tested by nested PCR, one or more DD treponemes were detected in 19/22 of swabs taken from ITN lesions. By comparison, all tissue samples and swabs taken from the same sites on unaffected animals were negative for DD treponemes (Table 2).

TABLE 2: PCR detection of digital dermatitis (DD) treponemes in swabs taken from ischaemic teat necrosis (ITN) lesions

N	Treponeme PCR	DD1	DD2	DD3
1	+	+	–	–
2	+	+	+	+
2	+	+	+	–
3	+	–	–	–
4	+	–	+	–
5	+	–	+	+
5	+	+	–	+

An additional 10 swabs not shown in the table were taken from unaffected animals, and were all negative by PCR
DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is *Treponema medium* phylogroup, DD2 is *Treponema phagedenis* phylogroup and DD3 is *Treponema pedis* phylogroup

All tissue samples were tested for presence of herpesviruses using a pan-herpesvirus PCR (Ehlers and others 1999, Hughes and others 2010). In all cases (ITN lesions and control samples), the results were negative.

Of the 22 swabs taken from cases of ITN, three were negative for DD treponemes, and were also negative when tested using the 16S rRNA gene PCR detecting all *Treponema* species.

16S rRNA gene analysis

Six pure treponeme culture isolates were obtained from lesions taken from cases of ITN and their 16S rRNA gene amplification PCR products were sequenced. In addition, two 16S rRNA gene PCR product sequences were obtained directly from two ITN lesion tissues using the eubacterial primers, and the sequence was of high enough quality to analyse, strongly suggesting that treponemes were the predominant bacteria in these tissues. To determine the relationship of the ITN treponeme isolates to those commonly found in DD lesions in cattle, the 16S rRNA gene sequences were compared with other previously sequenced isolates by phylogenetic analysis (Fig 2).

The 16S rRNA gene sequences of the six isolated treponemes and two sequences obtained directly from tissues showed high similarity to previously isolated treponemes from cattle DD lesions. The three *T. pedis* isolates from the teat lesions clustered together phylogenetically (Fig 2), with their 16S rRNA gene sequences differing from the type strain (T3552B^T) by 23 SNPs (1.5 per cent). The 16S rRNA gene sequence from the other isolates were either identical to T320A (a common bovine DD isolate from the *T. phagedenis* phylogroup) or a single nucleotide different to T19, a bovine DD isolate from the *T. medium* phylogroup.

Discussion

This study describes the typical clinical presentation of ITN associated with this disease and reports an apparent association with treponeme bacteria.

The authors tested for evidence of herpesvirus infection but found none, indicating that BHV (previously implicated in the development of udder lesions) was not associated with ITN pathology on the study farms.

By contrast, DD treponemes were commonly found in association with ITN lesions. The remaining samples may have been

