Identifying Digital Dermatitis infection reservoirs in beef cattle and sheep

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Abstract

Digital dermatitis (DD) is a superficial infectious dermatitis of the digital skin of cattle and sheep that can be very painful, causing severe lameness in affected animals. Bovine digital dermatitis (BDD) in dairy cattle has now been reported in most countries they are farmed, and DD in sheep, known as contagious ovine digital dermatitis (CODD) is rapidly emerging as a severe infectious foot disease since first reports from the UK in 1997. Spirochaetes, of the genus *Treponema* have frequently been found in large numbers in BDD lesions and are now considered the primary causative bacteria of BDD. Three treponeme phylogroups are consistently isolated from dairy cattle BDD in the UK and the USA, which are known as *Treponema medium*- like, *Treponema phagedenis*-like spirochaetes and *Treponema pedis*.

Over the past 40 years research has focused on dairy cattle BDD and overlooked whether the disease exists in beef cattle herds in the UK, and whether the same aetiological agents are causal. There is also limited information on the causative bacteriological agents of CODD. Furthermore, no definitive transmission routes or infection reservoirs of DD in either cattle or sheep had thus far been delineated, with only a single study finding a potential reservoir site of DD treponemes in the dairy cattle gastrointestinal (GI) tract.

Using molecular bacteriological studies it was found that CODD and beef cattle BDD, as in dairy cattle BDD, show a high association with the three DD treponeme phylogroups. All CODD and beef BDD lesions investigated had at least one of the three DD treponeme phylogroups present in the lesions and these treponemes were also isolated from a high proportion of lesions. No DD treponemes were detected in healthy sheep or beef cattle foot tissue. Upon 16S rRNA gene sequence analysis all isolates showed a high similarity, if not 100% identity, to representatives of each treponeme phylogroup isolated from dairy cattle BDD lesions, indicating a shared aetiology between DD in all three animals. Additionally, the same treponeme bacteria were detected and isolated

from a new undefined foot disease in dairy goats in the UK indicating that cross-species transmission of DD may have occurred causing DD infection in a previously unaffected domestic livestock species.

To understand potential transmission routes and infection reservoirs of DD, the host GI tract and hoof trimming equipment were investigated. Of the sheep gingival (n= 40) and rectal tissues (n= 40), 1/40 gingival tissues were positive for DD- associated treponemes (*T. pedis*), and 3/40 rectal tissues (one containing *T. medium- like* and two tissues containing *T. pedis*). No DD-associated treponeme DNA was amplified from beef cattle rectal tissues (n= 40), however 4/40 beef gingival tissues were positive for DD- associated treponemes (all containing *T. phagedenis*- like). A *T. phagedenis*- like DD treponeme was isolated from the rectal tissue of a CODD symptomatic sheep. Beef cattle (n= 41) and sheep (n= 79) faeces failed to amplify DD- associated *Treponema* DNA. Twenty two treponemes were isolated from sheep faeces; however, upon phylogenetic analysis these clustered with considered non-pathogenic treponemes, which interestingly exhibited farm specific diversity in their 16S rRNA gene. Trimming equipment was tested after being used to trim cattle and sheep hooves, and subsequently after disinfection of equipment. Of the blades used to trim DD symptomatic animals (n= 26, cattle and sheep combined), 25/26 were found to be positive for at least one of the DD *Treponema* phylotypes. This figure was reduced to 10/26 (38%) after disinfection of the blades. Following culture of a swab, an isolate belonging to the *T. phagedenis*- like spirochaetes was isolated from a knife sample after trimming a DD positive cow.

Beef cattle sera from DD positive and negative farms were investigated to understand whether beef cattle's perceived lower prevalence of BDD in the UK is due to a lack of exposure to treponemes, or a protective immune response. Beef cattle from DD positive farms appeared to produce a strong immunological response to treponemes, compared with DD negative farm animal sera. Therefore the perceived lower prevalence of DD in beef cattle does not appear to be due to a protective response in these animals, but more likely due to a lack of exposure to DD treponemes.

In conclusion, these studies have produced vital information describing DD in beef cattle and sheep and their respective aetiological agents allowing for more appropriate treatments in the future. Additionally, given the two potential transmission routes delineated from the data, effective actions can be taken to prevent the spread of DD within current hosts and to limit emergence into yet unknown additional host species.

Abbreviations

1D	1- dimensional
2D	2- dimensional
3D	3- dimensional
BB	Basil body
APS	Ammonium persulphate
BDD	Bovine digital dermatitis
BLAST	Basic local alignment search tool
Bp	Base pair
DD	Digital dermatitis
ddH_2O	Deionized water
DDT	dithiothreitol
CF	Cytoplasmic filament
Cl	Chloride
CODD	Contagious ovine digital dermatitis
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
EGTA	Ethyleneglycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAA	Fastidious anaerobic agar
FSC	Fallen stock centre
g	grams

gf	gram force
HCl	Hydrogen chloride
IgG_1	Immunoblogulin class G subclass 1
IgG_2	Immunoblogulin class G subclass 2
KCl	Potassium chloride
kDa	kilodalton
KH_2PO_4	Potassium dihydrogen phosphate
Kg	Kilogram
KOH	Potassium hydroxide
L	litre
Lkta	Leukotoxin
MEGA	Molecular evolutionary genetics analysis
Mg	milligram
$MgCl_2$	Magnesium chloride
Min	minute
mL	milliliter
MLST	Multilocus sequence typing
mM	Millimolar
Na_2HPO_4	disodium hydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCS	Nitrocellulose sheet
OD	Optical density
OTEB	Oral treponeme enrichment broth
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline tween

PCR	Polymerase chain reaction
PFF	Periplasmic flagella filaments
PP	Percentage positive
rcf	Relative centrifugal force
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TEMED	N, N, N', N' - tetramethylethylenediamine
TMB	3, 3', 5,5'Tetramethylbenzidine
Tris	2-Amino-2-hydroxymethylpropane-1,3-diol
V	Volts
v/v	Volume/ volume
w/v	Weight/ volume
µL	Microlitre
µm	Micrometre
µM	Micromolar

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Chapter 1

Introduction

1.1 Digital Dermatitis

1.1.1 General Introduction

Bovine Digital dermatitis (BDD) is a multifactorial, superficial dermatitis of the digital skin of domestic cattle (*Bos taurus*) that can be very painful, causing severe lameness in affected animals (Cheli and Mortellaro 1974; Blowey and Sharp 1988). Digital dermatitis (DD) can affect both dairy and beef herds; dairy cattle estimates have been found to be between 20-30% in the UK and USA (Brown *et al.* 2000, Cramer *et al.* 2008; Barker *et al.* 2009) and 4% in beef cattle populations (Brown *et al.* 2000). The sheep (*Ovis aries*) manifestation of DD, known as CODD, has been found to have a prevalence of 25% in some sheep flocks (Kaler and Green 2008), and within the first year of a CODD outbreak 30-40% of the flock can be affected (Defra 2003a).

The first case of BDD was reported in Italy in 1974 (Cheli and Mortellaro 1974), and the first case in the UK was reported 13 years later, in 1987 (Blowey and Sharp 1988). CODD was not reported until a much later 1997 (Harwood *et al.* 1997), but has since spread throughout the UK at a rapid rate and it has become an important welfare issue for the UK sheep flock. Equally, it has spread throughout dairy populations globally (Evans *et al.* 2008) and is now the most common lesion associated with lameness in UK dairy cows (Laven and Logue 2006) with approximately 25% of all cattle lameness being attributable to DD (ADAS 2001).

Spirochaetes, of the genus *Treponema* have frequently been found in large numbers in BDD lesions (Blowey *et al.* 1992; Demirkan *et al.* 1998), and are now considered widely as the primary causative bacteria of BDD. The exact aetiology of CODD is still partially uncertain, however *Treponema* have also been isolated from and detected in CODD lesions (Naylor *et al.* 1998; Demirkan *et al.* 2001; Moore *et al.* 2005; Sayers *et al.* 2009), suggesting BDD and CODD may have similar aetiologies.

Very little information is available regarding BDD in beef cattle, in terms of clinical signs, aetiology, UK prevalence or treatment, with most information coming from anecdotal sources.

Due to the severe lameness BDD and CODD causes, and welfare and cost implications involved, it is imperative to know more on the exact causation, transmission and carriage sites of the disease.

1.2 Lameness in ruminants - definition, causes and magnitude of the problem

1.2.1 Definition and observations

Lameness is defined as a departure from normal locomotion, causing an observable deviation in gait. Clinical lameness in cattle can be a manifestation of pain, weakness, a form of deformity, or a musculoskeletal problem (FAWC 2009).

Reliable nationwide data on the incidence of lameness in the UK are limited but it has generally been agreed that it has increased over the past 40 years, coinciding with changes in production including breed, size, nutrition, productivity and housing (Mill and Ward 1994; Clarkson *et al.* 1996).

Lameness involves the avoidance of full weight-bearing on one or more limbs, signalling pain and discomfort, which indicates suffering. By restricting the mobility of an animal, lameness reduces the physical and even social interactivity between the animal and its environment and social group. The incidence of lameness is extremely high and represents one of the most painful group of disorders to affect cattle and sheep, and is therefore a major welfare issue. A number of reports on the welfare of farm animals, and often with an emphasis on dairy cows, have highlighted the importance of lameness. These have been published by both the UK Farm Animal Welfare Council and the European Food Safety Authority (FAWC 2009; EFSA 2009a, 2009b).

1.2.2 Dairy cattle lameness in the UK

A 1980 study of cases of lameness treated by veterinary surgeons on 150 farms recorded an annual incidence of 7.3% (Eddy and Scott 1980). A 1982 survey of records kept by 48 veterinary practices throughout the UK yielded an average annual lameness incidence of 5.5% (Russell *et al.* 1982). In a broader study of lameness records kept by farmers and veterinarians for 185 herds, an average annual incidence of 25% (Whitaker *et al.* 1983) was found. A survey conducted by the University of Liverpool between 1989 and 1991, which combined observations made by farmers, foot trimmers and veterinarians on 37 farms, noted a mean annual lameness incidence of 54.6% (Clarkson *et al.* 1996). Most recently the mean prevalence of lameness in dairy herds was 36.8% (Barker *et al.* 2010).

Lameness is associated with delayed ovarian activity in Holstein cows during the early postpartum period. In a study by Garbarino *et al.* (2004), lame cows were 3.5 times more likely to have delayed cyclicity, compared with non-lame cows. Additionally, it was identified that milk yield was reduced from up to four months before lameness was diagnosed and for up to five months after treatment.

The detrimental effects of lameness on productivity along with its high incidence make dairy cattle lameness of large economic importance. Enting *et*

al. (1997) estimated an economic loss of €104 per case of clinical lameness. In a simulation study, Ettema and Østergaard (2006) estimated the costs per case of clinical lameness per cow-year to be €192 (£160) in a typical Danish dairy herd. Halving disease risk of all three lameness causing diseases in a herd with average and poor reproduction increased total gross margin by € 24,840 (£20,617) and €38,820 (£30,820), respectively.

In the UK in 2013, the average cost of an incidence of lameness, in terms of treatment costs, loss of yield and potential for shortened productive life of the cow may be in the region of £180; at current levels of incidence this could equate to a financial loss of nearly £15,000 for an average-sized herd (a cost of well over 1p per litre of milk produced on the farm (Dairy Co 2013).

1.2.3 Beef cattle lameness in the UK

It is very problematical when lameness affects beef cattle especially a stud bull or breeding cows during the breeding season, and is a serious welfare issue in all animals affected.

There is little reported on beef cattle lameness in the UK. This may be because beef cattle are not as easily observed as dairy cattle, which are closely monitored at daily milking practices. Various sources claim beef cattle lameness is found, however there is very little published literature focusing on beef cattle lameness in the UK. There are however, studies conducted outside the UK which give an idea of the levels of lameness found in beef cattle herds.

A study by Nicholson *et al.* (2013) conducted in Texas found that dairy cows had numerically more lameness than beef cows. They found that in 2007, dairy cows studied had lameness rates of 48.7% compared with 16.3% in beef cattle. A Norwegian study recorded lameness in only 1.1% of the animals (n= 362 beef animals), and only in hind claws. However in total, claw and limb disorders including lameness were recorded in 29.6% of the animals, 4.1% with front and 28.2% with hind limb disorders, respectively (Fjeldaas *et al.* 2007).

Roeber *et al.* (2000), in their USA study concluded, that the incidence of lameness of cattle was 26.6% for beef cows and 30.2% for dairy cows. Additionally, an Italian study (Cozzi *et al.* 2013), found 5 of 48 (10.4%) male beef animals to be lame in their investigation.

Although little is recorded for beef cattle in the UK, the studies conducted globally give an idea of what levels of lameness are commonly found in beef cattle herds vs. dairy cattle herds, of which it appears beef cattle suffer less from lameness problems.

Lame beef cattle are often cattle which have lower production levels, whether breeding animals or fattening stock. Weight loss is a common consequence in grazing cattle, with delayed heat and poor conception a possibility in suckler cows. Infertility is likely to one of the single biggest cost implications (Eblex 2015). Although no figures are available for the exact economic cost of beef cattle lameness in UK, using dairy cattle costs it is possible to get an estimate of the cost of beef cattle lameness. The amount of beef cattle in the UK in June 2012 was 9.9 million (Eblex 2013c), and taking an average lameness based on the figures above of 13.6%, and the estimated cost of lameness in a dairy cow of £180 (Dairy Co 2013), the cost of lameness in beef cattle in the UK could be around £13.1 million.

1.2.4 Sheep lameness in the UK

Lameness is one of the most widespread welfare problems in the UK sheep flock. It is a significant cause of discomfort and pain and is a major source of economic loss to the sheep industry.

The level of lameness varies in sheep flocks across the UK according to season and management. Reported studies show that the average level of lameness is around 5% of the flock. Farmers who have comprehensively managed lameness achieve levels as low as 2% (Eblex 2013a). In 2011, the Farm Animal Welfare Council recommended that the level of lameness in flocks should be

an average of 5% by 2016, and 2% by 2021 using currently available management practices (Eblex 2013a).

In 1994, the estimated prevalence of lameness in sheep was 8% (Grogono-Thomas and Johnston 1997) and in 2000 it was 10% (Wassink *et al.* 2004). In a questionnaire study by Kaler and Green (2008), out of 264,076 sheep 27,468 (10.4%) were estimated to be lame in 2004 (using farmer's estimate of lameness).

From the entire range of sheep diseases, sheep lameness has the highest cost to the sheep industry economy (Eblex 2013b). Although no exact figures are available for the cost of total lameness to the sheep industry, one of the main causes of lameness, footrot, costs around £90 per ewe which equates to costing around £24 million to the British sheep industry per year (Nieuwhof and Bishop 2005). With the addition of the cost of other causes of lameness to this figure, it is very clear that lameness in sheep is an extremely important economic issue for the UK sheep industry.

1.2.5 Common causes of cattle lameness

There are several common lameness conditions found in dairy cattle herds. There are very few studies conducted using beef cattle herds, but from the limited information available it appears both dairy and beef cattle are able to contract most of the same disorders.

Table 1.1 shows a summary of the most prevalent causes of lameness in dairy cattle.

Sole ulcers, white line disease and DD are recognised as the main lameness associated conditions seen on dairy farms in the UK, but there are several other common problems seen on a regular basis and several different lesions and problems can be present on a single foot at any one time.

Table 1.1: A summary of the most prevalent causes of lameness in dairy cattle.

Condition	Details	Causes
Digital dermatitis	Infection of the epidermis of the hoof skin (Mortellaro and Cheli 1974). Moist, grey/brown area of exudate between the heel bulbs of the foot.	Spirochaetal bacteria of the genus *Treponema* (Evans *et al.* 2008).
Foul of the foot	Inflammation between the claws (of the dermal layers of the interdigital space and adjacent coronary band).	Associated with *Fusobacterium necrophorum* (Blowey and Weaver 2003).
Laminitis	Inflammation of the laminae – below the outer horny wall of the foot.	Caused by physical injury and infection, (Vermunt and Greenough 1994; Stone 2004).
Sole hemorrhage and bruising	Inflammation of the corium, leading to increased blood flow. Blood pools with poor oxygenation leading to tissue damage and poor horn formation. Corium becomes fragile.	Physical damage due to overloading and pressure on the claw (Swalve *et al.* 2013).
Interdigital hyperplasia	Proliferation of the interdigital skin	Reaction to long-lasting inflammation of the interdigital cleft (Enevoldsen *et al.* 1991; Somers *et al.* 2003).
White line disease	Separation of the wall from the sole at the white line	Corium penetrated by external influences (Smith and Broderson, 1998; Manske *et al.* 2002).
Sole ulcer	Disruption of horn formation, early stages show fluid under the sole horn, which after infection shows when damaged horn gets to the surface.	Trauma to the hoof (Lischer and Ossent 2000).
Heel and toe ulcers	Small dark red/black marks in the sole area, can lead to under-running of horn at the sole-heel junction.	Thought to occur when the pedal bone sinks with the hoof (Cramer *et al.* 2009).
Slurry heel	Heel horn becomes pitted and in extreme cases totally eroded and internal changes can occur.	Feet exposed to slurry for long periods of time and horn erodes (Peterse 1985; Somers *et al.* 2003).

Additionally, "new" disorders affecting the bovine digit have been reported named non-healing lesions (Evans *et al.* 2011a). These disorders can be very severe and are defined as lesions with a pungent smell and a typically moist granular topical appearance. They do not heal well, and require amputation of the affected claw in many cases (Blowey 2012). These "new" non-healing disorders have been categorized into three main groups; toe necrosis (TN), non-healing white line disease (nhWLD) and non-healing sole ulcer (nhSU),

and are thought to have at least a partial spirochaetal bacteria aetiology (Evans *et al.* 2011a).

All of the foot disorders listed (Table 1.1) are well documented in dairy cattle, and the studies have focused on symptoms and causes in these animals.

Claw and limb disorders were studied in 12 Norwegian beef-cow herds (Fjeldaas *et al.* 2007) whereby a low prevalence of haemorrhages was observed (0.6% on front claws, 7.8% hind claws), and the prevalence of sole ulcers was found to be low (0.3% of front claws, 1.4% of hind claws), compared to what has been found in dairy cattle studies (Smits *et al.* 1992; Manske *et al.* 2002). White line disease (white line fissures) was found to be the most frequent laminitis-related lesion in their study, which agrees with Smith and Brodersen (1998) who found that white line disease was the most frequent external lesion in lame beef cattle. The prevalence of white-line fissures was as high as 36.4% in one herd. The prevalence of heel-horn erosions was found to be low compared to Norwegian free-stall dairy herds (39.6%).

The organisation for beef and lamb levy payers in England, Eblex, published in their beef cattle disease directory (Eblex 2015), that foot lameness in beef cattle is usually due to any of the following conditions:

•Foul of the foot

•Interdigital hyperplasia, hereditary

•Sole abscesses/white line disease

1.2.6 Common causes of sheep lameness

The most common infectious causes of lameness in sheep are footrot and interdigital dermatitis (ID), also known as scald (Grogono-Thomas and Johnston 1997), and more recently concern has been raised over the newly emerging infectious disease, CODD (Wassink *et al.* 2003). In addition to these infectious causes of lameness, non-infectious causes include white line degeneration (shelly hoof), foot abscesses and toe granulomas. These are

generally considered to be of low prevalence (Grogono-Thomas and Johnston 1997; Winter 2004a, 2004b).

More recently, footrot has been found to be attributable for over 90% of lameness in sheep (Kaler and Green 2008). Footrot is a highly contagious disease affecting the skin between the digits (interdigital skin) of a hoof resulting in lameness. Footrot is characterised by two pathological presentations: inflammation of the interdigital skin, ID, and separation of the hoof horn from the sensitive underlying tissue, severe footrot (SFR) (Beveridge 1941; Egerton *et al.* 1969; Witcomb *et al.* 2014). In 1941, Beveridge produced his seminal work on footrot in which he provided evidence that *Dichelobacter nodosus*, a Gram-negative anaerobe, was the primary aetiological agent of footrot rather than *Fusobacterium necrophorum*. A number of authors have investigated the presence of *D. nodosus* and *F. necrophorum* in sheep with healthy and diseased feet. *Dichelobacter nodosus* is recovered more frequently from feet with ID or SFR than healthy feet (La Fontaine *et al.* 1993; Moore *et al.* 2005; Bennett *et al.* 2009). There is still debate to which of these bacteria is the primary causative agent of footrot, but a recent study by Witcomb *et al.* (2014) found there was an increase in *D. nodosus* load the week prior to development of ID and SFR and during an episode of ID. In contrast, *F. necrophorum* load was not associated with ID before or during an episode, and was only associated with SFR once present. Therefore this study concluded that D. *nodosus* load plays the primary role in disease initiation and progression, with *F. necrophorum* load playing a secondary role.

Although footrot can be a very severe disease and of great economic importance, CODD in sheep is becoming more of an issue in sheep flocks throughout the UK, and according to recent figures, is very rapidly becoming as prevalent as footrot.

In a study by Kaler and Green (2008), the prevalence's of the main causes of lameness were investigated. They found that the average lameness in a flock

was 10.4% and determined the approximate percentage of lameness which was attributable to each disorder. Table 1.2 shows the results from this study, which gives a good indication, due to the high sample number, of the levels of the main lameness associated disorders in the UK sheep industry at the time of sampling (2005). Interdigital dermatitis, footrot and CODD appear, from this data, to be the most prevalent cause of lameness in sheep.

Table 1.2: Percentage of lame sheep with each foot disorder from the study by Kaler and Green (2008).

Cause of Lameness	Percentage of lame sheep
Interdigital dermatitis	69
Footrot	37
CODD	24
Shelly hoof	19
Foot abscess	9
Toe granuloma	8

1.3 Importance of bovine digital dermatitis (BDD) and contagious ovine digital dermatitis (CODD)

1.3.1 Economic impact of BDD and CODD

Bovine digital dermatitis has large economic implications such as; reductions in milk yield (Relun *et al.* 2013) and reproductive performance (Argaez-Rodriguez *et al.* 1997; Hernandez *et al.* 2001, 2002; Whay *et al.* 1997) as well as treatment costs. BDD remains the main cause of infectious lameness and costs on average between £76 and £84 per case (Esslemont 2005; Cha *et al.* 2010). Treatment costs were found by Cha *et al.* (2010) to be the main

contributor to the total cost per case (~£34), then costs of fertility loss (~ £25 per case), and lastly costs resulting from milk loss (~ £21 per case).

As the current prevalence of BDD in beef cattle is unknown, based on the economic impact of BDD in dairy cattle (Cha *et al.* 2010), on a 4% prevalence (Brown *et al.* 2000), and the amount of beef cattle in the UK, (Eblex 2013c) equates to costing the UK at least £5-6 million/year.

Although not calculated, with the economic impact of footrot estimated at £24 million annually (Nieuwhof and Bishop 2005), and CODD showing similar prevalence levels and lesions potentially considered to be of higher severity, we can assume CODD costs are at least that of footrot.

1.3.2 Welfare implications

Foot disorders, and in particular in cases of DD, are important health problems in cattle and sheep, in terms of the resulting animal welfare concerns. Foot disorders are the main cause of dairy cow lameness, and are considered to have a major impact on the welfare of animals affected (Galindo and Broom 2002). These consequences are largely due to the pain caused by foot disorders, which likely affect the movement of the cow. Pain can also cause cows to be reluctant to show normal cow specific behaviours (O'Callaghan *et al.* 2003). These behaviours help to achieve physiological needs and allow natural stimulation (Broom and Johnston 1993; Dawkins 2003). Some of these which can be influenced by the presence of foot disorders include; resting and moving freely to feed and drink (Walker *et al.* 2008). Generally, the impact on animal welfare depends on severity, duration and incidence of the foot disorder and therefore the reoccurrence and persistence of DD in many cases poses a large welfare concern. When the disease is untreated or chronic the condition can persist for months or reoccur, which can cause welfare problems (Laven and Proven 2000). In addition to this, the fact that there is still no single effective treatment for DD, increases the welfare burden of this disease in both cattle and sheep, along with the risk of premature culling arising from DD (Bruijnis *et al.* 2012).

A study by Bruijnis *et al.* (2012) estimated the welfare impact of foot disorders for individual dairy cows, based on the simulated incidence and duration of the disorders and pain involved. They found that from seven foot disorders, BDD had the highest impact on dairy cow welfare.

1.4 Epidemiology

1.4.1 Introduction and spread of DD

How BDD and CODD may be introduced onto previously disease-free farms is still unknown, however it is assumed it may be through a breach in biosecurity.

For BDD, buying in stock seems to be particularly important (Rodríguez-Lainz *et al.* 1999), even if the purchased animals may not appear to have clinical lesions, an epidemic outbreak may occur some weeks later. Contamination from individuals who come on to farm is also a possibility, such as veterinarians and foot trimmers who visit farms without cleaning their instruments have been implicated in spreading the infection (Wells *et al.* 1999; Losinger 2006). All cattle appear to be susceptible to contracting BDD, although some research suggests that first lactation cows are specifically at risk (Brentrup and Adams 1990; Frankena *et al.* 1991). After the initial outbreak, the infection tends to become more endemic in nature; more chronic lesions are observed at a lower prevalence, and periodic fluctuations in incidence may occur. Young cattle kept under unhygienic conditions on farms that purchase cattle from infected premises are prone to BDD lesions that can accumulate over time to produce outbreaks associated with lameness and production losses (Rodriguez-Lainz *et al.* 1996, 1999).

Once infection has been introduced onto a farm, BDD has never been reported as successfully eradicated through application of the existing control measures, which suggests that there is a reservoir of the causative organism.

A recent study by Angell *et al.* (2014) looked at farmer reported prevalence and factors associated with CODD in Wales using a questionnaire of 511 sheep farmers. They reported that CODD now appears to be endemic and widespread in Wales, UK. Additionally, they found that buying in animals appeared to be a risk factor for CODD, implying that this may be one of the mechanisms how CODD arrives on farms (Angell *et al.* 2014).

1.4.2 Prevalence

As the previous data suggested, BDD and CODD are extremely important lameness causing diseases and two of the most prevalent diseases in cattle and sheep, respectively.

Surveys indicate a farm level prevalence of between 8% and 53% of BDD in dairy cattle (Murray *et al.* 1996; Whay *et al.* 2002). Murray *et al.* (1996), from 1989 to 1992, studied lameness on 37 dairy farms in four regions of England and Wales and found that BDD was the primary cause of lameness, 8% of all lameness being attributed to the disease. Whay *et al.* (2002) collected data from 53 dairy farms, which showed that BDD occurred on 39 farms, and affected farms had a significantly higher lameness prevalence. However, the current prevalence of BDD in beef cattle is unknown, with only one study providing a prevalence estimate of 4% based on an abattoir study in the USA (Brown *et al.* 2000).

Farmer reported data in England provided prevalence estimates of lameness of 10.4 percent with the prevalence of CODD making up an estimated 2.4 percent, and footrot a 3.7 percent (Kaler and Green 2008). The study by Angell *et al.* (2014) reported a lower between farm prevalence of CODD across Wales (35%) compared to that across England (53%) (Kaler and Green 2008).

1.4.3 Seasonality

A seasonal effect has commonly been reported in BDD, with peak morbidity during the housing period which could probably be associated with poor hygiene and overcrowded conditions within the building (Blowey and Sharp 1988; Nutter and Moffitt 1990). Frankena *et al.* (1991) reported a population prevalence of 8.1% vs. 13.8%, during the grazing period and housing period respectively. Somers *et al.* (2005) reported a slightly higher prevalence of 28.5% during the housing period compared with 27.3% in the pasture period.

1.4.4 Risk factors associated with the prevalence of BDD and CODD

Previous studies in this area has suggested many contributing factors and more importantly, contradictory results. Although more studies have focused on cattle, it is also apparent they have focused primarily on dairy cattle. Beef cattle may not be too different from dairy cattle, and are often mixed hybrids of dairy and beef cattle breeds, but they can be exposed to different environments and nutritional differences which could affect levels of DD in beef herds. Table 1.3 summarises the possible risk factors for BDD found over the years in dairy cattle.

There is significantly less information on risk factors associated with CODD, possibly due to the more recent reporting of the disease. However, risk factors for CODD identified include; the presence of BDD in cattle on the farm, larger flocks, buying in sheep, adult sheep, time of year and housing (Angell *et al.* 2014).

There are, as shown in Table 1.3, a very large list of possible risk factors associated with DD and in many cases what is found in one study, was not found in another. However, a consistent risk factor associated with BDD is low standards of hygiene.

Table 1.3: Risk factors associated with digital dermatitis in cattle

Author/s	**Animal studied**	**Risk factors for DD**
Barker *et al.* 2009	Dairy cattle	Increase in herd size Low parity Months from calving Milking cows Housing 24hr/day Concrete tracks or roadways Solid grooved concrete floor surface Reduced bedding availability
Enevoldsen *et al.* 1991	Dairy cattle	Lactation stage 1
Nielson *et al.* 2012	Dairy cattle	Early lactation associated with reduced risk of DD. Reduced risk in parity 3 cows vs. Parity 1 cows. Increased risk in parity 2 vs. Parity 1.
Somers *et al.* 2003	Dairy cattle	Concrete flooring Length of time grazing
Hultgren and Bergsten 2001	Dairy cattle	Significantly lower DD on rubber slats vs. traditional concrete stalls
Somers *et al.* 2005	Dairy cattle	Lower parity Lactation stage Restricted grazing time Fast rise in concentrate amount after calving Feeding by-products Herd trimming only at long intervals Introduction of dry cows into the lactating herd before calving Cubicle size Length of walking path Type of soil/soil pH
Argáez-Rodríguez *et al.* 1997	Dairy cattle	First month of lactation Summer and autumn rates higher than in winter and spring
Nowrouzian and Radgohar 2011	Dairy cattle	Lower hygiene scores for the lower portion of the hind limbs significantly associated
Holzhauer *et al.* 2006	Dairy cattle	Low parity

		Cows at peak lactation (30 to 60 d in milk) and in the third parity. Cows trimmed >12 mo before the study (during regular trimming of the entire herd) were at lower risk than were cows trimmed at shorter intervals Cows with >8 h of access to pasture were at higher risk vs. No access to pasture
Barker *et al.* 2009	Dairy cattle	Restricted grazing/zero grazing Sparse bedding Concrete tracks Housing cattle Milking cattle vs. Dry cattle Herd size (larger herds associated with higher DD prevalence). Concrete tracks or roadways
Cramer *et al.* 2009	Dairy cattle	Trimming in summer or fall Access all year round to outside areas
Rodríguez-Lainz *et al.* 1999	Dairy and dual purpose cattle	Cattle calving in winter Cattle on farm with heifers bought in <10 years ago vs. Farm which never bought in heifers. Low parity Muddier environment Odds increased with increasing days in lactation Loose-housed cows had a higher risk followed by cows in free stalls than cows on pasture.
Scholey *et al.* 2013	Dairy cattle	Genetic susceptibility to DD
Wells *et al.* 1999	Dairy Cattle	Incidence of DD increased with shorter hoof trimming intervals and if the primary hoof trimmer worked on other farms. If the trimming equipment was not washed with water between cows, the percentage of herds with DD significantly increased.

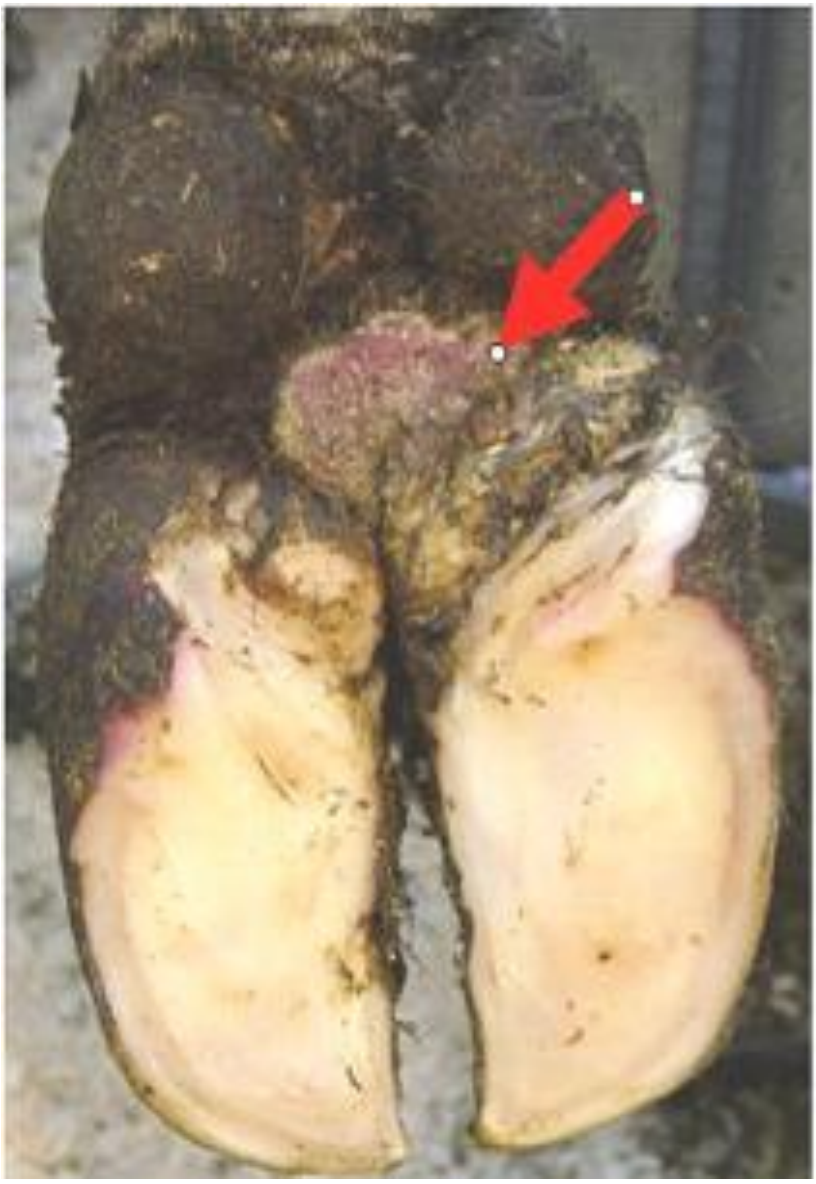
1.5 Clinical signs and management

1.5.1 Clinical manifestations of BDD in dairy cattle

Bovine digital dermatitis is an ulcerative foot disease (Cheli and Mortellaro 1974) with the main clinical feature being lameness resulting from a lesion immediately above the coronet on the rear feet between the heel bulbs (Blowey and Sharp 1988), as shown in Figure 1.1. Digital dermatitis can affect other sites such as the skin of the interdigital cleft found on interdigital hyperplasias, skin around the dew claws, heels, and the dorsal aspect of the coronary band (Döpfer 2009). Weaver *et al.* (1981) defined BDD as a diffuse or circumscribed superficial epidermitis of the digit at the coronary margin. Lesions may occur all around the coronary margin (Döpfer and Willemen 1998; Weaver *et al.* 1981), but are seen most commonly on the plantar or palmar aspect of the foot, midway between the heel bulbs, on the posterior border of the interdigital space (Rebhun *et al.* 1980; Blowey and Sharp 1988; Read *et al.* 1992; Kimura *et al.* 1993; Sauvageau *et al.* 1994). Less common lesion sites include the skin on the anterior margin of the interdigital space, and very occasionally on the coronary band at the abaxial wall (Blowey 1990). Approximately 80-90% of lesions occur in the hind feet and often affected cattle have the lesion concurrently in both hind feet (Kyllar *et al.* 1985; Nutter and Moffitt 1990). Holzhauer *et al.* (2006), reported that 30.1% of affected cows studied, presented lesions bilaterally.

The diameter of the lesions are usually small and vary in size from <1 cm to >6 cm. They are frequently seen as an irregular circular area of epidermal inflammation in the skin immediately above the coronet between the bulbs of the heel, but the shape is variable depending on location (Blowey and Sharp 1988). The presentation of the lesions change during their development and regression, and it is therefore useful to describe the stage of the infection.

Figure 1.1: Digital dermatitis lesion situated between the heel bulbs (Source: NADIS 2013)

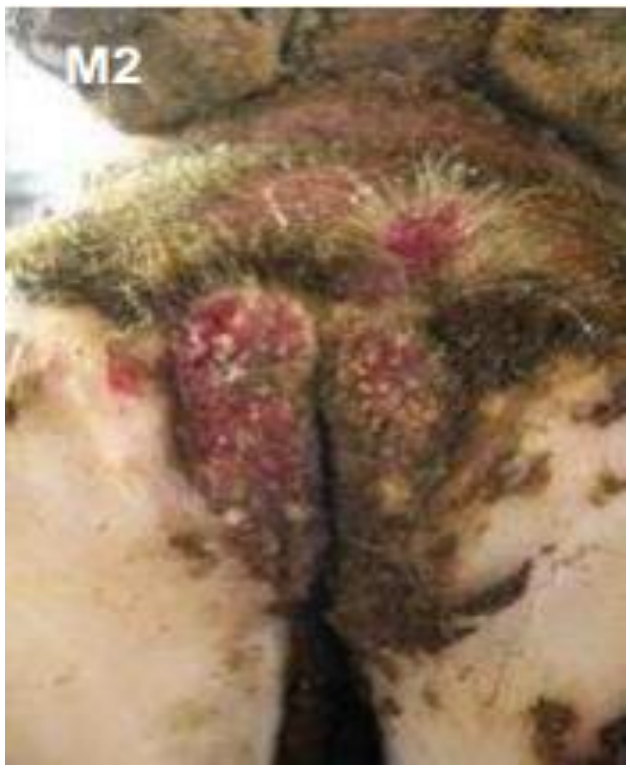


Cows with BDD are often severely lame and may walk on their toes (Blowey and Sharp 1988; Read and Walker 1998). Sometimes the animals may shake the affected foot or shift their weight from one foot to another (Bassett *et al.* 1990). These symptoms can be a good way to distinguish affected animals. However, in some cases the presence of a BDD lesion is not accompanied by obvious lameness in the animal affected.

As the clinical appearance of BDD lesions varies over the course of the disease, further descriptions of lesions is needed to define the severity and status of a BDD lesion (Holzhauer *et al.* 2008). Routinely used is a 5 M-stage scoring system, based on the one first described by Döpfer *et al.* (1997) which was recently amended by a consortium of international experts (Greenough *et al.* 2008). M stands for Mortellaro (one of two people to first report the disease in 1974), and the M-stages represent stages of DD that range from M0 = no lesion to M4 = chronic stage (See Figure 1.2).

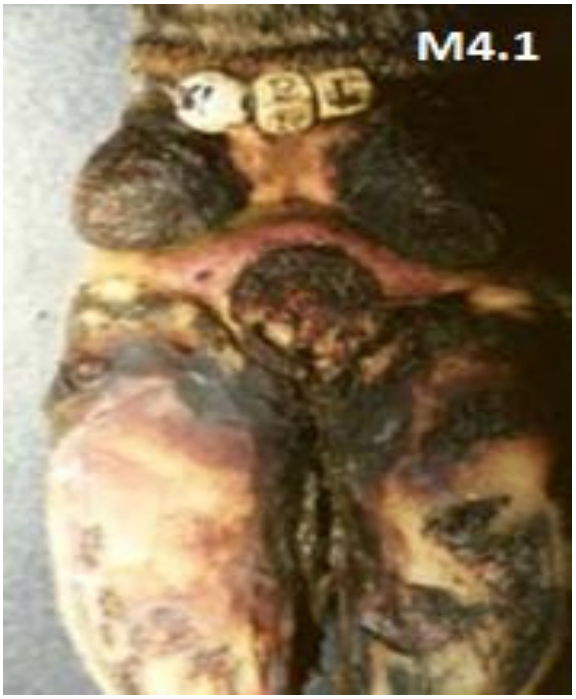
M0 refers to feet where no circumscribed skin lesions are present, ie BDD negative; M1 is an early stage lesion with a small, circumscribed, red to gray epithelial defect of less than 2 cm in diameter (subclinical infections); M2 is the classic ulcerative (bright red) or granulomatous (red-gray) stage with a diameter >2 cm; M3 is the healing stage where an acute BDD lesion is covered with a firm, scab-like material; and M4 is the late chronic stage characterized by a dyskeratotic lesion (mostly thickened epithelium), surface proliferation, or both. An extra 6^{th} stage (M4.1) is sometimes used to describe a chronic lesion which is showing signs of a subclinical infection again (Döpfer *et al.* 1997).

Figure 1.2: The Different presentations of BDD lesions described by the M0-M4.1 stages; detailed descriptions given in the text (M1, M2, M3, M4, M4.1, Source: Döpfer et al. 1997).




M3


M4

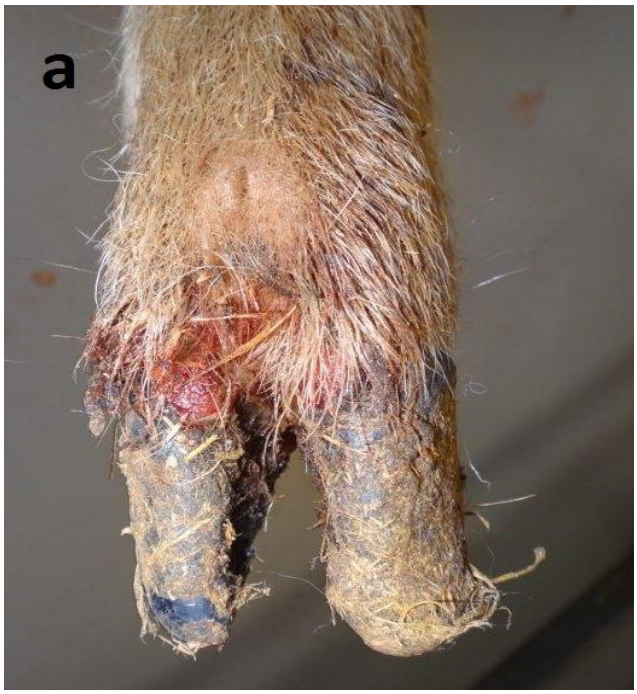


1.5.2 Clinical manifestations of CODD in sheep

Contagious ovine digital dermatitis is a disease of the ovine hoof resulting in acute, severe lameness, and tends to be more severe than BDD (Sayers *et al.* 2009). In contrast to footrot, characterized clinically by lesions involving the interdigital area and the heel, CODD can be characterized by ulcerative lesions of the coronary band which result in disruption of the abaxial wall lining the hoof and possibly the loss of the horn case in many cases (Abbott and Lewis 2005; Naylor *et al.* 1998; Davies *et al.* 1999). Similarly to BDD, the lesion begins with ulceration and loss of hair at the lesion site (Winter 2008).

The disease causes severe foot pathology characterised by under-running of the hoof wall starting at the coronary band where the lesion is present (see Figure 1.3), exposure of sensitive laminae and ultimately in a lot of cases avulsion of the hoof capsule (Winter 2008), an example of this can be seen in Figure 1.3 picture b.

Figure 1.3: a) shows a typical CODD lesion present on the coronary band, b) shows a more severe stage of CODD where loss of the horn capsule is seen.



This can be in contrast to BDD where the classical lesional site is usually on the bulb of the heel, although similar presentations as seen in sheep are increasingly reported in dairy cattle. Additionally, although the lesional site on the foot may be different, the tendency for cattle to contract the disease on the back feet may also be similar to sheep, as Duncan *et al.* (2011) found significantly more CODD lesions in the hind feet (62.5 percent) than front feet (37.5 percent) of sheep.

It is generally accepted that lameness associated with CODD is severe, and furthermore, animals can be affected in more than one foot, compounding the welfare compromise to affected sheep (Duncan *et al.* 2011). In untreated chronic cases there may be irreversible changes to the affected claw, with failure of regrowth of normal horn (Winter 2008).

The clinical appearance of CODD is usually sufficiently distinct to distinguish the condition from other common forms of lameness in sheep, and although the same M-stage system can be applied to the lesions seen in sheep, it is generally less routinely used. Using written descriptions and pictures of the feet of sheep with typical CODD and other foot diseases, Kaler and Green (2008) found that 94 percent of a group of 47 sheep specialists were able to identify the clinical manifestations of CODD. However, only 36 percent of farmers correctly identified the disease.

1.5.3 Diagnosis and treatment of BDD

Diagnosis of BDD can often be difficult when lameness is not seen, and then the lifting of the feet to look for a lesion is not prompted. However, several behaviours have been found to be associated with the disease; shaking of the affected foot, walking on toes and shifting weight over from one foot back to the other (Bassett *et al.* 1990). Therefore, diagnosis is generally made by clinical examination looking for the clinical features of BDD, whereby a lesion is located and scored.

There is some evidence that systemic antibiotic treatment can be effective, such as penicillin, ceftiofur and cefquinome (Read and Walker 1998; Rutter *et al.* 2001), however several other reports suggest that systemic antibiotics are

ineffective (Blowey and Sharp 1988; Britt *et al.* 1996). The perceived lack of effectiveness of injected antibiotics, combined with their cost, and the requirement for milk or meat withdrawal after treatment for many of these, has meant that topical antibiotic treatment and footbathing is far more commonly used for the treatment of BDD. The effectiveness of topical oxytetracycline as a treatment for BDD seems clearly established by many reports (Blowey and Sharp 1988; Nutter and Moffitt 1990; Cruz *et al.* 2001) and a number of antibiotic footbath solutions have been used to treat BDD, such as formalin and erythromycin (Watson 1999). Additionally, both a 5% copper sulphate footbath and a non-heavy metal-based proprietary dip have been shown to reduce lesion scores (Logue *et al.* 2012). Unfortunately however, there is no single effective treatment for BDD that can eliminate the disease (Laven and Logue 2006) and mass treatment using footbaths is generally the most common treatment in the UK (Laven and Logue 2006; Döpfer 2009).

1.5.4 Diagnosis and treatment of CODD

Several authors have described the clinical features of CODD, and currently they are still the routinely adopted way of disease diagnosis, as with BDD in cattle (Winter 2008). However in sheep, common themes include those features which seem important in distinguishing it from footrot such as lesions tending to commence at the coronary band (compared to the interdigital space) and then quickly under running the hoof horn capsule.

There is significantly less information available on the treatment of CODD. Anecdotal evidence suggests topically applied antibiotic treatments either through a hand-held sprayer or using foot baths are an effective treatment (Davies *et al.* 1999). Sawyer (2010) reported good results following the use of whole flock treatment with tilmicosin administered systemically and Judson (2010), showed the use of parenteral oxytetracycline with topical tylosin applied using a footbath to be effective. Duncan *et al.* (2011) found that amoxicillin treatment may have a preventive effect by reducing the rate of establishment of new CODD infections from 2.5 percent for foot bathing alone

to 1 percent when the systemic beta-lactam treatment was also used. However, like BDD, there is no single reported effective treatment for CODD.