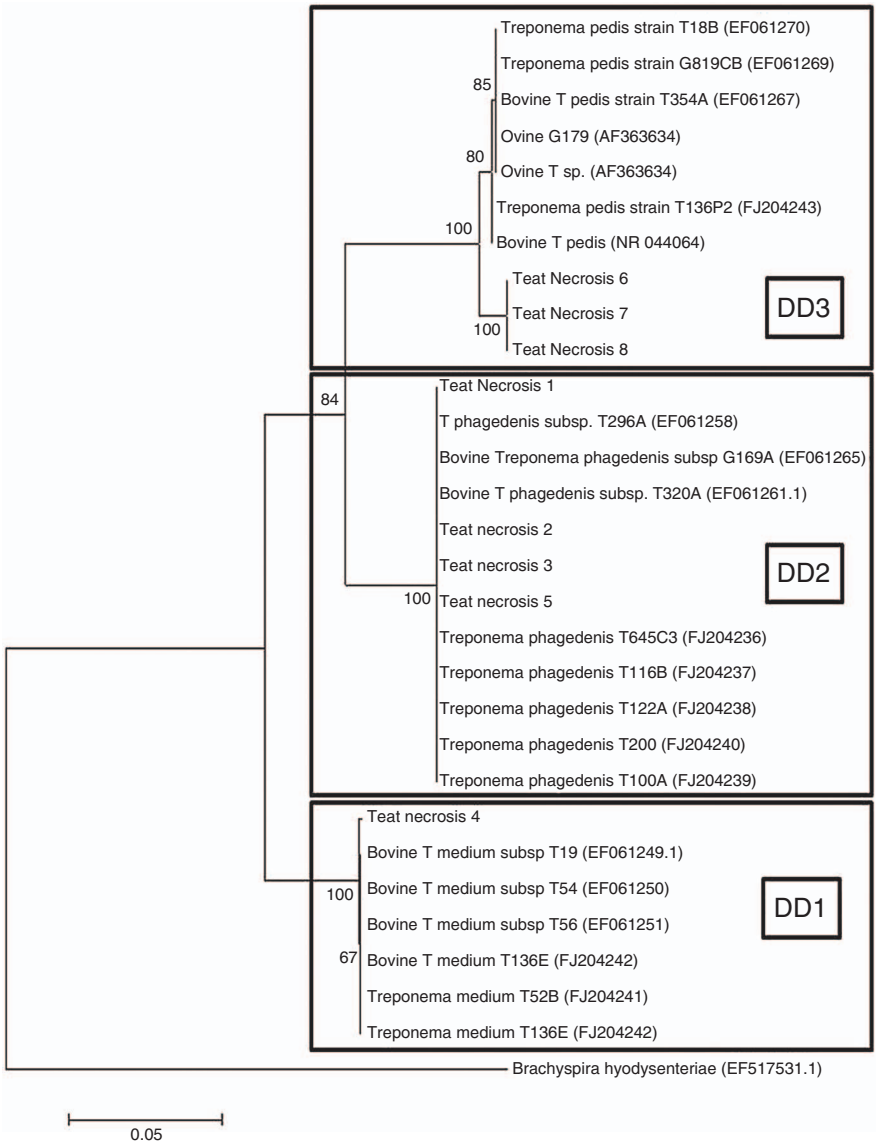

FIG 2: Molecular phylogeny of treponemes cultured from ischaemic teat necrosis (ITN) lesions. Comparison of treponeme 16S rRNA gene sequences isolated from ITN lesions in this study to those isolated from digital dermatitis (DD) lesions in cattle (for clarity, bootstrap values below 65 were removed). Sequences from Genbank of other cattle DD treponemes, are also shown, with the accession number in parentheses. The sequences from isolates in this study are labelled with ITN isolate number (as shown in Table 1). (Sequences 3, 5 and 6 were obtained directly from tissue). DD1, DD2 and DD3 refer to the DD treponeme phylogroups, where DD1 is *Treponema medium* phylogroup, DD2 is *Treponema phagedenis* phylogroup and DD3 is *Treponema pedis phylogroup*

degraded, or were lacking sufficient DNA to allow for PCR amplification. The generation of a clean 16S rRNA gene DD treponeme sequence directly from a tissue sample suggests that treponemes are the most abundant bacterial taxon present and that they are likely to have a role in disease. However, other bacterial species were also present and it should be considered that the treponemes may not be the sole pathogen involved in the disease, and further studies will be needed to identify whether other microbial agents may be involved.

Of the treponemes isolated from cases of ITN, one was very similar to other bovine *T. medium*-like isolates from clinical cases of DD, and isolates 2, 3 and 5 were most similar to bovine *T. phagedenis*-like cases isolated previously from bovine DD lesions (Evans and others 2009). This is analogous to other studies where similar DD-associated treponemes have been isolated from skin lesions from cattle and other hosts worldwide (Dawson 1998, Dhawi and others 2005, Sayers and others 2009, Evans and others 2011, Svartstrom and others 2013, Clegg and others 2014, Duncan and others 2014, Sullivan and others 2014a, b).

In conclusion, the authors have identified species of treponemes previously associated with DD in the majority of samples from ITN lesions from cattle, findings suggestive that they may play a role in the pathogenesis of this condition. Future work will require systematic investigation of other microorganisms and underlying pathogenesis of the condition.

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Bovine ischaemic teat necrosis: a further potential role for digital dermatitis treponemes

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