1.6 Pathogenesis of digital dermatitis

Although BDD has been reported now for many years the exact pathogenesis is still not completely clear (Logue 2011). Many bacteria are found in lesions, particularly in severe ones, but only one type is found consistently in all lesions, being absent from normal skin tissues. Treponemal bacteria are evident in large numbers in the lesional tissues in deeper layers of the dermis (Blowey and Sharp 1988; Read *et al.* 1992; Demirkan *et al.* 1998). Fluorescent *in situ* hybridization (FISH) experiments, using probes specifically for treponemes, have revealed a stratification of treponemes within the epidermis of DD lesions (Moter *et al.* 1998). More recently *Treponema* species were identified in large numbers deep within the lesion at the interface between the healthy tissues and necrotic tissue (Nordhoff *et al.* 2008; Evans *et al.* 2009a; Klitgaard *et al.* 2013).

Evans *et al.* (2009a) made an extremely interesting discovery, using immunohistochemistry, where they showed that the infecting treponemes may be entering cattle feet via hair follicles and/or sebaceous glands. This would explain how the treponemes are able to make their way through the physical barrier of the skin to establish infection deep into the tissue.

An experimental infection model to induce acute BDD lesions in a controlled environment was developed by Gomez *et al.* (2012) in the USA. Hind feet of four dairy cattle were wrapped to mimic the conditions that are known to be associated with DD on farms, such as prolonged moisture and reduced access to air and inoculated at the heel and dewclaw areas with a BDD lesion skin biopsy or a culture broth of *Treponema* species. After 12 to 25 days, BDD was confirmed histopathologically in four of six dewclaws inoculated with a fresh BDD biopsy and in 1 of 4 of dewclaws inoculated with *Treponema* spp. broth culture. Subsequently, *Treponema* spp. were detected by PCR in inoculation

sites. This strongly suggests that along with the correct environmental conditions, *Treponema* spp. play an important role in the pathogenesis of BDD.

High numbers of the invasive spirochaetes can be observed within BDD lesions suggesting an active contribution of treponemes to the pathogenesis of BDD (Choi *et al.* 1997; Collighan and Woodward 1997; Döpfer *et al.* 1997; Demirkan *et al.* 1998; Moter *et al.* 1998). This hypothesis is further substantiated by the fact that serum samples from cattle infected with BDD contain elevated levels of antibody to *Treponema* antigens (Demirkan *et al.* 1999; Murray *et al.* 2002; Trott *et al.* 2003).

More recently, work carried out by Scholey *et al.* (2013) found that the expression of genes for keratin and several associated proteins was reduced in BDD, but there was increased expression of genes for keratin 6 and IL1, both of which are involved in keratinocyte activation (Freedberg *et al.* 2001). So, it is possible that defects in keratin or keratin-associated protein transcription could negatively affect the hair/skin barrier allowing treponemes to penetrate via this route. Additionally, Scholey *et al.* (2013) found that BDD lesions had down regulation of filaggrin-2, which plays a part in the formation of the epidermal barrier and resistance to invasion of bacteria via the skin (Wu *et al.* 2009), and highlighted that MMPs (a family of enzymes that contribute to normal tissue turnover and are implicated in many disease processes) (Koskinen *et al.* 2011) may be linked to keratin associated molecules and therefore could play a part in the hyperkeratosis in BDD.

In adaptive immunity, the presentation of peptides to T cells by MHC class II molecules is critical for specific recognition of antigens (Ting and Trowsdale 2002). In BDD lesions, there is reduced expression of the major histocompatibility class MHC II (genes DYα), suggesting downregulation of the local adaptive immune response (Scholey *et al.* 2013)

Whilst antibodies against treponemes in cattle with BDD develop early following infection and reach high levels, they do not appear to be protective

(Walker *et al.* 1997; Demirkan *et al.* 1999; Vink 2006). This may be explained by Scholey *et al.* (2013) findings that in BDD a significant upregulation of anti-inflammatory cytokines occurred that could suppress immune responses. This may explain why systemic immunity to treponemes appears to have a small (or no) protective effect against the development or persistence of BDD. Additionally, *Treponema phagedenis*- like spirochaetes (a phylogroup highly associated with BDD) have been found to have an immunosuppressive effect on bovine macrophages and have a negative effect on the innate immune response, as well as wound repair, which may explain the persistent nature of the lesions (Zuerner *et al.* 2007).

As CODD has only been reported in the last 20 years, and the potential of a spirochaetal aetiology only even more recently discovered, little is known on the pathogenesis of CODD in sheep. However, given that the same bacteria are being isolated from both foot lesions and clinical appearance is very similar, it is likely the same/closely related series of events are occurring. However, for both sheep and cattle, more information is needed to fully understand the pathogenesis and the role the immune system plays in the formation/persistence of BDD and CODD lesions. Dhawi *et al.* (2005) attempted to test the hypothesis that the two diseases may have a shared spirochaetal aetiology. An enzyme-linked immunosorbent assay (ELISA) was developed to detect anti-treponeme antibodies in the sera of cattle and sheep against the two treponeme isolates and sera tested for antigen reactivity by Western blotting. Cattle and sheep with BDD and CODD, respectively, had increased seropositivity rates to both treponeme isolates. In some cattle herds, significant correlations were shown between antibodies to BDD treponemes and CODD treponemes and in other herds, there was no cross reaction, suggesting the presence of more than one treponeme in BDD. There was no significant correlation between the two treponeme isolates when ELISA-tested against sheep sera from CODD cases; sheep showed evidence of reactivity to one or the other treponeme antigens, never to both. Western blotting against both treponeme antigens showed that they frequently displayed different antigen epitopes, but some minor bands were common to both organisms. This

data suggests that there are a number of treponemes in UK farms, which could be involved in the pathogenesis of either BDD or CODD.

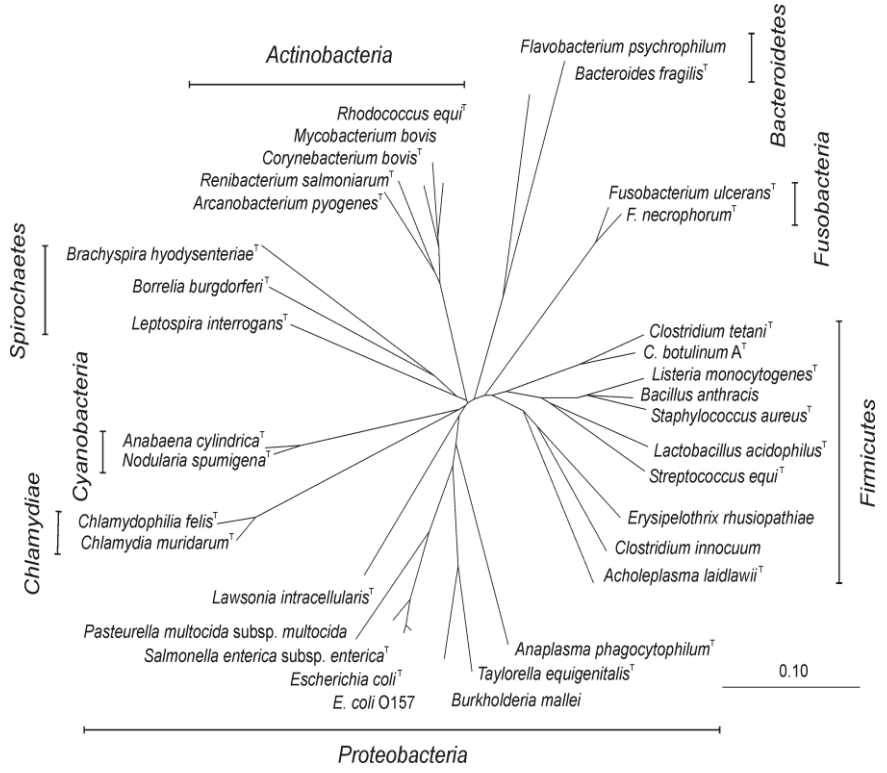
1.7 Introduction to the Spirochaetes

1.7.1 Spirochaete phylogeny

The *Spirochaetes* represent one phylum in the domain bacteria (Figure 1.4), representing one of around 40 major bacteria phyla, based on comparative analysis of 16S rRNA sequences (Hugenholtz *et al.* 1998). 16S rRNA is a highly conserved molecule present in all prokaryotic organisms, which is highly useful in measuring phylogenetic relationships due to its functional consistency and slow changes in sequence (Woese 1987).

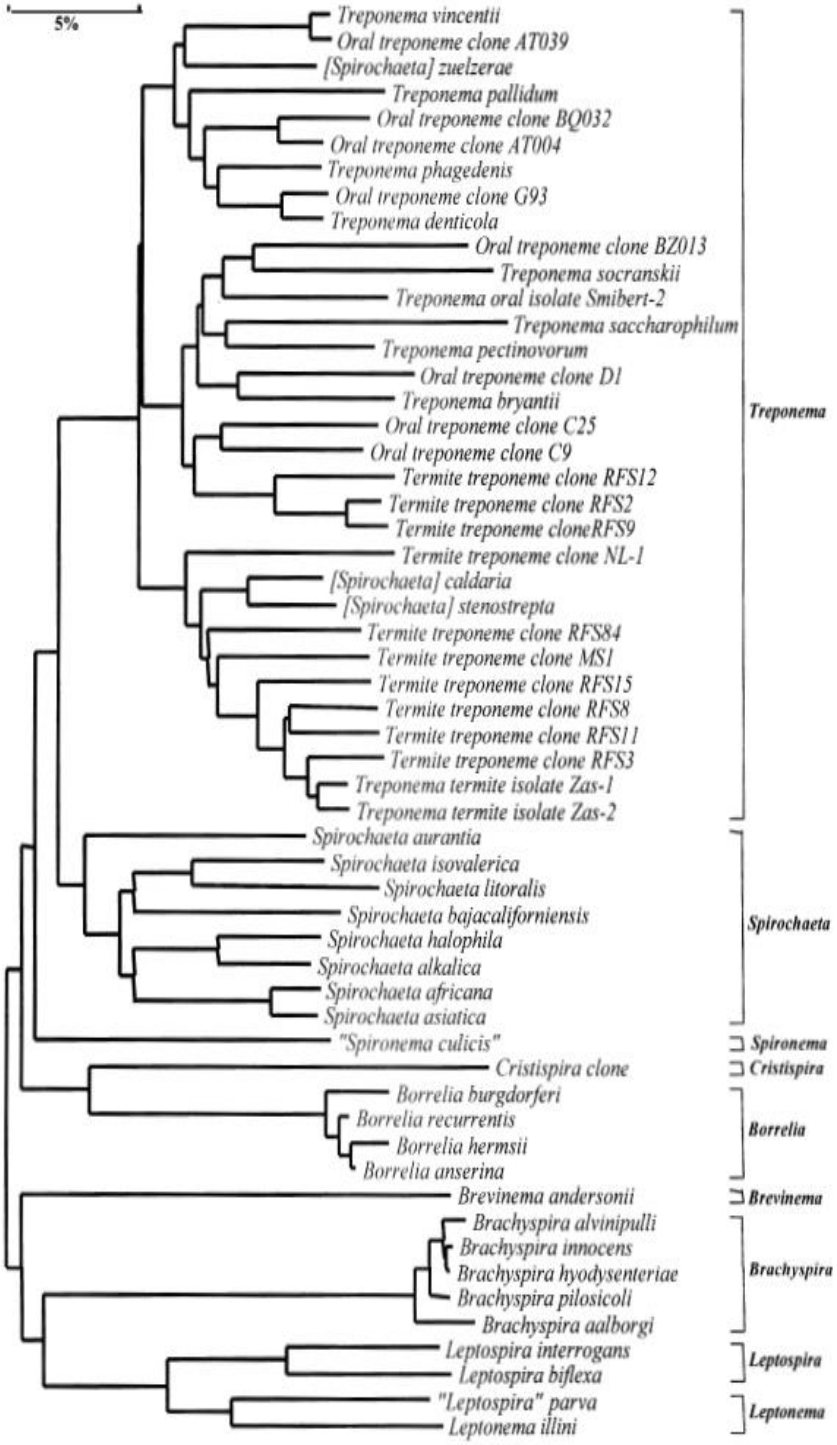
The spirochaetes are presently classified in the Class Spirochaetes in the order Spirochaetales that divides into three families; the *Brachyspiraceae,* the *Leptospiraceae,* and the *Spirochaetaceae*. The *Brachyspiraceae* family only includes one genus, the *Brachyspira,* which contains the important pathogenic species that causes dysentery in swine (Fernie *et al.* 1983) and the *Leptospiraceae* family includes two genera *Leptospira* and *Leptonema,* the former of which causes leptospirosis (Sakula and Moore 1969). The *Spirochaetaceae* family includes the genera *Spirochaeta, Borrelia, Brevinema, Clevelandina, Cristispira*, *Diplocalyx*, *Hollandina*, *Pillotina* and *Treponema* (Paster and Dewhirst 2000). New genera of termite spirochaetes, such as *Clevelandina, Diplocalyx,* and *Hollandina,* have been described due to differences in ultrastructural traits (Breznak 1984). Species of the genera *Borrelia* include host-associated spirochaetes that are transmitted by an arthropod vector to animals and humans (e.g. Lyme disease) (Wang *et al.* 1999). *Brevinema* includes infectious spirochaetes of the white footed mouse (Defosse *et al.* 1995). *Cristispira* contains spirochaetes which are large in size and live in aquatic environments (Leschine *et al.* 2001). *Spironema* includes spirochaetes from the mosquito (Paster and Dewhirst 2000).

Figure 1.4: A radial tree illustrating bacterial phylogeny based on 16S rDNA. Only some phyla are represented and each phylum is based on selected genera and strains. Members of the genus Anabaena and Nodularis were used as out groups. (Source: Råsbäck 2007).



The phylogenetic relationships of representatives of the Spirochaetal genera are shown in Figure 1.5

Figure 1.5: 16S rRNA dendrogram demonstrating the phylogenetic relationships of representatives of spirochaetal genera. The sequences of the species shown may be obtained through GenBank (Source: Paster 2000).

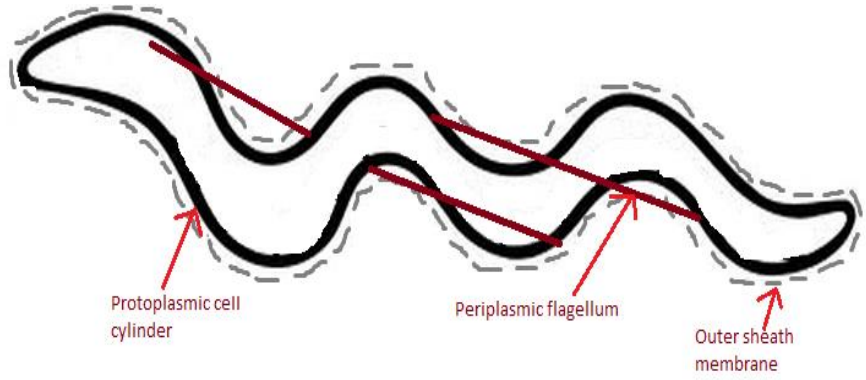


1.7.2 Spirochaete structure and biology

In the early years of bacteriology the observations of spirochaetal morphology were made on organisms involved in medicine such as *Treponema pallidum*. Early investigators, notably Zuelzer (1911) and Noguchi (1928), began the investigations of spirochaetes morphology by using light microscopy. Later, Morton and Anderson (1942), first used the electron microscope to examine spirochaetes.

The spirochaetes possess a cellular ultrastructure that is unique amongst bacteria (Paster *et al.* 1991). They are a group of flexuous, thin, gram-negative, helical shaped bacteria, which differ from other prokaryotes by the presence of axial fibril, known also as the endoflagellum. Members of the group also possess an outer sheath surrounding the cell, a protoplasmic cylinder, which consists of the cell wall, cell membrane, and the enclosed cytoplasm. The endoflagella (usually two or more) arise from opposite poles of the cell, which together constitute the "axial filament", located within the periplasmic space between the flexible cell wall and an outer sheath (Smibert 1974) and often overlap in the central region of the cell (Radolf and Lukehart 2006). A schematic diagram of a spirochaete showing the outer membrane sheath, protoplasmic cell cylinder, and periplasmic flagella is shown in Fig 1.6.

Figure 1.6: A schematic diagram of a spirochaete showing the outer membrane sheath, protoplasmic cell cylinder, and periplasmic flagella.



The cell dimensions of the spirochaetes vary from diameters of 0.2-0.75 μm to lengths of 5-500 μm (Brock *et al.* 1994). Some spirochaetes are quite large; for example, *Cristispira* are 0.5–3 μm wide and 30–180 μm in length, with over 100 periplasmic flagella attached to each end of the cell. Whereas, the Leptospiraceae (which includes *Leptospira* and *Leptonema* species) are only approximately 0.1 μm in diameter, 10–20 μm in length, with only one periplasmic flagellum at each end of its cell (Charon and Golstein 2002).

Spirochaetes change their form in response to osmolarity variations in the environment. Hypertonic conditions cause some spirochaetes (e.g. *Leptospira*) outer envelope to separate from the protoplasmic cylinder, changing its shape to a sphere (Johnson 1977). Within the sphere the protoplasmic cylinder maintains it's helical form (Auran *et al.* 1972). Other spirochaetes (e.g. *Treponema*) retain their spiral shapes when exposed to hypertonic conditions, but when in a hypotonic environment, change to the spherical shape (Hardy and Nell 1961).

1.7.3 Spirochaete motility

The endoflagella are the organelles which allow the spirochaete cell motility (Bromley and Charon 1979) and depending on the species, the number of flagella can be anything from two to hundreds per cell (Johnson 1977). Non-translational movement in free liquid gives the organism the appearance of spinning and rotating on its axis. However, the bacteria do not spin, the body remains relatively stationary but there is contra-rotation of the hooked ends (Charon and Goldstein 2002). Translational movement is effected by helical waves travelling for a short distance near the trailing end of the cell. The broad hook at the trailing end waves in the opposite direction to the propulsive helical wave as to prevent rotation of the body (Charon and Golstein 2002). For this movement to be possible, the flagella must extend along the axis of the body but not be wound helically around the cell body (Goldstein *et al.* 1994). Consequently, they have swimming modalities that are very complex. It is this motility which plays a role in the pathogenesis of the diseases of many spirochaetes, including *Treponema, Borrelia*, and *Leptospira* (Ruby *et al.* 1997; Motaleb *et al.* 2000; Lux *et al.* 2001).

1.7.4 Metabolic requirements of spirochaetes

Spirochaetes are a metabolically diverse group of bacteria. They vary, for example, with respect to their oxygen requirements. Some are aerobic, such as *Leptospira* species which are obligate aerobes. *Spirochaeta* are often facultative, *Brachyspira*, and *Borrelia* microaerophilic, and most *Treponema* spp. are obligate anaerobes (Radolf and Lukehart 2006).

Spirochaetes also vary with respect to their nitrogen utilization. The free-living *Spirochaeta aurantia* and certain *Treponema* species from the guts of termites utilize atmospheric nitrogen as a nutrient source (Lilburn *et al.* 2001).

1.7.5 Antibiotic resistance of spirochaetes

A useful trait of the spirochaetes is their resistance to the antibiotic Rifampicin (Stanton *et al.* 1979; Nelson *et al.* 1991), excluding *Leptospira* (Leschine and Canale-Parola 1986). This antibiotic is therefore used as a selective agent in the isolation of spirochaetes from a variety of environments to eliminate other bacterial growth in culture.

1.7.6 Free living and host-associated non-pathogenic spirochaetes

Both free-living and commensal (non-pathogenic) spirochaetes are widespread in nature. Saprophytic *Leptospira, Spirochaeta,* and other species are found in freshwater, saltwater and soil (Harwood and Canale-Parola 1984). They have also been detected in fluidized bed reactors in wastewater treatment plants (von Wintzingerode *et al.* 1999) and contaminated aquifers (Dojka *et al.* 1998).

Spirochaetes can also be found in a range of animal hosts. The digestive tract of insects, like the wood-eating types such as termites, contains spirochaetes (Breznak 1973). They are found attached to the surface of protozoa present in the insect gut (Johnson 1977). *Cristispira* lives in the crystalline style of bivalve molluscs (Harwood and Canale-Parola 1984) and spirochaete-like organisms have also been observed in *Diptera*, including flies, fleas, mosquitoes, keds, gnats, and butterflies (Breznak 1973).

The first compartment of the multi-chambered stomach of ruminants (known as the rumen) also can contain spirochaetes (Stanton and Canale-Parola 1979;

Paster and Canale-Parola 1982). Ultrastructural studies have established a close association of spirochaetes with the epithelial cells of large intestines of humans, monkeys (Takeuchi *et al.* 1974), dogs (Leach *et al.* 1973), rats (Davis *et al.* 1972), and mice (Savage *et al.* 1971).

1.7.7 Host-associated pathogenic spirochaetes

Several species of spirochaetes cause medically important diseases, some of which are quite prevalent and can have grave consequences. *Borrelia burgdorferi* causes Lyme disease, which is the most prevalent vector-borne disease in the United States (Diuk-Wasser *et al.* 2012). *Borrelia hermsii* and other closely related *Borrelia* species cause relapsing fever (Kraiczy *et al.* 2003) and various *Leptospira* species can cause leptospirosis (Gravekamp *et al.* 1993). The latter is a potentially fatal waterborne zoonosis which has many possible manifestations and occurs worldwide. *Brachyspira hyodysenteriae* causes swine dysentery, and *Brachyspira pilosicoli* and *Brachyspira aalborgi* are associated with human intestinal infections in developing countries and in immunocompromised individuals (Mikosza *et al.* 2003). Spirochaetes of the genus *Treponema* first came to popular knowledge as associated with the sexually transmitted disease syphilis (Gray *et al.* 2010; Shields *et al.* 2012) *Treponema denticola* and other oral treponemes can be associated with periodontal disease (Simonson *et al.* 1988; Sela 2001). More recently treponemes have been associated with animal diseases such as BDD in cattle (Blowey *et al.* 1992, Demirkan *et al.* 1998; Evans *et al.* 2008) and ear necrosis in swine (Svartström *et al.* 2013).

1.8 The Genus *Treponema*

1.8.1 Introduction

Species of the genus *Treponema* are considered in general to be anaerobic, spirochaetes that represent one of the nine spirochaetal genera of the spirochaetal phylum (Radolf and Lukehart 2006). Using conventional genotypic and phenotypic traits, the treponemes have been characterised, and

now comparative genomic analysis has forwarded our knowledge of the evolutionary information of treponemes.

Treponema are typically host-associated spirochaetes (Norris et al. 2002). They contain both pathogenic and non-pathogenic species with hundreds of species found in the human and animal oral cavities, gastrointestinal tract (GI), and are the causative bacteria of many debilitating diseases (Radolf and Lukehart 2006). However, due to the fastidious nature of the treponemes it is likely that there remains a large number of uncharacterized species.

In *Treponema*, the number of flagella ranges from one to eight per cell (Edwards *et al.* 2003). Consequently, these flagella impart a motility mechanism that allows them to swim through highly viscous environments and play a part in the pathogenesis of treponemal diseases.

Human oral *Treponema* species such as *Treponema denticola* can now be routinely cultured *in vitro***.** PCR amplification and sequence comparison of 16S rRNA genes, immunohistology, immunocytochemistry, and electron microscopy have allowed treponemes to be detected and also confirmed pathogenic when they are associated with disease (Choi *et al.* 1994, 1996; Dewhirst *et al.* 2000; Riviere *et al.* 1999; Paster *et al.* 1998; Edwards *et al.* 2003).

However, it is still thought that about 75% of oral *Treponema* species have yet to be cultured (Dewhirst *et al.* 2000). The invention of new isolation and culture techniques, together with molecular and immunological techniques, has made it possible to classify many treponemes, but the cultivability of these organisms remains an issue. Comparison of 16S rRNA gene sequences following PCR amplification from spirochaetal DNA, or from colonized lesions, has allowed the identification and detection of not-yet-cultivated organisms, and preliminary associations of these with various disease conditions of animals and humans.

1.8.2 Non-pathogenic *Treponema*

Spirochaetes are commonly found in the rumen, caecum, colon and faeces of ruminants. A physiologically and morphologically diverse population of spirochaetes has been identified. Species include *Treponema bryantii* and *Treponema saccharophilum*. Both are obligatory anaerobic symbionts which live off the digesta contents found in the rumen (they are cellulolytic), and occur in high population densities (Ziolecki 1979; Paster and Canale-Parola 1982; Stanton and Canale-Parola 1979). These treponemes have not been associated with GI disease, and it is likely that they play a substantial role in the degradation of ingested plant materials (Paster and Canale-Parola 1979).

Treponemes are also present in healthy tissue of the oral cavity of healthy dogs, cats and humans, including, *Treponema socranskii* (Valdez *et al.* 2000; Takeuchi *et al.* 2001). Other treponemal sites include the hindgut of the termite (including *Treponema primitia* and *Treponema azotonutricum*) (Graber *et al.* 2004), and the intestines of horses (Simpson *et al.* 2004) and pigs (Leser *et al.* 2002).

1.8.3 Pathogenic *Treponema*

Treponema pallidum subspecies *pallidum* causes the serious sexually transmitted disease syphilis. The disease is systemic and initially involves rashes and ulcers, but later stages can include cardiovascular and neurological diseases (Zetola *et al.* 2007). This treponemal disease is of special concern as a recognized cofactor in the acquisition and transmission of human immunodeficiency virus (HIV) (Shields *et al.* 2012).

Other closely related treponemes cause yaws, bejel, and pinta. Unlike syphilis, these infections are transmitted by nonsexual contact, mainly between children living in conditions of poor hygiene. Yaws is a tropical infection of the skin, bones and joints caused by the spirochaete *Treponema pallidum* subsp. *pertenue*, which is considered to be transmitted by skin-to-skin contact with an infective lesion (Mitjà *et al.* 2012, 2013). Bejel (*Treponema pallidum* subsp. *endemicum***)** causes mouth sores and destructive lumps in

bone, and pinta (*Treponema carateum*) causes itchy patches on the skin (Harper *et al.* 2008).

Periodontal diseases, such as periodontitis, are chronic inflammatory infections affecting the gingival tissue (gums), underlying connective tissues and bone that supports the teeth of the human mouth. Oral treponemes are widely-considered to play important roles in periodontal disease etiology and pathogenesis (You *et al.* 2013). *Treponema denticola* is the most characterized oral treponeme, in terms of virulence factors and interaction with host cells *in vitro* (Sela 2001). PCR amplification and sequence comparisons of spirochaete 16S rRNA genes (Dewhirst *et al.* 2000), as well as immunological and microscopic studies, has provided convincing evidence that the treponemes in the oral cavity are directly associated with active disease. Numerous other species have been isolated from diseased sites, including *Treponema vincentii, Treponema socranskii, Treponema maltophilum, Treponema amylovorum, Treponema lecithinolyticum, Treponema pectinovorum* and *Treponema parvum* (Wyss *et al*. 1996, 1997, 1999, 2001; Walker *et al*. 1997).

Treponemes have also been implicated in swine diseases such as ear necrosis and shoulder ulcers (Pringle *et al.* 2009; Pringle and Fellström 2010). These are serious welfare problems that can cause significant economic losses for producers. Previously, spirochaetes had been observed microscopically in scrapings from pig ulcers (Dodd 1906). More recently in a study by Svartström *et al.* (2013), twelve *Treponema* species isolates, belonging to three different phylogroups were cultured from porcine ear necrosis, shoulder ulcers and gingiva.

1.9 Treponemes and Digital Dermatitis

1.9.1 Aetiology of BDD

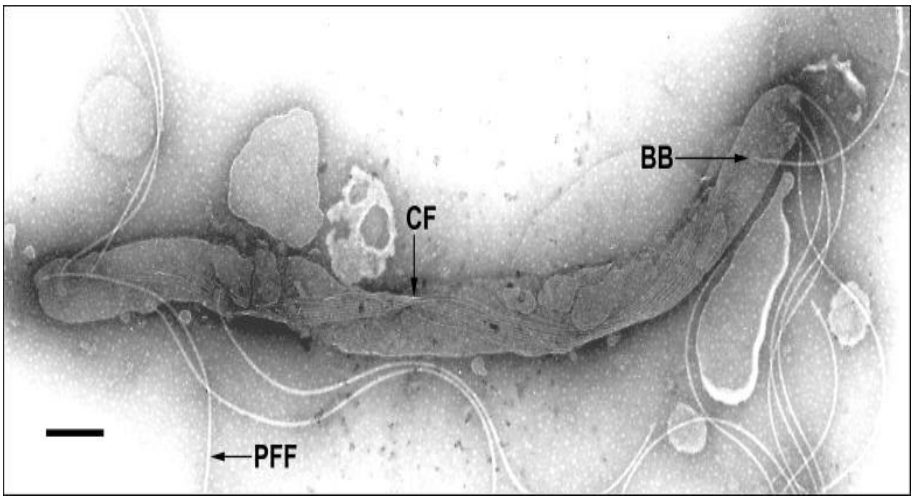
The precise aetiology of BDD is extremely complex and is not yet completely understood. The rapid spread of BDD suggests it has a highly contagious nature. Bacteria have been consistently identified in histological examination

of lesions, and lesions demonstrate a response to antimicrobial agents (Read *et al.* 1992).

Spirochaetes, of the genus *Treponema* have frequently been found in large numbers in BDD lesions (Demirkan *et al.* 1998), and are now known as the primary causative bacteria of BDD.

The cloning of bacterial 16S rRNA genes, in Germany, identified five phylotypic groups of spirochaetes in lesions of BDD (Choi *et al.* 1997). Since then, three of these have been isolated in dairy cattle in the UK and the USA, which are known as *Treponema* Group 1, *Treponema medium*- like, Group 2, *Treponema phagedenis*- like spirochaetes and Group 3, *T. denticola*/*T. putidum*-like, (Walker *et al.* 1995; Stamm *et al.* 2002; Evans *et al.* 2008; Nordhoff *et al.* 2008) with the latter now recognised as a new species, *Treponema pedis* (Evans *et al.* 2009a). *T. phagedenis-like* (the group 2 treponeme phylogroup) can be seen in Figure 1.7. It was suspected that BDD lesions may contain more than one *Treponema* phylogroup at one time and Evans *et al.* (2009b) highlighted the extent to which this disease is in fact polytreponemal. In BDD samples tested, they found that BDD treponeme group 1, group 2 and group 3, were present in 96.1%, 98%, and 76.5% of BDD lesions, respectively.

Figure 1.7- Treponema phagedenis. The cytoplasmic filament (CF), the periplasmic flagellar filaments (PFF) and the basil body (BB) are highlighted in the image (Source: Izard et al. 1999).



To further cement the treponemal aetiology of BDD lesions, an experimental model developed by Gomez *et al.* (2012) found that BDD-like lesions developed after inoculation of the dew claw region of the bovine foot with *Treponema* spp.

1.9.2 Aetiology of CODD

The aetiology of CODD is still partially uncertain, and initial evidence of a response to antibiotic therapy suggests that a bacterial aetiology is likely (Davies *et al.* 1999). The roles of spirochaetes, particularly treponemes, and *D. nodosus*, the causative agent of footrot, have been investigated by several authors.

Initial investigations suggested that *D. nodosus* was not present in CODD lesions (Davies *et al.* 1999). Later, work using cultural techniques demonstrated *D. nodosus* in 38 percent of sheep CODD lesions compared with 20 percent of healthy sheep feet (Wassink *et al.* 2003). Similarly, Moore *et al.* (2005) identified *D nodosus* in 44 percent of CODD lesions by culture and 74 percent by PCR compared with 7.7 percent and 31 percent, respectively, of apparently healthy feet.

Interest in the possible involvement of spirochaetes in CODD, particularly those belonging to the genus *Treponema*, followed the isolation of a spirochaete from a severe ovine foot disease, which was yet to be known as CODD (Naylor *et al.* 1998). Collighan *et al.* (2000), by comparison of 16S rRNA gene sequences, showed that this spirochaete was closely related to a treponeme isolated from human periodontitis and BDD. The next year Demirkan *et al.* (2001) also isolated a spirochaete from a case and showed on the basis of 16S rDNA analysis that the organism was most closely related to a spirochaete isolated from cases of BDD in the USA. *T. medium*- like and *T. phagedenis*- like spirochaetes, previously found to be associated with BDD lesions, were isolated from CODD- affected sheep on a farm in Ireland (Sayers *et al.* 2009). This study also found their treponemal cultures from CODD lesions to be mixed with different co-cultures of *T. medium*- like, *T. phagedenis*- like, and *T. pedis*. The possible association between CODD and treponemes was also supported by Moore *et al.* (2005), who, using PCR analysis, found treponemes in 70 percent of CODD lesions compared with 38 percent of healthy feet.

The relatedness of spirochaetes from severe ovine foot lesions and BDD suggests the potential for their involvement in the disease process in sheep. However, a clear link between infection in cattle and sheep has not yet been demonstrated. Whilst the role of treponemes as primary agents in BDD appears convincing, a comprehensive bacterial molecular survey of CODD lesions has not yet been carried out to determine if there is a shared spirochaetal aetiopathogenesis between BDD and CODD as well as a large survey into the role *D. nodosus* and *F. necrophorum* in the lesions.

1.9.3 Isolation and detection of BDD and CODD associated treponemes

Blowey and Sharp (1988) demonstrated spirochaete- like, filamentous organisms in lesions by culture methods, however these cultural techniques were yet to be improved to consistently isolate treponemes from the lesions.

Culture of these organisms has been problematic due to their fastidious anaerobic nature, but the isolation of two new spirochaetes from BDD cases in California was successfully performed by Walker *et al.* (1995). Due to the fastidious nature of these organisms, the use of these cultural techniques may have been underestimating their prevalence in CODD and BDD lesions. However, the development of more sensitive molecular techniques that do not rely on the presence of viable organisms has provided the opportunity to improve detection rates.

In 1997, Rijpkema *et al.* tested typical lesions of BDD in two dairy cows by the polymerase chain reaction (PCR) for the presence of spirochaetal 16S rRNA gene and follow up work by this group has defined two treponemes by complete 16S rRNA gene sequence analysis (Collighan and Woodward 1997). In Germany, cloning of bacterial 16S rRNA genes identified five phylogroups of spirochaetes present within BDD lesions (Choi *et al.* 1997). Three of these have since been isolated using anaerobic cultural techniques in the USA and UK (Walker *et al.* 1995; Stamm *et al.* 2002; Evans *et al.* 2008).

Although other techniques such as immunochemistry have been used to detect treponemes in lesions, the most commonly used method is a combination of cultural techniques alongside PCR analysis for the three associated *Treponema* groups. Recently, PCR's for the three BDD associated *Treponema* phylogroups were developed by Evans *et al.* (2009b), using twenty-three strains isolated and biochemically phenotyped from BDD lesions to validate the PCR tests. This development has enabled quick and effective detection of the BDD treponemes in lesions and other tissues that are under investigation.

1.9.4 Transmission and carriage sites of DD treponemes

Due to the difficulties of bacterial isolation and culture, little is known about the distribution of the DD- associated *Treponema* species in the farm environment and the transmission routes of BDD and CODD.

Thus far, attempts at detecting the causative *Treponema* phylogroups in the farm environment have proved largely unsuccessful, although the bovine

gingiva and rectal tissues have been identified as potential infection reservoirs (Evans *et al.* 2012). Evans *et al.* (2012) identified BDD treponemes in the oral cavity (14.3% of cattle) and the rectum (14.8% of cattle). There has been much debate about the GI tracts role as a reservoir of infection of DD treponemes, with the bovine gingival and rectal tissues, rumen fluid and faeces identified as potential infection reservoirs. More recent work detected DD treponeme phylogroups in rumen fluid, faecal samples and slurry (Klitgaard *et al.* 2014; Nascimento *et al.* 2015; Zinicola *et al.* 2015).

However, to date, isolations of DD treponemes from the bovine GI tract have failed. The sheep GI tract as a reservoir of infection has yet to be investigated, and the tendency of beef cattle to be different breeds, fed different diets and subjected to different housing regimes than dairy cattle, gives reason for further investigations into both these animals GI tracts. However**,** the contribution of the GI tract to DD transmission needs further investigation to understand how these bacteria could be transmitted from the GI tract to infecting cattle feet.

It may be possible that transmission of infection is achieved by dissipation of infectious material from lesions into the environment, thus infecting other animals by indirect contact, or that ruminant DD transmission may in fact be more similar to the non-venereal human treponematoses such as yaws, with direct touch as a major route of transmission.

No transmission routes for DD have yet been identified. Without this information, preventing the spread of DD between animals and between farms is virtually impossible, and once the disease is present on the farm it is even harder to eliminate. Various risk factors have been found to be associated with BDD, such as early stages of lactation, first and second parity cows (Somers *et al.* 2005; Holzhauer *et al.* 2006; Barker *et al.* 2009), poor hygiene (Barker *et al.* 2009; Somers *et al.* 2005; Rodriguez-Lainz *et al.* 1999; Nowrouzan and Radgohar 2011) flooring type (Somers *et al.* 2003; Barker *et al.* 2009) and frequency of trimming (Holzhauer *et al.* 2006; Wells *et al.* 1999). However, studies have failed to find any definitive transmission routes of the disease.

It remains under discussion whether foot tissues could be the primary infection reservoir for the disease or if there are other BDD treponeme carriage sites in the cow or somewhere in the farm environment. From data so far it is unclear how the disease is being spread, and where the bacteria may harbour. From the limited success of studies so far to detect *Treponema* on the farm environment and elsewhere it is hard to say what the most likely route of transmission may be. However, from the study previously spoken about, it would suggest that there may be more clues hidden in the GI tract, but further studies are vital to determine the potential contribution of this route.

1.10 Aims of the project

1. Further characterise DD treponemes involved in beef cattle BDD and sheep CODD to understand their relatedness to dairy cattle BDD treponemes, and to understand whether a shared aetiology between many livestock species may be apparent.
- Analyse BDD lesions and CODD lesions from beef cattle and sheep, respectively, for DD treponemes and aim to compare these to previously collected dairy cattle BDD treponeme isolates.
- Investigate an unknown foot disease in goats for the presence of treponemes to understand whether DD has spread to another host species. Attempt the isolation of treponemes from these lesions to compare with the 16S rRNA sequences of treponemes already collected from DD lesions in sheep and cattle.

2. Identify possible carriage sites and transmission routes of DD in cattle and sheep.
- Analyse previously collected bacterial databases from studies which investigated the diversity of 16s rRNA gene sequences in various niches such as the ruminant GI tract and faeces for the presence of treponemes.
- Investigate these previously collected bacterial databases to delineate whether there is an association between the ruminant diet and levels of

treponemes in the GI tract, with the aim of understanding whether certain diets promote the growth of treponemes within the rumen.
- Collect and investigate sheep and beef cattle GI tract tissues; rectal tissue and gingival tissue for the presence DD treponemes.
- Collect and analyse sheep and beef cattle faeces for the presence of DD treponemes.
- Analyse equipment used to trim both sheep and cattle feet for the presence of treponemes to investigate whether this equipment could be a potential route of transmission of treponemes from foot to foot.

4. Investigate beef cattle's immune response to DD treponemes to understand their exposure and reaction to BDD treponemes.
-Analyse beef cattle blood samples from BDD positive and BDD negative herds and investigate their immunological response to dairy cattle BDD, beef cattle BDD and sheep CODD treponeme isolates.

The above aims involve animal sampling hence ethical approval and licensing is necessary for these to be carried out and important to the welfare of animals involved.

All sampling carried out within this thesis was conducted in accordance with United Kingdom legislation. All sampling was carried out either using Home Office Project License PPL40/3275 and/or were approved by the University of Liverpool ethical review process with approved ethics application number VREC137. The ethical review process involves each proposed study being examined by an expert committee. Each study must satisfy criteria which ensure that the study will yield maximum benefits and minimise risk of harm. Informed consent was obtained from the owners prior to inclusion of respective samples in the study.

Chapter 2

Materials and methods

This chapter consists of the specific, detailed methods used throughout this thesis.

2.1 Reagents and buffers

Reagents and buffers used within this thesis and their corresponding preparation are listed in Table 2.1.

Table 2.1: Reagents and buffers used throughout this thesis and their corresponding preparation method.

Solution/buffer	Preparation
1X sodium dodecyl sulfate (SDS) gel-loading buffer	100 mM Tris-Cl (pH 6.8) (see below) 4% (weight/volume (w/v)) SDS (electrophoresis grade) (Sigma-Aldrich, Dorset, UK). 0.2% (w/v) bromophenol blue (Sigma-Aldrich, Dorset, UK). 20% (volume/volume (v/v)) glycerol 200 mM dithiothreitol (DTT) (Sigma-Aldrich, Dorset, UK).
10X phosphate buffered saline (PBS) stock	80 g NaCl (Sigma-Aldrich, Dorset, UK) 2 g KCl (Sigma-Aldrich, Dorset, UK) 11.5 g Na2HPO4 (Sigma-Aldrich, Dorset, UK) 2 g KH2PO4 (Sigma-Aldrich, Dorset, UK) All the above was dissolved in 900 ml of ddH2O and pH adjusted to 7.2 using hydrochloric acid (HCl) (Sigma-Aldrich, Dorset, UK). This was then made up to 1 L with ddH2O. 10X stock solution could then be diluted with ddH2O to make a working concentration of 1X PBS.
Acrylamide solution 30% (w/v)	A 30% (w/v) Acrylamide solution was obtained from Severn Biotech Ltd, Worcestershire, UK.

Agarose 1.0% (w/v)	1 g of agarose powder (Biorad, Hemel Hempstead, UK) was added to 100 ml 1X TAE buffer.
Ammonium persulfate (APS) stock solution 10% (w/v)	1 g ammonium persulfate (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml of ddH_2O and stored at 4 °C. Ammonium persulfate decayed slowly in solution, so was replaced every 2-3 weeks.
Chelex-100 resin 5% (w/v)	5 g of Chelex-100 resin (BioRad, Hemel Hempstead, UK) was dissolved in 10 ml of ddH_2O.
Color Prestained Protein marker	Color Prestained Protein Standard, broad range 11-245 kilodalton (kDa) were purchased from NEB, Hertfordshire, UK.
Dntps	20 mM of stock solutions of dATP, dTTP, dCTP, and dGTP (5 mM each) were obtained from Thermo Scientific (Hemel Hempstead, UK). Stored at – 20 ˚C and diluted as required.
Dithiothreitol (DTT)	1 M DTT was prepared by dissolving 3.09 g of DTT in 20 ml of water, sterilised by filtration (0.22 µm pore filter) and stored at –20 ºC in 1 ml aliquots.
Enrofloxacin	50 mg of Enrofloxacin powder (Sigma-Aldrich, Dorset, UK) was added to 5 ml of 1 M potassium hydroxide (KOH) (Sigma-Aldrich, Dorset, UK) and balanced with equal 1 M HCl (Sigma-Aldrich, Dorset, UK). Enrofloxacin and balancing solution were then sterilized by filtration (0.22 µm pore filter) and stored at 4 ºC in 1 ml aliquots.
Ethyleneglycol tetraacetic acid (EGTA) buffer	100 mM EGTA (in 1 M NaOH) 0.380 g of the EGTA (Sigma-Aldrich, Dorset, UK) was dissolved in 100 ml of 1 M NaOH (BDH, Dorset, UK).
Ethidium bromide (Etbr)	Ethidium bromide solution was provided by the supplier GibcoBRL as a 10 mg/ml solution in ethanol.
Foetal calf serum (FCS) 10% (v/v)	10% (v/v) FCS (Sigma-Aldrich, Dorset, UK) was heated activated at 56 °C for 30 minutes (min) in a water bath and stored at -20 °C in 10 ml aliquots.
Glycerol	10 ml of Glycerol (BDH, Dorset, UK) was sterilized by autoclaving and replaced every month with freshly autoclaved glycerol.
Isobutanol	Isobutanol (2-Propanol) was obtained from Biorad, Hemel Hempstead, UK.
Magnesium Chloride; $MgCl_2$ (5 mM)	0.203 g of $MgCl_2$ (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml of 1X PBS to get a 100 mM solution. 1 ml of $MgCl_2$ solution was added to 9 ml of 1X PBS for a concentration of 10 mM $MgCl_2$. Finally 5 ml of 10 mM $MgCl_2$ was added to 95 ml 1X PBS for final concentration of 5 mM.
Marvel 5% (w/v)	5% (w/v) Marvel: 5 g Marvel (Chivers, Dublin, ROI) in 100 ml PBST.
PageBlue Protein staining solution	PageBlue Protein staining solution purchased from Thermo Scientific, Hemel Hempstead, UK.

Phosphate buffered saline with Tween® 20 (PBST)	0.05% (v/v) PBST: 100 ml 10X PBS 900 ml ddH2O 500 uL Tween® 20 (BDH, Dorset, UK)
Protein standard molecular-weight marker	SigmaMarker, wide range 6.5-200 kDa was obtained from Sigma-Aldrich, Dorset, UK.
Rifampicin	50 mg of Rifampicin powder (Sigma-Aldrich, Dorset, UK) was dissolved in 10 ml of 1 M methanol (Sigma-Aldrich, Dorset, UK). Rifampicin was then sterilized by filtration (0.22 μm pore filter) and stored at -20 ^{o}C in 1 ml aliquots.
Sample buffer	3 ml 10% (w/v) SDS, 2 ml 0.5 M Tris-Cl (pH 6.8), 2-4 mg of Bromophenol Blue, 2 ml glycerol and 50 mM DTT, ddH_2O was added to a total volume of 10 ml.
SDS 10% stock solution	SDS stock solution (10% w/v, electrophoresis grade)- 10 g of SDS (sodium dodecyl sulphate) was dissolved in 80 ml of ddH_2O, and then ddH_2O added to 100 ml volume.
Stain solution	0.1% (w/v) Coomassie Brilliant Blue, 40% (v/v) methanol, 10% (v/v) glacial acetic acid was added into 1 L ddH_2O.
Stopping solution	25 ml HCl (Sigma-Aldrich, Dorset, UK) was added to 475 ml ddH20.
TAE (1X) electrophoresis buffer	100 ml of TAE (40X) (molecular grade) (Sigma-Aldrich, Dorset, UK) was added to 3900 ml of ddH_2O to give a working solution of 1X TAE.
TEMED	N,N,N′,N′-Tetramethylethylenediamine (TEMED) (electrophoresis grade) (Sigma-Aldrich, Dorset, UK)
Transfer buffer	3.03g Trizma Base (Sigma-Aldrich, Dorset, UK) 14.4 g Glycine (Sigma-Aldrich, Dorset, UK) 200 ml Methanol (analytical grade) (Thermo Scientific, Hemel Hempstead, UK). When all above reagents had been added, the solution was then made up to 1 L with ddH2O.
Trichloroacetic acid (TCA) 5% (w/v)	25g of TCA powder (Sigma-Aldrich, Dorset, UK) was added to 2.25 ml of ddh2O to obtain a 10% (w/v) stock solution. This was then added to an equal volume of sample which gives the working concentration of 5% TCA (w/v).
Tris-Cl (1.0 M, pH 6.8) and (1.5 M, pH 8.8)	To prepare a 1 M solution, 121.1 g of Tris base was dissolved in 800 ml of H_2O. The pH was adjusted to the desired value by adding concentrated HCl. The solution was allowed to cool to room temperature before making final adjustments to the pH. The volume of the solution was adjusted to 1 L with H_2O then dispensed into aliquots and sterilized by autoclaving.

Tris- glycine electrophoresis running buffer	A 5x stock solution was prepared in 1 L of ddH_2O: 15.1 g Trizma Base (Sigma-Aldrich, Dorset, UK) 94 g Glycine (electrophoresis grade) (Sigma-Aldrich, Dorset, UK) 50 ml of 10% (w/v) SDS (electrophoresis grade) (Sigma-Aldrich, Dorset, UK). The 1× working solution was 25 mM Tris-Cl/250 mM glycine/0.1% (w/v) SDS.

2.2 Collection and transportation of clinical samples

2.2.1 Collection of BDD and CODD lesion samples and ruminant healthy foot tissues

The surface of the BDD or CODD lesion (or healthy foot) being collected was firstly cleaned by brushing and washing with 1X PBS, pH 7.4, (see Table 2.1). Tissue samples were then obtained using a 3 mm punch biopsy (William H Neeshams & Associates Ltd, Derbyshire, UK) taken from the centre of the lesion and washed in sterile 1X PBS. If these were samples from live animals, this was done under local anaesthesia (Lignavet Injection, C-Vet Ltd, Lancashire, UK) administered by the attending veterinary surgeon. Tissue samples from the abattoir or fallen stock centre (FSC) were collected using the same technique but no local anaesthesia was administered. Tissue samples were then divided using a scalpel via a cross-sectional cut to gain two representative halves of the sample. One half of the sample was then transferred into a 1.5 ml Eppendorf (Eppendorf, Stevenage, UK) of transport medium and placed on ice for subsequent *Treponema* culture. Transport medium consisted of oral treponeme enrichment broth (OTEB; Anaerobe Systems, Morgan Hill, CA, USA) and contained the antibiotics rifampicin (5 μg/ml) and enrofloxacin (5 μg/ml) (both Sigma-Aldrich, Dorset, UK; please see Table 2.1 for stock solutions). The remaining half of the tissue from lesions, for PCR analysis, was placed in a sealed sterilin container and also transported on ice and then stored at −20 °C. Samples for isolation were inoculated immediately.

2.2.2 Rectal tissue

The recto-anal junction was removed from the animal (sheep/beef cattle) using appropriate instruments e.g. clean scalpel blades. An approximately 3 cm^2 piece of the recto-anal junction was then removed from this using scalpel blades, inclusive of the intestinal mucosa. The tissue sample was then washed with 1X PBS (pH 7.4). Using 3-4 mm punch biopsy, a biopsy from the piece of rectal tissue was then taken and the biopsy removed with sterile tweezers. This rectal tissue sample was then halved and transported as per BDD and CODD samples ready for bacterial culture and PCR analysis (Method 2.2.1).

2.2.3 Gingival tissue

The gingival tissue was removed from the mouth of the animal (sheep/beef cattle) using appropriate instruments e.g. clean scalpel blades. An approximately 1-2 cm squared piece of tissue was removed where the gum meets the first or second molar. The tissue sample was then washed briefly to remove blood with sterile 1X PBS. This gingival tissue sample was then halved and transported as per BDD and CODD samples ready for bacterial culture and PCR analysis (Method 2.2.1).

2.2.4 Faecal samples

Fresh faeces samples from animals were collected either rectally, using gloved hands, or by collecting the top portion of fresh faeces using a sterile inoculating loop or small sterile scoop. In either case approximately 10 g of faeces was collected and placed into a sterilin container. A small portion, approximately 1 g, of faeces was then placed into a 1.5 ml eppendorf of transport medium and placed on ice for subsequent *Treponema* culture (Method 2.1.1). The remaining faeces in the sterilin container was also transported on ice and then stored at −20 °C.

2.2.5 Swab samples

In some cases BDD and CODD lesions (and other surfaces) were sampled using a plain sterile cotton swabs with re-attachable caps to avoid

contamination after sampling (Copan Italia, BS, Italy). This was done by passing the swab over the centre of the lesion approximately 3-4 times to expose the entire swab surface to the lesion. The cotton portion of the swab (the part exposed to the lesion) was then halved and half placed into a 1.5 ml microcentrifuge tube of transport medium and half into a sealed sterilin container. Samples were then transported as per BDD and CODD samples ready for bacterial culture and PCR analysis (Method 2.2.1).

2.3 Bacterial culture

2.3.1 Inoculation into liquid and solid media

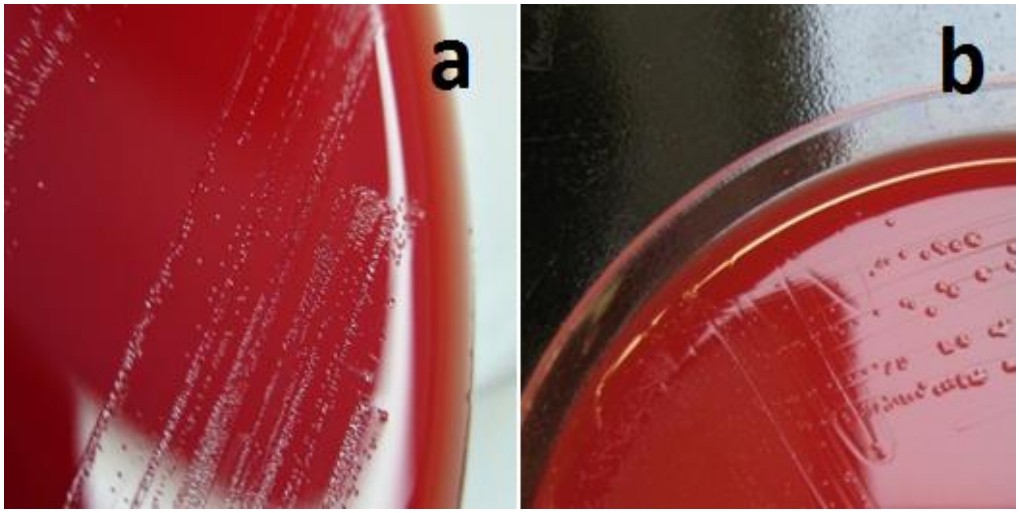
A standard culture technique was used in all bacterial isolations which was designed specifically for the isolation of treponemes (Evans *et al.* 2008).

A piece of tissue/swab (1-1.5 mm) is transferred from the transport medium into an anaerobic cabinet (85% N_2, 10% H_2 and 5% CO_2, 36 °C) (Whitley A35 anaerobic workstation, Don Whitley, Bradford, UK). Each was placed into a sterile petri-dish and cut into approximately 6-8 pieces using scalpel blades. These were then inoculated into a tube of OTEB with 10% foetal calf serum (FCS) (Sigma-Aldrich, Dorset, UK) and the antibiotics rifampicin (5 μg/ml) and enrofloxacin (5 μg/ml) (Evans *et al.* 2008). Tubes were checked for treponeme growth every 1-2 days. This was carried out by removing a small portion of culture (80 μl) and viewing the sample under phase-contrast microscopy. Bacterial cells in liquid media could be identified as spirochaetal on the basis of their spiral morphology demonstrating high motility, showing both rotational and translational movement as well as jerky flexing movements. Additionally the spirochaete cells were typically found sedimenting towards the bottom of the tube which provided an indicator for treponeme growth (Figure 2.1).

After 2-5 days, or when good growth was observed as above, bacteria were sub-cultured on fastidious anaerobe agar (FAA) plates (LabM, Bury, UK) with 5% defibrinated sheep blood (TCS Biosciences, Buckingham, UK) 10% FCS and antibiotics as above. This was done by adding 1-2 drops of the bacterial culture onto the plate using a 150 mm plugged disposable glass pasteur pipette (Volac, Essex, UK) and spreading using an inoculating loop. Single colonies, identified after 1-2 weeks, appeared as translucent, circular, convex single colonies ~0.5-2.0 mm in diameter (Evans *et al.* 2009b) (Figure 2.2). Single colonies were inoculated into growth media (OTEB and FCS) and checked for pure culture by phase contrast microscopy.

Figure 2.1: Sediment formation at the base of an OTEB tube typical of spirochaete growth in culture.

Figure 2.2: a) and b) show translucent, circular, convex colonies typical of treponeme colonies on blood agar plates.



2.3.2 Storage of treponeme cultures

For general storage cultures were stored at -80 °C after addition of 10% (v/v) glycerol (Sigma-Aldrich, Dorset, UK).

2.4 DNA extraction

2.4.1 Treponeme cultures

DNA was extracted from the treponeme cultures using Chelex-100 resin (BioRad, Hemel Hempstead, UK). The culture is centrifuged at room temperature (23 °C), and the pellet suspended in 250 µl of 5% (w/v) Chelex-100 resin. This suspension is then boiled for 10 min, followed by centrifugation at 13,000 *g* at 23 °C, for 10 min; then supernatant removed and stored at –20 °C until used.

2.4.2 Tissues and swabs

For PCR analysis, all animal tissues and swab samples were thawed and DNA extracted using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK), according to the manufacturer's instructions, and genomic DNA stored at -20°C.

2.4.3 Faeces

For PCR analysis, all faeces were thawed and DNA extracted using a DNeasy stool kit (Qiagen, Manchester, UK), according to the manufacturer's instructions, and genomic DNA stored at -20°C.

2.5 Polymerase Chain Reaction (PCR) assays

2.5.1 Primers

All primer sequences used are listed in Table 2.2. All primers were synthesised by and purchased from Eurofins MWG, Ebersberg, Germany.

Table 2.2: Primers used to detect DD specific treponeme phylogroups, all treponeme species, D. nodosus and F. necrophorum.

Primer	**Primer sequence**	**Predicted band size (bp)**	**Gene targeted**	**Region of gene targeted (positions)[a]**	**Source**
Universal	16S F (5'-AGAGTTTGATCCTGG-3′)			7-26	Rurangirwa *et al.* 1999
	16S R (5′-TACCTTGTTACGACTT-3′)	1,526	16S rRNA gene	1491-1506	
Group 1 (*T. medium*- like)	TmF (5′-GAATGCTCATCTGATGACGGTAATCGACG-3′)			472-500	Evans *et al.* 2009b
	TmR (5′-CCGGCCTTATCTAAGACCTTCTACTAG-3′)	475	16S rRNA gene	1001-1029	

Group 2 (*T. phagedenis*-like)	TbF (5′-GAAATACTCAAGCTTAACTTGAGAATTGC-3′)			612-640	Evans *et al.* 2009b
	TbR (5′-CTACGCTACCATATCTCTATAATATTGC-3′)	400	16S rRNA gene	1006-1029	
Group 3 (*T. pedis)*	TpF (5′-GGAGATGAGGGAATGCGTCTTCGATG-3′)			459-484	Evans *et al.* 2009b
	TpR (5′-CAAGAGTCGTATTGCTACGCTGATATATC-3′)	475	16S rRNA gene	1017-1045	
Treponema sp.	TPF (5′-AARCATGCAAGTCGARCGGCAAG-3′)			49-71	Moore *et al.* 2005
	TPR_1 (5′-TCCATTGCGGAATATTCTTA-3′)	335	16S rRNA gene	365-384	
D. nodosus	DnF: (5'-TGAAGAATGAAAGCGGGGGC -3')			179-198	Sullivan *et al.* 2015b
	DnR: (5'-CTAATCCTGTTTGCTACCCACG-3')	583	16S rRNA gene	762-783	
F. necrophorum	lktA-up (5'-ACAATCGGAGTAGTAGGTTC-3')			6332-6350	Bennett *et al.* 2009
	lktA-dn (5'-ATTTGGTAACTGCCACTGC-3')	402	lktA gene	6715-6732	

[a] Locations relative to those for *Escherichia coli* 16S rRNA gene sequence (GenBank accession number: M25588) (Ehresmann *et al.* 1975) except for *F. necrophorum*) which is respective to *F. necrophorum* strain A25 lktA gene sequence (Narayanan *et al.* 2001).

2.5.2 Universal bacterial 16S rRNA gene PCR

A universal bacterial primer pair encompassing the majority of the 16S rRNA gene was used (Rurangirwa *et al.* 1999) (Table 2.2). PCR mixtures used *Taq* polymerase (Qiagen, Manchester, UK) according to the manufacturers' instructions, with 1 µl of the DNA template and per 25 µl

reaction mixture volume and 20 mM of stock solutions of dATP, dTTP, dCTP, and dGTP (5 mM each). PCR assay conditions are as listed: incubation at 95 °C for 5 min, 25 cycles of 94 °C for 1 min, 55 °C for 3 min, and 72 °C for 3 min, with a final extension step at 72 °C for 7 min (Biometra thermocycler, Glasgow UK). If this PCR was used on DNA from treponeme cultures to subsequently sequence the 16S rRNA gene, then 40 cycles rather than 25 (94 °C for 1 min, 55 °C for 3 min, and 72 °C for 3 min) was used.

2.5.3 Treponeme phylogroup specific 16S rRNA gene PCR assays

The initial PCR step used for these assays was the universal bacterial 16S rRNA PCR assay (Method 2.5.2; 25 cycles). The nested treponeme phylogroup specific PCR step used primers encompassing smaller (300 to 500bp) regions within the 16S rRNA gene. These primers were previously developed using a 16S rRNA gene CLUSTALW alignment of a relevant BDD treponeme strain set to identify unique nucleotide regions shared by each of the three culturable DD treponeme phylogroups (Evans *et al.* 2008). The three treponeme phylogroup specific primer sets (Table 2.2) targeted each of the three phylogroups; *T. medium*- like, *T. phagedenis*- like and *T. pedis*. 25 µl reaction mixes were used as described (Method 2.5.1) with 1 µl PCR product template from the initial reaction. Temperature cycling entailed 95 °C for 5 min followed by 40 cycles of 95 °C for 1 min; annealing for either 2 min at 68 °C for group 1 primers, 1 min at 64 °C for group 2 primers, or 30 sec at 68 °C for group 3 primers; an extension step at 72 °C for 2 min; and then a final elongation step at 72 °C for 10 min. To ensure validity in each assay, water was used as a negative control, and positive controls included genomic DNA from each of the three treponeme groups.

2.5.4 Treponeme genus specific 16S rRNA gene PCR assay

The *Treponema* genus PCR assay detects all *Treponema* species, both pathogenic and commensal and was developed and implemented as described previously (Moore *et al.* 2005).

Taq DNA polymerase Master Mix (Qiagen, Manchester, UK) was used containing 1.5 mM $MgCl_2$, 8.2 µl double distilled (deionized) water (ddH_2O), 0.4 µl each primer (0.1 mM stock solutions) and 1 µl of template DNA. PCR assay conditions are as listed: 34 cycles of 95 °C (15 sec), 53 °C (30 sec) and 72 °C (30 sec per 500 bp of expected product) followed by 72 °C for 5 min.

2.5.5 *Dichelobacter nodosus* specific 16S rRNA gene PCR assay

A species-specific *D. nodosus* PCR assay was developed. Initial attempts to use a previous developed PCR (La Fontaine *et al.* 1993), failed to produce control PCR products and on using recent primer design programs these primers were identified as having poorly matching characteristics. Instead, species-specific *D. nodosus* primers (Table 2.2) were designed based on available 16S rRNA gene GenBank sequences. Representatives of *D. nodosus,* along with their nearest relatives (as identified using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*1990) on the NCBI website (NCBI 2013a) were aligned to identify unique primer regions, using ClustalW (Thompson *et al.* 1994) within Molecular Evolutionary Genetics Analysis 2 (MEGA2) (Kumar *et al.* 2001). The PCR assay primers were designed to amplify a 586bp region of the *D. nodosus* 16S rRNA gene with primer pairs matched for annealing temperatures and guanine-cytosine content using the oligonucleotide properties calculator, "OligoCalc" (Kibbe 2007).

PCR mixtures used *Taq* polymerase according to the manufacturer's instructions, with 1 µl of the DNA template and 1.5 mM $MgCl_2$ (Qiagen, Manchester, UK), per 25 µl reaction mixture volume. To ensure validity, water and the genomic DNA of the two closest relatives to *D. nododus* (based on 16s rRNA gene sequence similarity) were used as negative controls. These were *Suttonella indologenes* (DSM8309) (Genbank accession: AJ247267) and *Cardiobacterium hominis* (DSM8339) (Genbank accession: AY360343). The genomic DNA of *D. nodosus* (DSM23057) was used as a positive control. The genomic DNA of *Suttonella indologenes, Cardiobacterium hominis and D. nodosus* were purchased from DSMZ, Braunschweig, Germany.

PCR conditions were as follows: incubation at 95 °C for 3 min, 35 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 2 min, with a final extension step at 72 °C for 10 min. These PCR conditions were previously optimised using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). To further ensure validity of the PCR assay, a subset of PCR products were sequenced to ensure positive PCR bands were produced by the presence of *D. nodosus*.

2.5.6 Fusobacterium necrophorum specific lktA PCR assay

A species-specific *F. necrophorum* PCR assay was used as described originally (Bennett *et al.* 2009). The primers used in this assay (Table 2.2) target the leukotoxin (*lktA*) gene which appears to be unique to *F. necrophorum*, not being present in other *Fusobacterium* species (Oelke *et al.* 2005).

To ensure validity, water and the genomic DNA of *Fusobacterium varium*, a closely related species of *Fusobacterium* isolated by our laboratory and subsequently gene sequenced, were used as negative controls. The genomic DNA of *F. necrophorum* subsp. *necrophorum* (DSM21784) (DSMZ, Braunschweig, Germany) was used as a positive control.

The PCR thermal profile consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 59 °C for 30 sec and 72 °C for 30 sec. A final extension of 5 min at 72 °C was performed.

To ensure validity of the PCR assay, a subset of PCR products were sequenced to ensure positive PCR bands were produced by the presence of *F. necrophorum*.

2.5.7 Agarose Gel Electrophoresis

PCR assay results were visualized by electrophoresis through 1.0% (w/v) Agarose (Biorad, Hemel Hempstead, UK), in a Geneflow electrophoresis tank (GeneFlow Ltd, Staffordshire, UK) with TAE (1X) electrophoresis buffer.

Gels were stained with 0.5 mg/ml ethidium bromide (GibcoBRL, Hemel Hempstead, UK). For visual tracking of DNA migration during electrophoresis 6X Orange DNA Loading Dye (Thermo Scientific, Hemel Hempstead, UK) was used to prepare samples and two ladders. The ladders added to the gel, were a 100bp and 1kb (Thermo Scientific, Hemel Hempstead, UK). Biorad Powerpac 300 (Biorad, Hemel Hempstead, UK) was used to supply the electric current and gels were run at 110 volts (V) for 40 min. Gels were then visualised using a UV-transilluminator and images recorded using Geldoc gel documentation instrument (Bio-Rad, Hemel Hempstead, UK).

2.6 Gene sequencing and assembly

2.6.1 Purification of PCR products

PCR products for gene sequencing were gene cleaned using QIAquick PCR Purification Kit (Qiagen, Manchester, UK) according to the manufacturer's protocol.

2.6.2 Gene sequencing and assembly

Amplified PCR products are sequenced commercially (Cogenics Inc, Surrey, UK) and sequences were assembled into a double stranded consensus sequence using Chromas Pro 1.41 (Technelysium Pty Ltd). Sanger based DNA sequencers generate a four-colour chromatogram which depicts the results of the sequencing run, and the program's interpretation of the data. When disagreements between two sequences in an assembly are found, the original sequencing chromatograms are referred to, to see whether the error is genuine, or a base calling problem. After curation of sequences they were then exported as a '.fasta' file. A nucleotide BLAST against the NCBI nucleotide database was used to confirm identity of the gene sequence.

2.7 Phylogenetic analysis of bacterial 16S rRNA gene sequences

To understand the relationship of isolated spirochaetes with other treponemes, phylogenetic trees were produced from the aligned and trimmed near-entire 16S rRNA gene sequences of the isolates produced together with relevant micro-organisms available in GenBank and identified using BLAST (Altschul *et al.* 1990). Consensus sequences were aligned by ClustalW (Thompson *et al.* 1994) in Mega 5.2 (Kumar *et al.* 2001). For tree analysis, the most appropriate evolution model was predicted using "model test" as implemented in the Topali programme (Milne *et al.* 2009). The final model for nucleotide substitutions chosen by the model test (dependant on the 16S rRNA gene sequences being analysed in the corresponding study), and used to infer a bootstrapped maximum likelihood tree (bootstrapping was performed 10,000 times).

2.8 Statistical analyses

2.8.1 Chi-square test for significance

The chi-square test was used to determine whether there was a significant difference between expected values and observed values in one or more categories.

Pearson's chi-square is denoted as X^2 and the formula used is given as:

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$ (Pearson 1900)

Whereby:

χ^2 = Pearson's test statistic

O_i = observed value

E_i = expected value

n = the number of cells in the table.

Yates correction was used when performing the chi squared tests (Yates 1934). The effect of Yates' correction is to prevent overestimation of statistical significance for small data.

The following is Yates' corrected version of Pearson's chi-square equation:

$$\chi^2_{\text{Yates}} = \sum_{i=1}^{N} \frac{(|O_i - E_i| - 0.5)^2}{E_i}$$

This was carried out using GraphPad InStat Software, Version 3.10 (GraphPad Software, USA). In all analyses, an associated probability (P-value) of < 0.05 was considered significant.

2.8.2 Fisher's exact test for significance

Fisher's exact test (Fisher 1922) is used to determine whether there was a significant association between categories, the same as a chi-square test, but is specifically used when one or more group frequencies are <5. With larger frequencies, a chi-squared test can be used. The significance value provided by a chi- square test is only an approximation which is inadequate when sample sizes are small, or the data is extremely unequally distributed, resulting in low cell counts predicted (expected values). Therefore, a Fisher's exact test was used in these circumstances.

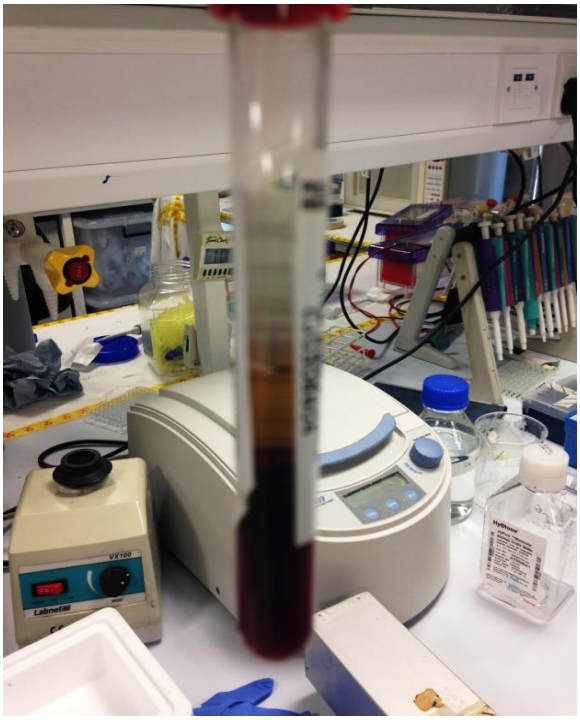
This was carried out using GraphPad InStat Software, Version 3.10 (GraphPad Software, USA). In all analyses, an associated probability (P-value) of < 0.05 was considered significant.

2.9 Serological methods

2.9.1 Blood collection

Whole blood was collected from the coccygeal vein of cows (the tail vein), by venepuncture using a BD Vacutainer® blood collection 10 ml tube with red hemogard closure (BD, Oxford, UK). Blood was transported vertically at room temperature and allowed to clot. At arrival at laboratory blood tubes were centrifuged within 24 hr of collection at 700 *g* (23 °C) for 15 min. Sera, now separated to sit at the top of the tube (Figure 2.3) was taken using a 150 mm plugged disposable glass pasteur pipette and stored in aliquots at -20 °C until analysed.

Figure 2.3: Blood tube after centrifugation. Serum layer can be seen as the top layer translucent layer.



2.9.2 Antigen preparation

Treponemes were cultured according to Method 2.3.1, using previously stored (-80 °C) treponeme cultures. When good growth was observed, usually 4 days (*T. pedis* phylogroups), 7 days (*T. phagedenis*- like) and 10 days (*T. medium*-like phylogroup), bacterial cultures were removed from the anaerobic cabinet into a laminar flow cabinet. For each antigen preparation 10 ml of cell culture was centrifuged at 10,000 *g* (23 °C) for 30 min at 20 ºC, and supernatant removed. 5 ml of 5 mM $MgCl_2$ (Sigma-Aldrich, Dorset, UK) in 1X PBS was added to cell pellet. This was then vortexed well (30 seconds (sec)) and a further 5 ml $MgCl_2$ was added and then vortexed again. The suspension was then centrifuged at 10,000 *g* (23 °C) for 30 min at 20 °C and the supernatant removed. Method repeated from the initial addition of 5 ml 5 mM $MgCl_2$ in 1X PBS. Pellet was resuspended in 1 ml 1X PBS, then sonicated (Fisherbrand FB 11021, Fisher, Loughborough, UK) on ice for 30 sec and put on ice for 20 sec. The sonication and ice step was repeated 3 more times. 20 µl of Nonidet P-40 (Sigma-Aldrich, Dorset, UK) was added and 10 µl of 100 mM EGTA (Sigma-Aldrich, Dorset, UK) (in 1 M NaOH (BDH, Dorset, UK)), to 1 ml of sonicated supernatant. Suspension was incubated at 37ºC for 4 hours (hr) with occasional mixing then frozen at -20 °C for 45 – 60 min. Suspension was thawed and centrifuged at 10,000 *g* (23 °C) for 15 min at 20 °C. Then supernatant was then dialysed against 1 L 1X PBS for 72 hr at 4 °C and 1X PBS replaced every 8 hr or after overnight period. The dialysis tubing used had a 12-14 kDa molecular weight cut off, 6.3 mm in thickness and ~30 cm tubing used (Medicell, London, UK). Dialysis tubing was soaked for 2 hr before use. Prepared antigens were then removed from dialysis tubing and stored in 1.5 µl aliquots at -20 °C.

2.9.3 Quantification of protein concentration in antigen preparations

Protein concentration of antigen preparations was quantified so to allow the correct concentration of protein to be used in Enzyme-linked immunosorbent assays (ELISA) assays. This method was carried out using a Qubit Protein

Assay Kit (Life Technologies, Paisley, UK) according to manufactures instructions using a Qubit 2.0 Fluorometer (Life Technologies, Paisley, UK). Briefly, three assay tubes for standards were set up and one tube for each antigen preparation sample. Qubit Working Solution was prepared by diluting the Qubit Protein Reagent 1:200 in Qubit Protein Buffer. 200 µl of Working Solution for each standard and sample was prepared.

Assay tubes were prepared (using 0.5 ml PCR tubes) according to the manufacturer's instructions and were vortexed for 2-3 sec and incubated at room temperature for 15 min prior to subjecting the sample to protein quantification in the Qubit 2.0 Fluorometer.

2.9.4 Trichloroacetic acid (TCA) protein precipitation

Protein fractions were TCA precipitated by using a final concentration of 5% (w/v) TCA. The solutions were mixed well, inverted, and placed on ice for 20 min and then centrifuged at 11,300 *g* (23 °C) for 15 min. The supernatant was discarded and 1 ml of ice-cold acetone added. The Eppendorf tube was inverted and centrifuged at 11,300 *g* (23 °C) for 1 min. The acetone was removed and the pellet was dried under vacuum for 12 min. Precipitates could then be solubilised in 50 µl of sample buffer and analysed by SDS-PAGE.

2.9.5 Enzyme-linked immunosorbent assays (ELISAs)

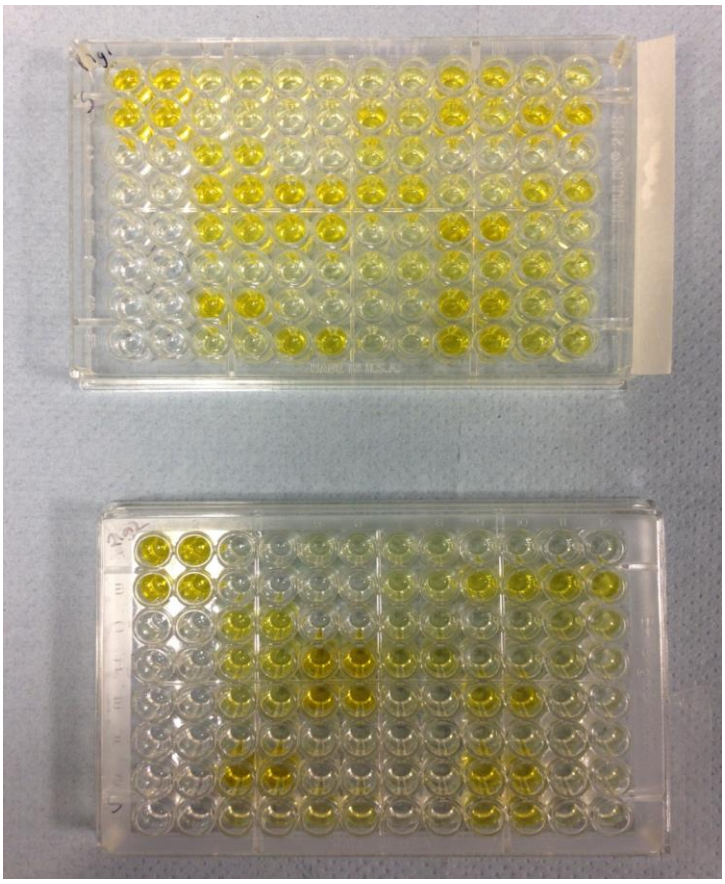
Non-activated, 96-well microtitre ELISA plates (Microplate Immunlon 2HB 96 well 128 mm x 86 mm (0.33 ml well volume 2.37 cm^2 per well) (ThermoFisher, Horsham, UK) were coated with 5 µg/ml of prepared antigen in PBS (1X), pH 7.2. Plates were then incubated for 1 h at 37 °C and overnight at 4 °C. Unbound antigen was removed by washing three times with PBST. Sera samples were diluted to 1/100 in PBST (0.05% (v/v)) (determined optimum dilution) and 100 µl pipetted into ELISA plate wells in duplicate. All plates included positive and negative control sera. Additionally, substrate blank wells (no substrate added) and conjugate blank wells (no conjugate added) were also used on all plates. Table 2.3 shows the ELISA plate layout used for all ELISA assays.

Table 2.3: ELISA plate layout. Each serum is analysed in duplicate shown by the duplication of each number in its parallel column. Abbreviations: CB, conjugate blank; SB, substrate blank. (+) indicates positive serum control, (-) indicates negative serum control.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	+	+	1	1	9	9	17	17	25	25	33	33
B	+	+	2	2	10	10	18	18	26	26	34	34
C	--	--	3	3	11	11	19	19	27	27	35	35
D	--	--	4	4	12	12	20	20	28	28	36	36
E	CB	CB	5	5	13	13	21	21	29	29	37	37
F	CB	CB	6	6	14	14	22	22	30	30	38	38
G	SB	SB	7	7	15	15	23	23	31	31	39	39
H	SB	SB	8	8	16	16	24	24	32	32	40	40

After incubation for 1 h at 37 °C, the plates were washed again. 100 µl of either Mouse Anti Bovine Immunoblogulin class G subclass 1 (IgG_1), clone IL-A60 monoclonal antibody or Mouse Anti Bovine Immunoblogulin class G subclass 2 (IgG_2), clone IL-A2 monoclonal antibody (Biorad, Hemel Hempstead, UK) was added to each well at a 1/1000 dilution in PBST (determined optimum dilution). Following this plates were washed with PBST as above, and bound antibodies were detected using 100 µl per well of Anti Mouse polyvalent immunoglobulins (peroxidise conjugate) (Sigma-Aldrich, Dorset, UK) at a 1/10,000 dilution with PBST. Conjugate was not added to the conjugate blank wells, instead 100 µl of PBST was added to these wells. The antibody-conjugated reaction was visualized with 3, 3 ’ , 5,5 ’ Tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, Dorset, UK). Substrate was not added to the substrate blank wells, instead 100 µl of PBST was added to these wells. After 15 min a stopping solution was added to stop the reaction. This produces a colour reaction (Figure 2.4).

Figure 2.4: Two ELISA plates after the colour reaction has occurred when the TMB liquid substrate and stopping solution has been added to the ELISA plates. As can be seen from the plate negative control wells (those on the left hand two columns of the plate with the exception of the first two rows of these columns) have shown no colour reaction.



2.9.6 One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE)

Protein electrophoresis was carried out based on the original described method (Laemmli 1970). A 12% (v/v) resolving SDS-polyacrylamide gel was used, solutions used in the preparation for this are in Table 2.4. Acrylamide resolving gels were overlayed carefully with isobutanol. After polymerization was complete (30 min), overlay was poured off and top of the gel washed several times with ddH_2O to remove any unpolymerized acrylamide. 5% (v/v) SDS-

polyacrylamide stacking gels were used, with solutions used in this preparation in Table 2.5, and Teflon combs (mini protean combs 0.75 mm, Biorad, Hemel Hempstead, UK) were added to produce the wells of the gel. 15 lane combs were used for 1D SDS-PAGE viewing of proteins and one lane combs (not including the marker lane) used for Western Blots. After polymerization is complete (30 min), Teflon combs were removed carefully and wells washed immediately with ddH_2O to remove any unpolymerized acrylamide.

The gels were cast in a mini-gel system (Mini-PROTEAN electrophoresis system, Biorad, Hemel Hempstead, UK) with gel cassettes and glass plates (0.75 mm short plates and spacer plates) (Biorad, Hemel Hempstead, UK). The set gels were transferred to the electrophoresis tank and covered with the Tris-glycine electrophoresis running buffer. Protein samples were dissolved in 1X SDS gel-loading sample buffer by heating at 100 °C for 5 min using a water bath prior to loading into wells, and protein ladder according to manufacturers instructions was added.

The electrophoresis tank was run at 180V for 50 min using a Biorad Powerpac 300 (Biorad, Hemel Hempstead, UK). The gels were then removed from the plates and washed with 100 ml ddH_2O then microwaved on high power for 40 sec with gel still submerged in the water, and then was in repeated three times. Gels were then immersed in the PageBlue Protein staining solution and added to the microwave on high power for 30 sec, then left shaking on a rocking platform (ProBlot 35 delux rocking platform) (Appleton Woods, Birmingham, UK), for 15 min. Stain was then poured off and gels rinsed with ddH_2O three times, then placed back onto the rocking platform immersed in ddH_2O for another 10 min. Protein bands were now visible to assess.

Table 2.4: Solutions for preparing resolving gels for SDS-PAGE. Components are row descriptors and gel volumes are column descriptors. Solution volume is listed in ml.

12%								
	5 ml	**10 ml**	**15 ml**	**20 ml**	**25 ml**	**30 ml**	**40 ml**	**50 ml**
ddH_2O	1.600	3.300	4.900	6.600	8.200	9.900	13.200	16.500
30% (w/v) acrylamide mix	2.000	4.000	6.000	8.000	10.000	12.000	16.000	20.000
1.5 M Tris-Cl (pH 8.8)	1.300	2.500	3.800	5.000	6.300	7.500	10.000	12.500
10% (w/v) ammonium persulfate	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
10% (w/v) SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.400	0.500
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Table 2.5: Solutions for preparing 5% stacking gels for SDS-PAGE. Components are row descriptors and gel volumes are column descriptors. Solution volume is listed in ml.

5%								
	1 ml	**2 ml**	**3 ml**	**4 ml**	**5 ml**	**6 ml**	**8 ml**	**10 ml**
ddH_2O	0.680	1.400	2.100	2.700	3.400	4.100	5.50	6.800
30% (w/v) acrylamide mix	0.170	0.330	0.500	0.670	0.830	1.000	1.300	1.700
1.0 M Tris-Cl (pH 6.8)	0.130	0.250	0.380	0.500	0.630	0.750	1.000	1.250

10% (w/v) ammonium persulfate	0.010	0.020	0.030	0.040	0.050	0.060	0.080	0.100
10% (w/v) SDS	0.010	0.020	0.030	0.040	0.050	0.060	0.080	0.100
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.010

2.9.7 Western Blotting

For Western blotting, proteins were first separated using 1D SDS-PAGE as described Method 2.9.6.

Protein samples were diluted to a final working concentration of 1.5 mg/ml with 10X PBS. However, the protein marker used was Color Prestained Protein Standard, broad range 11-245 kDa (NEB, Hertfordshire, UK) according to manufacturer’ s instructions. After resolving spirochaete proteins in the SDS-PAGE gel, the electrophoretic transfer of proteins to a 0.2-µm nitrocellulose sheet (NCS) (Biorad, Hemel Hempstead, UK) was carried out as previously described (Towbin *et al.* 1979). This was done by firstly removing and disposing of the stacking part of the gel. Sponges, filter paper and cut NCS membranes were placed in transfer buffer. For the transfer components a Mini Trans-Blot Module (Biorad, Hemel Hempstead, UK) was used. The following components were arranged into a Mini Trans-Blot Module transfer cassette; black side of the transfer cassette, sponge, filter paper x 2, SDS-PAGE gel, NCS membrane, filter paper x 2, sponge, white side of the transfer cassette.

A pipette was used to roll in between layers to ensure no air bubbles were left in between the gel and the membrane. Once layered the cassette was locked and placed in the transfer holder and tank with an ice pack and a magnetic flea added and tank placed on a magnetic stirrer. The transfers were run at 100V, 240 milliamps (mA) for 1 hr and 20 min. Once transferred the membranes were removed and washed three times in PBST for 5 min on a rocking platform. Membranes were blocked overnight with an incubation in 5% (w/v)

Marvel/PBST at 4 °C on a rocking platform. Membranes were washed three times for 5 min in PBST on a rocking platform and membranes were cut into strips and each strip incubated at room temperature for 1 hr with sample sera (1 ml per strip). One strip was incubated with a negative sera and 1 strip with a positive sera for the appropriate treponeme antigens tested by ELISA. All sera were diluted 1/100 with PBST (determined optimum dilution). The strips were washed three times in PBST as above and incubated at room temperature on a rocking platform with 1 ml per strip of either Mouse Anti Bovine IgG_1, clone IL-A60 monoclonal antibody or Mouse Anti Bovine IgG_2, clone IL-A2 monoclonal antibody (Biorad, Hemel Hempstead, UK) at a 1/1000 dilution with PBST (determined optimum dilution). Membrane strips were again washed with PBST as above and then the reaction was detected with Anti Mouse polyvalent immunoglobulins (peroxidise conjugate) (Sigma-Aldrich, Dorset, UK) at a 1/10,000 dilution with PBST, 1 ml per strip, incubated for 1 hr on a rocking platform. The strips were washed three times in PBST as above and for development 1 ml of liquid substrate added per 20 strips TMB liquid substrate system for membranes (Sigma-Aldrich, Dorset, UK), for 10-15 min in darkness. After this time, the reaction was stopped with distilled water.

2.10 Ethical approval and project licencing

All experiments were conducted in accordance with United Kingdom legislation governing experimental animals under project license PPL40/3275 and were approved by the University of Liverpool ethical review process, ethics application number was: VREC137.

Chapter 3

The bacteriology of contagious ovine digital dermatitis lesions, and the presence of digital dermatitis treponemes in an unknown foot disease in dairy goats.

3.1 Introduction

Lameness in cattle and sheep has serious animal welfare and economic implications (Marshall *et al.* 1991; Enting *et al.* 1997; Hernandez *et al.* 2001; Warnick *et al.* 2001). Bovine digital dermatitis is now a worldwide problem and controlling BDD on dairy operations has proven difficult. Moreover, in the last 20 years, UK sheep have been identified with a form of DD, termed contagious ovine digital dermatitis (CODD), which is rapidly emerging as a severe infectious foot disease since being first reported in the UK in 1997 (Harwood *et al.* 1997; Davies *et al.* 1999). Now, CODD has spread into the Republic of Ireland (Sayers *et al.* 2009).

BDD in cattle manifests in several forms, but most frequently as an ulcerative lesion of the digital skin located immediately above the coronet between the heel bulbs which results in severe lameness (Cheli and Mortellaro 1974). The clinical features of CODD in sheep are slightly different, mainly because the

initial lesion site on the sheep foot is different. CODD lesions commence at the coronary band and then under run the hoof horn capsule dorsally and abaxially (Duncan *et al.* 2014). Frequently CODD presents as particularly severe lesions and subsequently the whole horn capsule can be lost (Harwood *et al.* 1997; Naylor *et al.* 1998; Wassink *et al.* 2003; Winter 2008). As a result of the severity of the lesions, affected sheep can be extremely lame, impacting their welfare (Duncan *et al.* 2011).

Previous studies have investigated the association of *Treponema* bacteria with CODD lesions (Moore *et al.* 2005; Sayers *et al.* 2009) and hypothesised CODD to have derived from BDD lesions in dairy cows (Dhawi *et al.* 2005). To date, little substantiating molecular evidence has been produced, and the possible involvement of other organisms such as *Dichelobacter nodosus* and *Fusobacterium necrophorum* has been proposed (Moore *et al.* 2005). In a previous study, *D. nodosus*, *F. necrophorum* and also treponemal bacteria were detected in a considerable proportion of CODD lesions (74%, 86% and 70% respectively) (Moore *et al.* 2005), but these listed bacteria were also detected in a substantial proportion of healthy foot tissue (31%, 46% and 38% respectively). Moreover, this study did not discriminate between *Treponema* species as their respective PCR assay detects both pathogenic and commensal treponemes, the latter of which are predominantly found in the ruminant gastrointestinal (GI) tract and faeces (Evans *et al.* 2012). Additionally, healthy bovine foot tissue samples often amplify treponemal DNA using the genus specific PCR assay but fail to produce products using DD treponeme phylogroup PCR assays (Evans *et al.* 2009b, 2012). Indeed, in a large survey of the dairy farm environment (Evans *et al.* 2012), nearly all samples were positive using the genus specific *Treponema* PCR assay, suggesting it has little diagnostic value for clarifying relevant associations with clinical disease. In BDD, there is a clear association with certain phylogroups of treponemes and these are only present in BDD lesions and are completely absent from healthy bovine tissues and most environmental and faecal samples (Evans *et al.* 2009b, 2012). Therefore, the previous CODD study (Moore *et al.* 2005) provides no

specific data on the presence/association of BDD associated *Treponema* species with CODD lesions. Similarly the remaining bacteriological data on CODD lesions comprised of a study entirely based on culture dependant methods, using only ten samples, of which only seven treponemal cultures were produced and only two pure treponeme isolates obtained (Sayers *et al.* 2009).

Thus, whilst the role of treponemes as primary causative agents in BDD appears convincing, a comprehensive bacterial molecular survey of CODD lesions is justified to determine if there is a shared spirochaetal aetiopathogenesis between BDD and CODD and to consider the role of other candidate bacteria.

Lameness is also reported in goats, although with fewer published studies. In France, Mazurek *et al.* (2007) reported that out of 108 goats, 12.5% were lame and Christodoulopoulos (2009) identified a prevalence of 24% in a herd of 170 goats in Greece. In the UK, Hill *et al.* (1997), found an average lameness prevalence of 9.1% (range 2.7- 23.4%) on four goat farms. In a study of 24 dairy goat farms in England and Wales, the mean lameness prevalence was 19.2% (inter-quartile range 7.7- 30.2%). More recently, similarly high levels of lameness (40% and 67%) were observed amongst dairy goats on two UK goat farms (Groenevelt *et al.* 2013).

Foot disorders recognised in goats are similar to those described in sheep (Winter 2011) and include; ID, footrot (Piriz Duran *et al.* 1990), heel horn erosion (Christodoulopoulos 2009), white line disease, foreign body penetrations (Mgasa and Arnbjerg 1993) and over grown feet (Hill *et al.* 1997). In the study by Groenevelt *et al.* (2013), laminitis accompanied by infection with *Treponema* bacteria was identified as the cause of lameness in two dairy goat herds. Although goats and sheep share many foot diseases, CODD is yet to be reported in goats; however, having possibly crossed into sheep from one host species, there was always the chance that this could happen again and infection of other domesticated species could occur.

The aims of this study are split into two parts. Firstly; further our understanding of CODD aetiology by surveying a large number of lesions and healthy foot tissue for the presence of the three DD treponeme phylogroups, as well as *D. nodosus* and *F. necrophorum*. Additionally, attempt to determine the range of spirochaetes present in CODD lesions from a number of farms across the UK, and successfully isolate and characterise a collection of purified spirochaete strains from CODD lesions for comparison with other relevant treponemes.

The second part of this study was to investigate the aetiology of an unknown foot disease present on a UK dairy goat farm and to compare the findings to our current knowledge of CODD. This study aimed to describe the clinical findings of a severe lameness problem in a UK dairy goat herd and to determine whether the same treponemes found in CODD lesions are also present, and therefore possibly an aetiological agent, in these goat lesions.

This study aims to uncover bacteriological information about CODD lesions and an unknown goat disease, with the hypothesis that treponemes highly associated with BDD in dairy cattle will also be present in a statistically significant proportion of the lesions tested.

3.2 Methods

3.2.1 CODD investigation

3.2.1.1 Sample collection

Surgical biopsies were collected (under a Home Office license as relevant) from 44 CODD lesions from six different farms between March 2013 and July 2014. These farms were in the following UK areas: Anglesey, Cheshire, Denbighshire, Shropshire and Conwy. Genomic DNA from a further 14 CODD lesion biopsies collected during 2009-2010 from two farms in Cheshire and Gloucestershire were also included in this investigation (see Table 3.1 for all sample origin information). All farms had between 300-1000 breeding ewes and were lowland farms except for a Conwy farm located on hill land. Sheep

breeds on farms included: Welsh Mountain, Scottish Blackface, Suffolk/Suffolk crosses, Lleyn/Lleyn crosses, Charolais crosses and Easy Care. Additional to lesional material, 56 healthy foot skin biopsies were collected. Of these, 16 biopsies were obtained from a farm in Meirionydd, from each foot of four Balwen sheep and eight were obtained from normal feet of three cross bred sheep sampled in this study with CODD lesions on their other feet. These sheep were all from the Cheshire farm and taken post-mortem after euthanasia for other reasons. The remaining 32 healthy foot tissue samples were obtained from sheep sent to slaughter that did not have any evidence of CODD or any other foot lesions. These unidentified sheep were sampled at an abattoir which received sheep from farms within Lancashire, Cheshire and South Cumbria.

Table 3.1: CODD and healthy tissue sample origin information

Tissue type	Origin of samples-Farm location	Number of samples obtained from location
CODD lesion	Anglesey	21
	Cheshire	11
	Denbighshire	1
	Shropshire	2
	Conwy Farm 1	12
	Conwy Farm 2	4
	Gloucestershire	7
Healthy foot tissue	Meirionydd	16
	Cheshire	8
	Abbatoir (services Lancashure, Cheshire and South Cumbria).	32

On all farms from which CODD biopsies were obtained from live sheep, the sheep were run through a race and identified lame animals then inspected for CODD lesions. A sheep was defined as having CODD if one or more feet had a clear lesion consistent with the clinical signs of CODD (Winter 2004a). These signs can be varied, but include: an ulcerative or granulomatous lesion at the coronary band which may extend dorsally and abaxially under the hoof wall and in severe cases lead to avulsion of the hoof capsule. The affected digit may be swollen and shortened. Sheep identified with classic CODD lesions were examined and the lesions were biopsied according to Method 2.1.1. All CODD lesions biopsied were active lesions shown by tissue appearing haemorrhagic, granulomatous and/or necrotic. Figures 3.1, 3.2 and 3.3 show typical lesions from which biopsies were collected. All CODD lesion and healthy foot tissue biopsy samples were transported and stored as described in Method 2.1.1.

3.2.1.2 Culture of spirochaetes

Treponema culture was attempted on all CODD tissue samples but was not attempted on sheep healthy foot tissue samples, primarily because of treponeme PCR data from these samples being consistently negative. The culture technique used, subsequent bacterial DNA extraction, gene cleaning and sequencing were carried out as described in Methods 2.2, 2.3.1, 2.5.1 and 2.5.2, respectively.

Figure 3.1: Shows a typical CODD lesion present on the coronary band of a sheep foot. The lesion appears granulomatous in appearance and the digit swollen. In addition to the CODD lesion, and possibly resulting from the infection, deformation of the hoof has occurred.

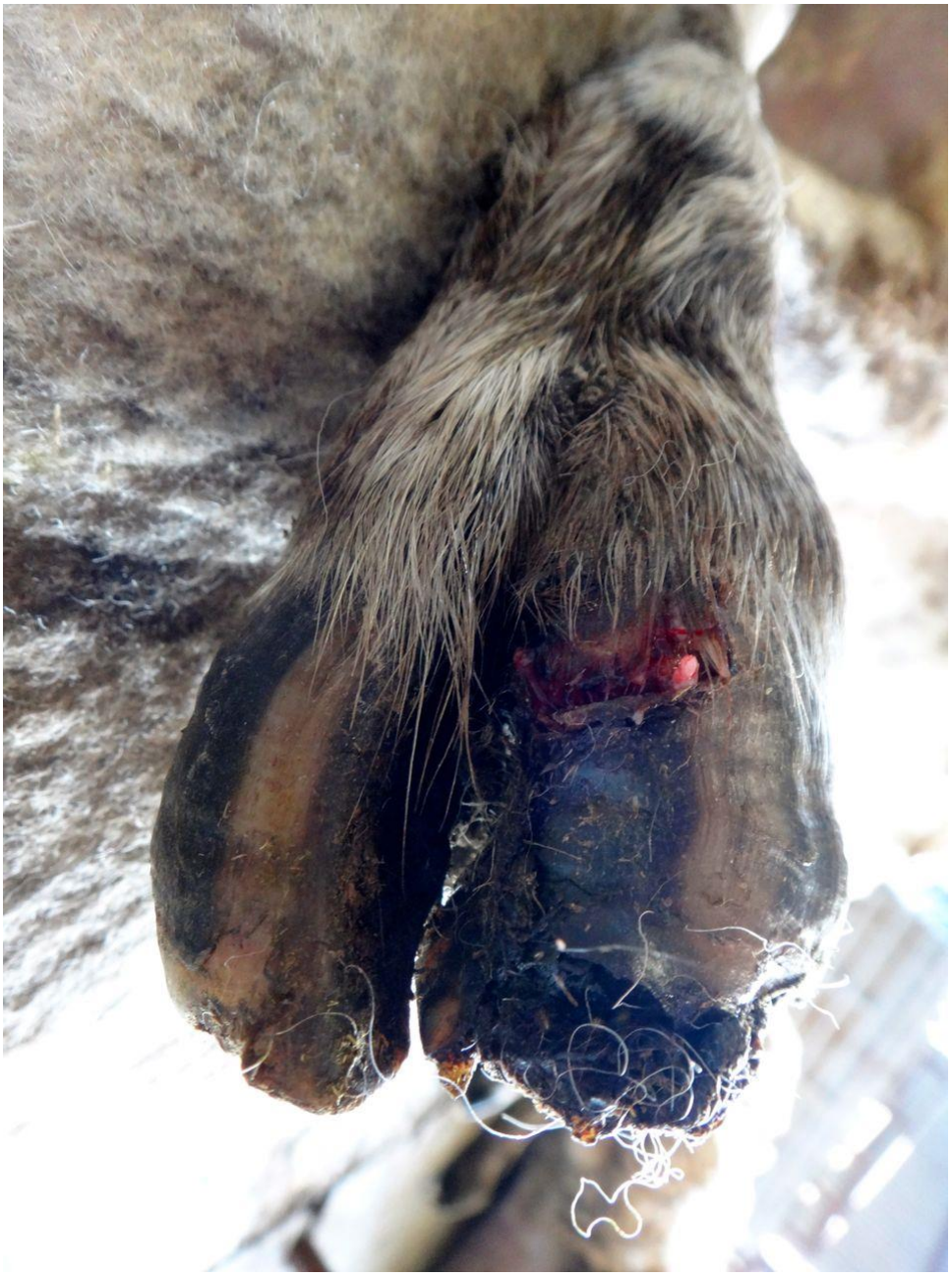


Figure 3.2: Shows a typical CODD lesion present on the coronary band of a sheep foot. The lesion appears ulcerative and the affected digit is swollen and deformation of the hoof has occurred.

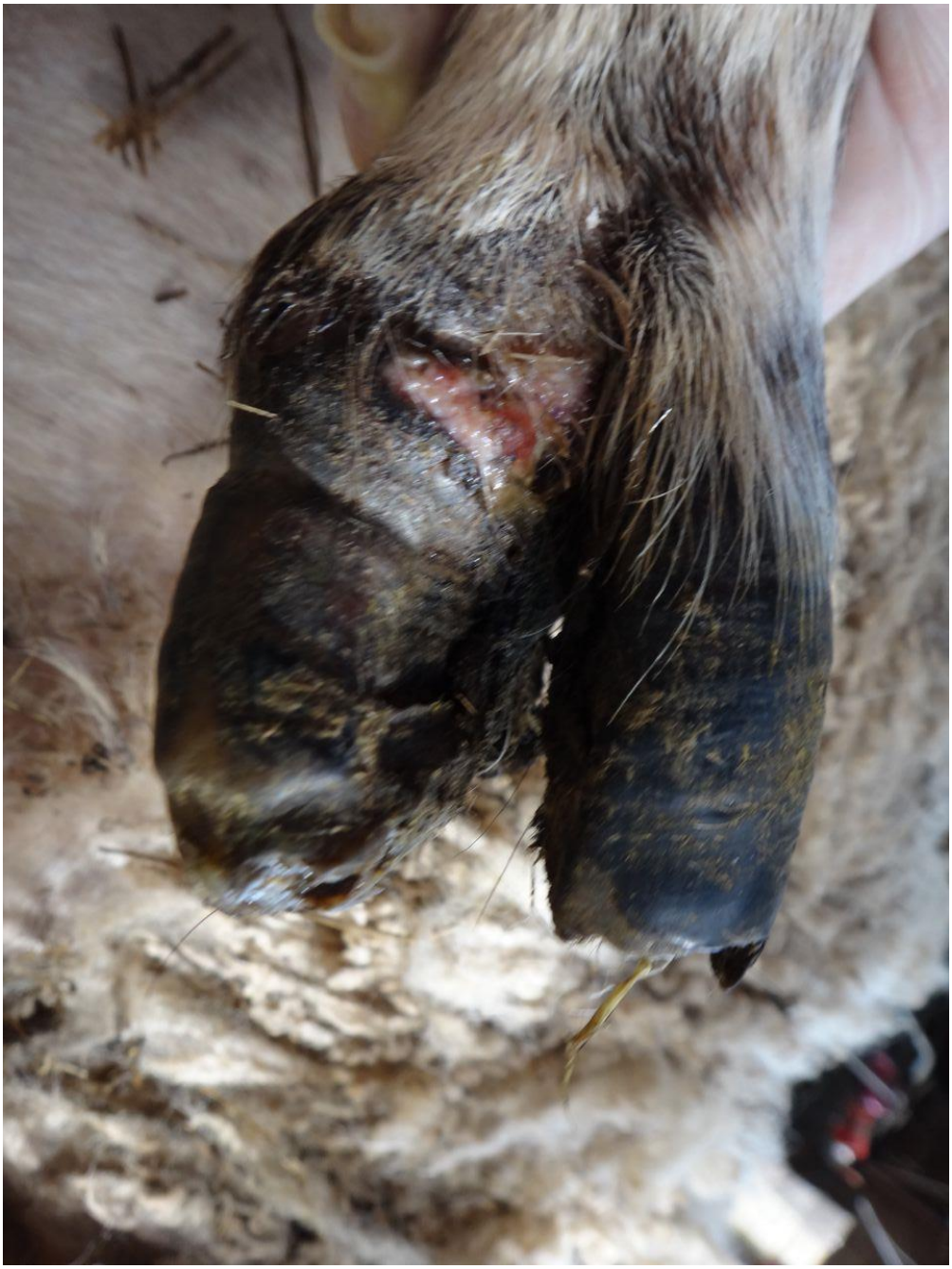
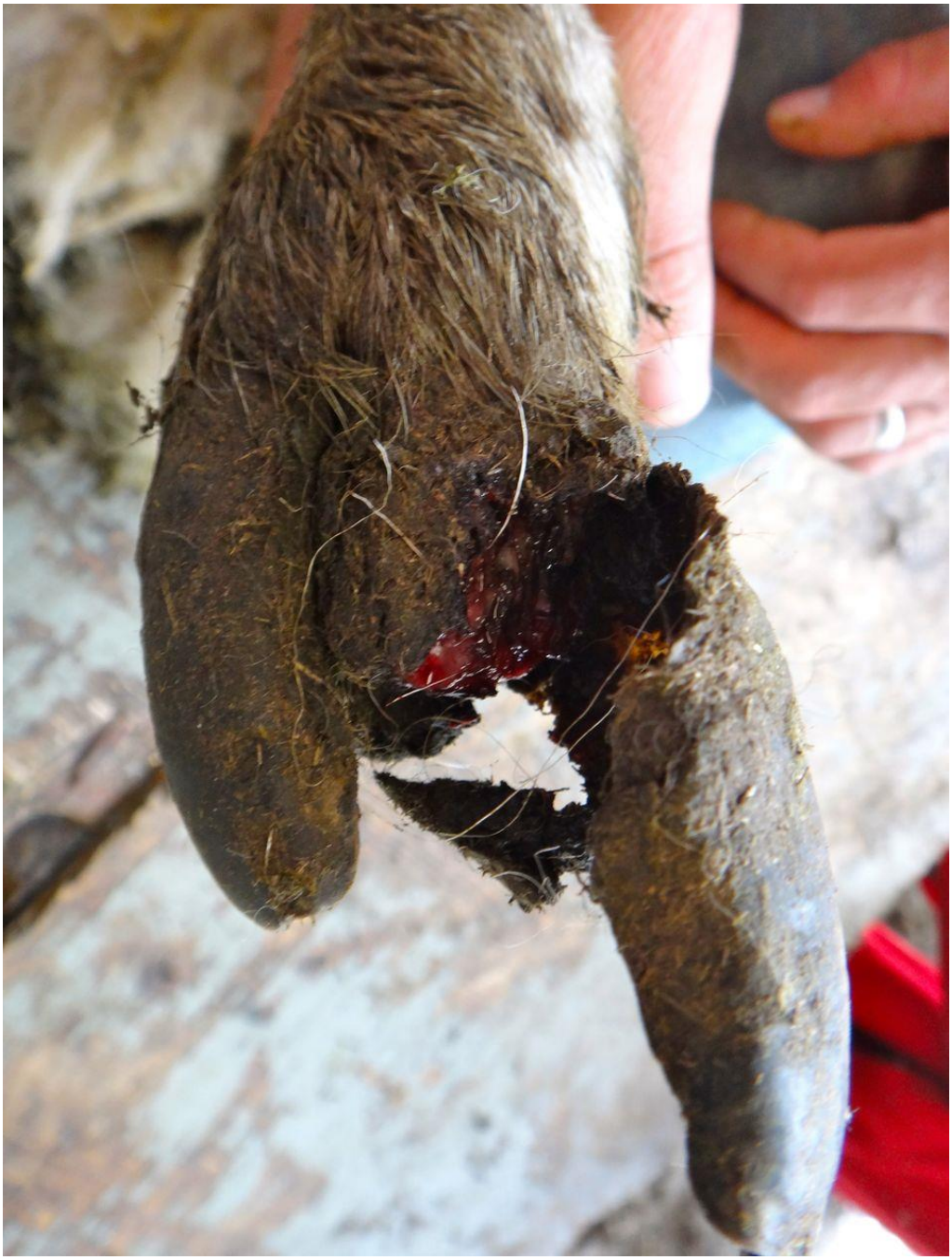


Figure 3.3: A severe CODD lesion which appears ulcerative and granulomatous and has underrun the hoof wall and lead to avulsion of the hoof capsule.



3.2.1.3 DNA extraction

All tissue samples, CODD lesions and healthy sheep foot tissues, were used for DNA extraction. DNA extraction was carried out as described in Method 2.3.2.

3.2.1.4 PCR assays

All CODD lesion samples and sheep healthy foot tissue samples were subjected to nested PCR assays specific for the three DD- associated treponeme groups, *T. medium*- like, *T. phagedenis- like*, *T. pedis* and the treponeme genus specific 16S rRNA PCR assay, as described in Methods 2.3.2 and 2.3.3, respectively. All samples were also subjected to the species-specific *D. nodosus* PCR assay and a species-specific *F. necrophorum* PCR assay (targeting the lktA gene), as described in Methods 2.3.4 and 2.3.5, respectively.

3.2.1.5 Phylogenetic analysis of spirochaete isolates from CODD lesions

To understand the relationship of the isolated spirochaetes with other treponemes, and in particular those previously isolated from dairy cattle BDD lesions and previous CODD lesions, a phylogenetic tree was produced according to Method 2.6. The most appropriate evolution model was predicted using "model test" in the Topali programme (Milne *et al.* 2009). The final model chosen for nucleotide substitutions was the TrN model (Tamura and Nei 1993). This was used to infer a bootstrapped maximum likelihood tree with bootstrapping performed 10,000 times.

3.2.1.6 Nucleotide Accession numbers

The 16S rRNA gene GenBank accession numbers determined as part of this study are KP063152 - KP063183.

3.2.1.7 Statistical Analysis

A chi-square test (with Yates correction) (Method 2.7.1) was used to identify associations between the presence of each bacteria: *T. medium*- like, *T. phagedenis*- like, *T. pedis*, *F. necrophorum*, *D. nodosus*, with presence or absence of CODD. Chi-square tests (with Yates correction) were also used to identify any co-associations between the presence of each DD- associated *Treponema* phylogroup and *D.nodosus* and *F.necrophorum* in CODD lesions. Additionally, the same statistical analysis was performed to identify any co-association between *D. nodosus* and *F. necrophorum* in both CODD and

healthy foot tissue samples. In all analyses, an associated probability (*P* value) of < 0.05 was considered significant.

3.2.2 Goat lesions with an unknown aetiology

3.2.2.1 Farm information

Following reports of a severe lameness problem of unknown aetiology, a UK goat herd was assessed by the attending veterinary surgeons (JSD and JEH) in October 2013.

The herd consisted of 1000 milking goats of Anglo Nubian and British Saanen breed. The goats were housed year round in four straw yards (250 goats per yard), which were cleaned out every six to eight weeks. Milking occurred twice daily and goats were fed a ration of grass silage and cereal based concentrate. Infection with Caprine Arthritis Encephalitis Virus had previously been diagnosed on the farm. All goats were routinely foot trimmed every three months and the herd walked through a copper sulphate footbath post milking, twice weekly. The farmer estimated that 30% of the herd was lame and his current treatment for lame goats was foot trimming and topical application of oxytetracycline spray; in some but not all lameness cases the goats were also be treated with a course of the parental antibiotic, ceftiofur (Naxcel, Zoetis, Catania, Italy). However, it appeared this was not controlling the disease effectively and a high level of lameness was apparent on the farm for several months.

The prevalence of lameness in this herd was estimated by the attending veterinary surgeon after lameness scoring the entire herd on exit from the milking parlour. Due to the rate and numbers of goats exiting the parlour, a simple lame/not lame scoring system was used (Phythian *et al.* 2013). On this basis, 65% of the herd were identified as lame. There appeared to be no observable difference in lameness prevalence between the four straw yards.

Fifteen lame goats were randomly selected for further examination of lesions causing the severe lameness.

3.2.2.2 Sample collection

Ten of the goats with foot lesions were chosen randomly from the goats examined and lesions were biopsied according to Method 2.1.1. Healthy goat foot tissues (n= 10) were used to serve as negative controls. Single rear-hoof skin biopsies were collected from each hind foot of five dairy goats from a fallen stock centre which received animals from farms within Lancashire, Cheshire and South Cumbria. All goats at this centre had no evidence of any foot lesions. All goat lesion biopsies and healthy foot tissue biopsy samples were taken, transported and stored as described in Method 2.1.1.

3.2.2.3 Culture of spirochaetes

Spirochaete culture was attempted on all goat lesion samples but was not attempted on healthy foot tissue samples. The culture technique used and subsequent DNA extraction from pure cultures was carried out as described in Methods 2.2 and 2.3.1, respectively.

3.2.2.4 Tissue DNA extraction

All goat lesion and healthy foot tissue samples were used for DNA extraction. DNA extraction was carried out as described in Method 2.3.2.

3.2.2.5 Treponeme PCR assays

All goat lesion samples and healthy foot tissue samples were subjected to nested PCR assays specific for the three DD- associated treponeme groups, *T. medium*- like, *T. phagedenis*- like, *T. pedis* and the treponeme genus specific 16S rRNA PCR assay, as described in Methods 2.4.2 and 2.4.3, respectively.

3.2.2.6 Phylogenetic analysis of spirochaete isolates from goat lesions

To understand the relationship of the isolated spirochaetes from the goat lesions with other treponemes, and in particular those previously isolated from sheep CODD lesions and BDD lesions, a phylogenetic tree was produced

according to Method 2.6. The most appropriate evolution model was predicted using "model test" as implemented in the Topali programme (Milne *et al.* 2009). The final model for nucleotide substitutions chosen was the GTR (general time reversal) model (Tavare 1986), used to infer a bootstrapped maximum likelihood tree; bootstrapping was performed 10,000 times.

3.2.2.7 Nucleotide Accession numbers

The 16S rRNA gene GenBank accession numbers determined as part of this study are KJ206528- KJ206532.

3.3 Results

3.3.1 CODD investigation

3.3.1.1 PCR assays

The results of the specific DD *Treponema* phylogroup PCR and *Treponema* genus-specific PCR assays in CODD lesions and healthy foot tissues are shown in Tables 3.2 and 3.3, respectively.

All CODD lesions (*n*= 58) were found to be positive for general *Treponema* DNA. The phylogroup specific PCR for *T. medium*- like, *T. phagedenis*- like and *T. pedis* DD spirochaetes, showed that they were individually present in 39/58 (67%), 49/58 (85%) and 41/58 (71%) of CODD lesions, respectively. All CODD lesions (100%) were positive for at least one or more of the DD-associated *Treponema* phylogroups, with 27/58 (47%) of CODD lesions positive for all three DD- associated *Treponema* phylogroups. Of the healthy foot tissues sampled (*n*= 56), 38/56 (68%) were positive for the presence of general treponemes (*Treponema* genus-specific PCR). However, all healthy foot tissues were negative for the three DD- associated *Treponema* phylogroups.

The *D. nodosus* and *F. necrophorum* specific PCR results for CODD lesions and healthy foot tissues are shown in Tables 3.2 and 3.3, respectively. *D. nodosus* was present in 34/58 (59%) of CODD lesions. In healthy tissues surveyed, *D. nodosus* was present in 22/56 (39%) of samples. *F. necrophorum* was present in 41/58 (71%) of CODD lesions and present in only 5/56 (9%), of healthy foot tissues.

Table 3.2: PCR detection of treponemes, D. nodosus and F. necrophorum in CODD lesion biopsies.

Sample	**Biopsy date (mo/yr)**	**Details (Farm location, sheep number[a])**	**Treponeme isolated[b]**	**Result**					
				Specific PCR for group[c]:			***Treponem -a* PCR**	***F. necroph -orum***	***D. nodos -us***
				1	**2**	**3**			
1	02/09	Cheshire, 51	G2S1F	+	+	+	+	+	-
2	02/09	Cheshire, 52	G2S2R	+	+	+	+	+	-
3	02/09	Cheshire, 53	G2S3R1	+	+	+	+	+	-
4	02/09	Cheshire, 54	G2S4F	+	+	+	+	+	-
5	02/09	Cheshire, 55	IF	+	-	+	+	+	-
6	08/09	Gloucestershire, 11	G1OV11	+	+	-	+	-	-
7	08/09	Gloucestershire, 14	IF	+	-	+	+	+	-
8	08/09	Gloucestershire, 17	IF	-	+	+	+	+	-
9	08/09	Gloucestershire, 18	IF	+	-	+	+	+	+
10	08/09	Gloucestershire, 20	IF	-	+	+	+	+	+
11	08/09	Gloucestershire, 21	IF	+	+	+	+	-	+
12	08/09	Gloucestershire, 22	IF	+	-	-	+	-	+
13	01/10	Cheshire, 28	IF	-	-	+	+	+	+
14	01/10	Cheshire, 29	IF	+	+	-	+	+	+
15	05/13	Anglesey, 1	G2SL1	+	+	+	+	-	+
16	06/13	Anglesey, 97	IF	-	+	-	+	-	+
17	06/13	Anglesey, 73	IF	-	+	-	+	-	+
18	06/13	Anglesey, 30	G2SL5	+	+	+	+	+	+
19	06/13	Anglesey, 63	G12F2	+	+	+	+	+	+
20	06/13	Anglesey, 229	G13F3, G23F1	+	+	+	+	+	+
21	06/13	Anglesey, 36 back left	IF	+	+	+	+	+	+
22	06/13	Anglesey, 36 back right	IF	+	+	+	+	-	-
23	06/13	Anglesey, 2	IF	+	+	+	+	+	+

24	06/13	Denbighshire, 3	G16F2, G26F1	+	+	-	+	+	-
25	07/13	Conwy farm 1, 218	IF	-	+	+	+	-	-
26	07/13	Conwy farm 1, 10	G2F2C10, G2ST24	-	+	-	+	+	+
27	07/13	Conwy farm 1, 49	G2F3C12, G2F3	+	+	+	+	+	-
28	07/13	Conwy farm 1, 4	G2F4C4	+	+	+	+	+	-
29	07/13	Conwy farm 1, 53	IF	+	+	+	+	+	-
30	07/13	Conwy farm 1, 12	G2F6C6	+	+	+	+	+	-
31	07/13	Conwy farm 1, 33	G1F7C5	+	+	+	+	+	-
32	07/13	Conwy farm 1, 8	IF	+	+	+	+	+	-
33	07/13	Conwy farm 1, 86	G1F9C27, G2F9	+	+	+	+	-	-
34	07/13	Conwy farm 1, 85	G2F10C10	+	+	+	+	+	-
35	07/13	Conwy farm 1, 62	G2F11C11	+	+	+	+	-	-
36	07/13	Conwy farm 1, 96	IF	+	+	+	+	+	-
37	08/13	Conwy farm 2, 5	G2138C	+	+	+	+	-	+
38	08/13	Conwy farm 2, 6	G2148C	-	+	+	+	+	+
39	08/13	Conwy farm 2, 900	G2158C	-	+	+	+	+	+
40	08/13	Conwy farm 2, 930	IF	+	+	+	+	+	+
41	08/13	Anglesey, 38	IF	-	+	-	+	-	+
42	08/13	Anglesey, 653	IF	+	-	-	+	+	+
43	08/13	Anglesey, 58	IF	+	+	-	+	+	+
44	08/13	Anglesey, 40	IF	-	+	-	+	+	+
45	08/13	Anglesey, 74	IF	+	+	+	+	+	+
46	08/13	Anglesey, 60	G21C11	-	+	-	+	+	-
47	08/13	Anglesey, 59	G22C4	+	+	+	+	-	-
48	08/13	Anglesey, 41	IF	-	+	-	+	-	+
49	08/13	Anglesey, 39	IF	+	+	-	+	+	+
50	08/13	Anglesey, 651	IF	+	-	+	+	+	+
51	08/13	Anglesey, 652	IF	-	+	-	+	+	-
52	08/13	Anglesey, 33	IF	-	+	+	+	-	+
53	12/13	Cheshire, 101*	G21LJ	-	+	-	+	+	+
54	12/13	Cheshire, 102*	IF	-	+	-	+	-	-
55	12/13	Cheshire, 103 front left*	G23LJ	+	+	+	+	+	-
56	12/13	Cheshire, 103 back right*	IF	+	-	+	+	+	-
57	07/14	Shropshire, 1	G3ST1	-	-	+	+	-	+
58	07/14	Shropshire, 4	G3S4S	-	+	+	+	+	+

[a]Sheep number given with additional foot information if animal had multiple feet sampled.
[b]All isolations are shown for comparison to PCR results. Abbreviations: IF, isolation failed. If isolation was successful the isolated strains are listed.
[c]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively which are routinely found in bovine DD lesions.
* Sheep from which healthy foot tissue was also obtained and investigated in this study with corresponding results in Table 3.3.

Table 3.3: PCR detection of treponemes, D. nodosus and F. necrophorum in healthy foot tissue biopsies.

Sample			**Result**

	Biopsy date	Details (Farm, location[a], sheep number[b])	Specific PCR for group[c]:			*Treponema* PCR	*F. necrophor-um*	*D. nodosus*
			1	2	3			
1	09/13	Meirionydd,1 front left	-	-	-	+	-	+
2	09/13	Meirionydd,1 front right	-	-	-	+	-	+
3	09/13	Meirionydd,1 back left	-	-	-	+	-	+
4	09/13	Meirionydd,1 back right	-	-	-	+	-	+
5	09/13	Meirionydd,2 front left	-	-	-	+	-	-
6	09/13	Meirionydd,2 front right	-	-	-	+	-	-
7	09/13	Meirionydd,2 back left	-	-	-	+	-	-
8	09/13	Meirionydd,2 back right	-	-	-	+	-	-
9	09/13	Meirionydd,3 front left	-	-	-	+	-	+
10	09/13	Meirionydd,3 front right	-	-	-	+	-	+
11	09/13	Meirionydd,3 back left	-	-	-	+	-	-
12	09/13	Meirionydd,3 back right	-	-	-	+	-	+
13	09/13	Meirionydd,4 front left	-	-	-	+	+	-
14	09/13	Meirionydd,4 front right	-	-	-	+	-	-
15	09/13	Meirionydd,4 back left	-	-	-	+	-	-
16	09/13	Meirionydd,4 back right	-	-	-	+	-	-
17	12/13	Cheshire, 101 front left*	-	-	-	+	-	+
18	12/13	Cheshire, 101 back left*	-	-	-	+	-	+
19	12/13	Cheshire,101 back right*	-	-	-	+	-	+
20	12/13	Cheshire, 102 front left*	-	-	-	+	-	+
21	12/13	Cheshire, 102 front right*	-	-	-	+	-	+
22	12/13	Cheshire, 102 back right*	-	-	-	-	+	-
23	12/13	Cheshire, 103 front right*	-	-	-	-	+	+
24	12/13	Cheshire, 103 back left*	-	-	-	+	-	-
25	03/14	26, front left	-	-	-	+	-	-
26	03/14	26, front right	-	-	-	+	-	-
27	03/14	26, back left	-	-	-	-	-	-
28	03/14	26, back right	-	-	-	-	-	-
29	03/14	83, front left	-	-	-	+	+	-
30	03/14	83, front right	-	-	-	+	-	-
31	03/14	31, front left	-	-	-	+	-	-
32	03/14	31, back left	-	-	-	+	-	-
33	03/14	89, front left	-	-	-	-	-	-
34	03/14	89, back left	-	-	-	-	-	-
35	04/14	8	-	-	-	-	-	-
36	04/14	5	-	-	-	-	-	-
37	04/14	6	-	-	-	-	-	-
38	04/14	79, front left	-	-	-	-	-	-
39	04/14	79, back right	-	-	-	-	-	-
40	04/14	80, front left	-	-	-	-	-	-
41	04/14	80, front right	-	-	-	-	-	-
42	04/14	80, back left	-	-	-	-	-	-
43	04/14	80, back right	-	-	-	+	-	-
44	04/14	7, front left	-	-	-	-	-	-
45	04/14	7, front right	-	-	-	+	-	+
46	04/14	7, back left	-	-	-	+	-	+
47	04/14	7, back right	-	-	-	+	-	+
48	04/14	87, front left	-	-	-	+	+	+
49	04/14	87, front right	-	-	-	+	-	+
50	04/14	87, back left	-	-	-	-	-	-
51	04/14	87, back right	-	-	-	+	-	-
52	04/14	987, front left	-	-	-	+	-	+
53	04/14	987, front right	-	-	-	-	-	+
54	04/14	987, back left	-	-	-	+	-	+
55	04/14	987, back right	-	-	-	+	-	+

56	04/14	9	-	-	-	-	-	-

[a]Farm location listed from sheep feet samples not obtained from sheep at the abattoir.
[b]Sheep number given with additional foot information if animal had multiple feet sampled.
[c]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively which are routinely found in bovine DD lesions.
* Sheep which also had a CODD lesion present on a different foot were also investigated in this study with corresponding results in Table 3.2.

3.3.1.2 Statistical Analysis

Chi-square analysis indicated that the proportion of samples positive for the three DD- associated *Treponema* phylogroups, *T. medium*- like, *T. phagedenis*- like and *T. pedis* DD spirochaetes, was significantly higher in CODD lesion samples than in healthy foot tissue samples (all $P < 0.0001$). The proportion of samples positive for *D. nodosus* was not significantly higher in CODD lesions than in healthy foot tissue samples ($P = 0.0605$); however, the proportion of samples positive for *F. necrophorum* was significantly higher in CODD lesions than in healthy foot tissue samples ($P < 0.0001$).

Following statistical analysis, all *P* values for co-associations between the presence of the different bacterial species in CODD lesions are listed in Table 3.4.

Table 3.4: Associations between bacteria present in CODD lesions from PCR data analysis (Chi-squared analysis (P values).

		***Treponema* group[a]:**			***D. nodosus***	***F. necrophorum***
		1	**2**	**3**		
***Treponema* group[a]:**	**1**	-	-	-	-	-
	2	0.7291	-	-	-	-
	3	0.0157*	0.9125	-	-	-
D. nodosus		0.0606	0.6161	0.1627	-	-
F. necrophorum		0.2353	0.9125	0.3363	0.8109	-

[a] Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively.
* *P* value shows statistical significance.

In CODD lesions, there was a statistically significant co-association between the presence of *T. medium*- like and *T. pedis* DD spirochaetes ($P = 0.0157$).

However, there were no statistically significant co-associations identified between any other bacterial species in CODD lesions.

The chi-square test revealed there was no statistically significant co-association between the two non-treponemal bacteria, *D. nodosus* and *F. necrophorum* in healthy foot tissue. It was not possible to perform statistical analysis to identify co-association between the treponemal bacterial in healthy foot tissue as no DD *Treponema* DNA was detected in healthy foot tissues.

3.3.1.3 Culture of spirochaetes and phylogenetic analysis of spirochaete isolates

Spirochaetes were successfully isolated from a high proportion of CODD lesions (Table 3.2). In several cases, multiple isolates were obtained from a single CODD lesion biopsy.

In total, 32 spirochaetes were successfully isolated from 27/58 CODD lesions (47%). Many of these isolates (*n*= 24, 75%), were identified as belonging to the *T. phagedenis*- like spirochaete group, with 23/24 sharing 100% 16S rRNA gene sequence identity with the *T. phagedenis*-like DD spirochaete strain T320A (Genbank accession: EF061261), previously isolated from a dairy cow DD lesion in the UK (Evans *et al.* 2008). The remaining *T. phagedenis*- like DD spirochaete isolate shared a higher sequence identity (100%) with the human *T. phagedenis* strain CIP62.29 (EF645248) which both differ from the dairy cow DD isolate, *T. phagedenis*- like DD spirochaete strain T320A, by a single nucleotide substitution.

Six isolates (19%) belonged to the *T. medium*- like spirochaetes and shared 100% 16S rRNA gene sequence identity with *T. medium*- like DD spirochaete strain T19 (Genbank accession: EF061249) previously isolated from a dairy cow DD lesion in the UK (Evans *et al.* 2008).

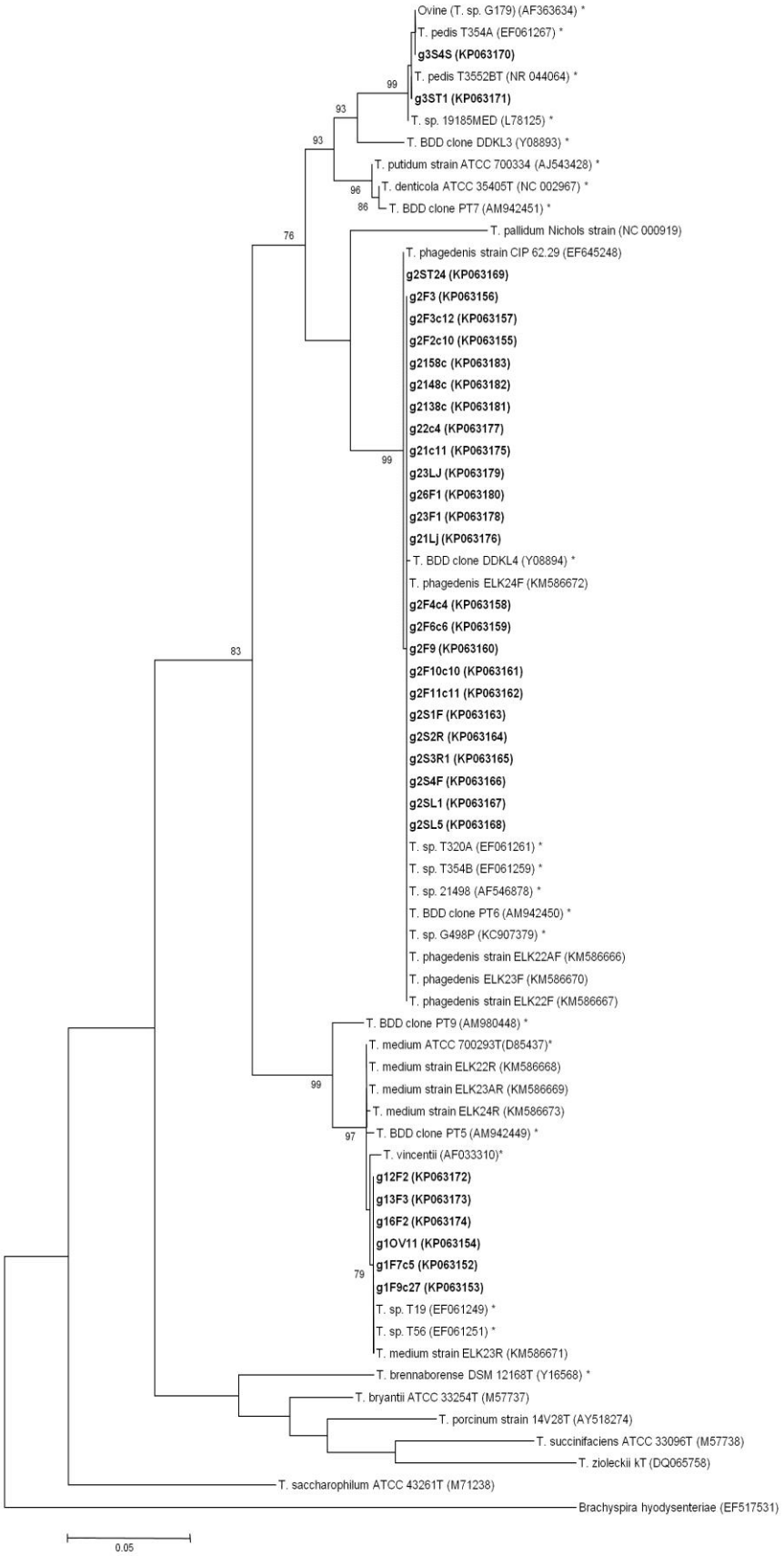
Two isolates (6%) belonged to the *T. pedis* spirochaetes. Spirochaete isolate G3ST1 (Genbank accession: KP063171), shared 100% 16S rRNA gene sequence identity with *T. pedis* T3552B (Genbank accession: NR044064),

previously isolated from a dairy cow DD lesion in the UK (Evans *et al.* 2008). The other *T. pedis* spirochaete isolate from this study, G3S4S (Genbank accession: KP063170), was found to share 100% 16S rRNA gene sequence identity with *T.* sp. G179 (Genbank accession: AF363634), which was similarly isolated from a sheep CODD lesion in the UK (Demirkan *et al.* 2001). These two *T. pedis* spirochaete groups differ by just three nucleotide substitutions.

Upon phylogenetic tree analysis of the 16S rRNA gene sequences, the 32 CODD treponeme isolates separated into three distinct phylogroups corresponding exactly to the three *Treponema* phylogroups commonly isolated from dairy cattle BDD lesions (Figure 3.4).

Figure 3.4: A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~1,200 aligned bases showing the relationship between the strains isolated here (shown in bold) from sheep foot CODD lesions and other DD associated and commensal treponeme 16S rRNA gene sequences.

Bootstrapped 10,000 times, and for clarity only bootstrap values above 70% are shown. = previously reported 16S rRNA gene sequences from BDD lesions.*


Ovine (T. sp. G179) (AF363634) *
T. pedis T354A (EF061267) *
g3S4S (KP063170)
T. pedis T3552BT (NR 044064) *
g3ST1 (KP063171)
T. sp. 19185MED (L78125) *
T. BDD clone DDKL3 (Y08893) *
T. putidum strain ATCC 700334 (AJ543428) *
T. denticola ATCC 35405T (NC 002967) *
T. BDD clone PT7 (AM942451) *
T. pallidum Nichols strain (NC 000919)
T. phagedenis strain CIP 62.29 (EF645248)
g2ST24 (KP063169)
g2F3 (KP063156)
g2F3c12 (KP063157)
g2F2c10 (KP063155)
g2158c (KP063183)
g2148c (KP063182)
g2138c (KP063181)
g22c4 (KP063177)
g21c11 (KP063175)
g23LJ (KP063179)
g26F1 (KP063180)
g23F1 (KP063178)
g21Lj (KP063176)
T. BDD clone DDKL4 (Y08894) *
T. phagedenis ELK24F (KM586672)
g2F4c4 (KP063158)
g2F6c6 (KP063159)
g2F9 (KP063160)
g2F10c10 (KP063161)
g2F11c11 (KP063162)
g2S1F (KP063163)
g2S2R (KP063164)
g2S3R1 (KP063165)
g2S4F (KP063166)
g2SL1 (KP063167)
g2SL5 (KP063168)
T. sp. T320A (EF061261) *
T. sp. T354B (EF061259) *
T. sp. 21498 (AF546878) *
T. BDD clone PT6 (AM942450) *
T. sp. G498P (KC907379) *
T. phagedenis strain ELK22AF (KM586666)
T. phagedenis ELK23F (KM586670)
T. phagedenis strain ELK22F (KM586667)
T. BDD clone PT9 (AM980448) *
T. medium ATCC 700293T(D85437)*
T. medium strain ELK22R (KM586668)
T. medium strain ELK23AR (KM586669)
T. medium strain ELK24R (KM586673)
T. BDD clone PT5 (AM942449) *
T. vincentii (AF033310)*
g12F2 (KP063172)
g13F3 (KP063173)
g16F2 (KP063174)
g1OV11 (KP063154)
g1F7c5 (KP063152)
g1F9c27 (KP063153)
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. medium strain ELK23R (KM586671)
T. brennaborense DSM 12168T (Y16568) *
T. bryantii ATCC 33254T (M57737)
T. porcinum strain 14V28T (AY518274)
T. succinifaciens ATCC 33096T (M57738)
T. zioleckii kT (DQ065758)
T. saccharophilum ATCC 43261T (M71238)
Brachyspira hyodysenteriae (EF517531)
99
93
93
96
86
76
99
83
99
97
79
0.05

3.3.2 Goat lesions with an unknown aetiology

3.3.2.1 Clinical description of goat foot lesions

All 15 goats examined were lame; non-weight bearing on the affected foot and were affected on one leg only. Eight of the 15 lame goats had foot lesions showing very close resemblance to the typical presentation of CODD in sheep. These goats displayed separation of the hoof capsule at the level of the coronary band with the underlying exposed tissue appearing haemorrhagic and granulomatous (Figures 3.5 and 3.6). These lesions appeared to originate at the coronary band. The lesions on the other seven goats examined demonstrated an even more severe presentation, with loss of solar horn accompanied by marked granulation and haemorrhage of the sole (Figure 3.7). The lesions on these seven goats therefore presented lesions similar to CODD, but the origin, development and progression of the lesions was not open to interpretation due to their consistent severity.

3.3.2.2 PCR assays

The results of the specific DD *Treponema* phylogroup PCR and *Treponema* genus-specific PCR assays in goat lesions and healthy foot tissues are shown in Table 3.5.

The 10 goat lesion biopsy samples were all positive for the *Treponema* genus specific PCR assay. The DD group- specific PCR assays found *T. medium*- like spirochaetes and *T. phagedenis*- like DD spirochaetes in 9/10 (90%) of lesion samples and 8/10 (80%) were positive for *T. pedis* spirochaetes. All lesional samples were positive for at least one or more of the DD- associated *Treponema* phylogroups upon PCR analysis.

All healthy foot tissues were negative for all three of the DD group-specific PCR assays. Of the ten healthy foot tissue samples, 7/10 (70%) were positive for the *Treponema* genus specific PCR.

Figure 3.5: A severe "CODD type lesion" in a dairy goat. The lesion has underrun the hoof causing avulsion of the hoof capsule. The granulation of the tissue is highly visible and the severity of the swelling caused by the lesion.

Figure 3.6: A severe "CODD type lesion" in a goat with separated hoof horn removed to show underlying granulomatous and haemorrhagic tissue.

Figure 3.7: Goat foot with a more severe "CODD type lesion" affecting the sole of foot. Although the clinical appearance of the lesion appeared the same as more typical "CODD type lesions" in the goats, due to the severity of the lesion it was undetermined where the lesion had originated.

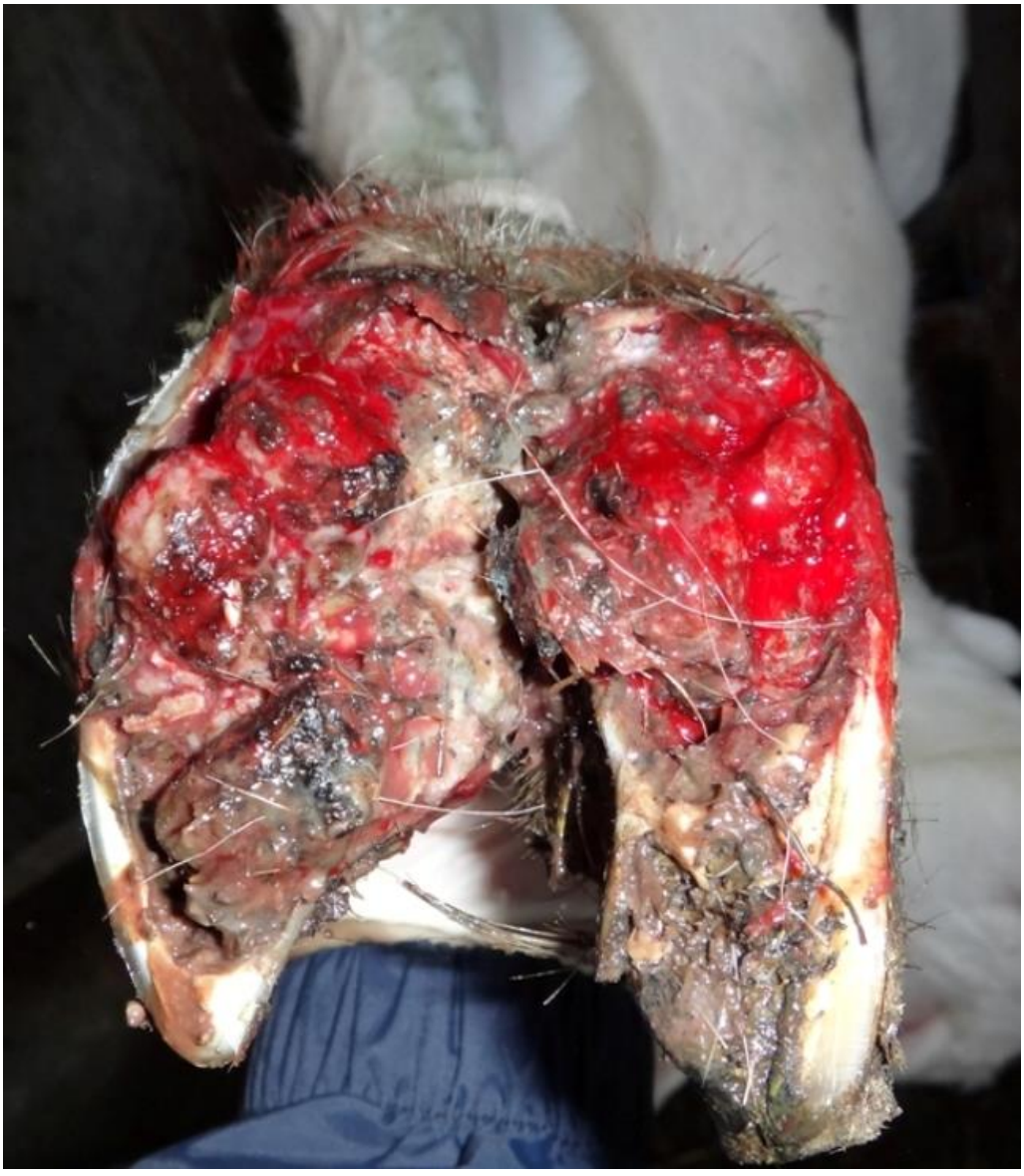


Table 3.5: PCR detection and isolation of treponemes in goat foot lesion biopsies (samples 1–10) and healthy goat foot tissues (samples 11–20).

Sample number	Biopsy site (foot)	Type	Group specific PCR[a]			*Treponema* genus-specific PCR	Treponemes isolated[b]
			1	2	3		
1	Hind left	CODD-like lesion	+	+	-	+	IF
2	Front right	CODD-like lesion	+	+	+	+	G2JD
3	Hind right	CODD-like lesion	+	+	+	+	IF
4	Hind right	CODD-like lesion	+	+	+	+	IF
5	Front left	CODD-like lesion	+	+	-	+	IF
6	Front left	CODD-like lesion	+	+	+	+	G6JD
7	Front left	CODD-like lesion	+	+	+	+	G7JD
8	Hind right	CODD-like lesion	-	-	+	+	IF
9	Hind right	Severe CODD-like with underrun sole	+	+	+	+	G9JD
10	Front left	Severe CODD-like with underrun sole	+	+	+	+	G10JD
11	Back left	Healthy tissue	-	-	-	+	N/A
12	Back right	Healthy tissue	-	-	-	+	N/A
13	Back left	Healthy tissue	-	-	-	+	N/A
14	Back right	Healthy tissue	-	-	-	+	N/A
15	Back left	Healthy tissue	-	-	-	+	N/A
16	Back right	Healthy tissue	-	-	-	-	N/A
17	Back left	Healthy tissue	-	-	-	+	N/A
18	Back right	Healthy tissue	-	-	-	-	N/A
19	Back left	Healthy tissue	-	-	-	+	N/A
20	Back right	Healthy tissue	-	-	-	-	N/A

[a] Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively which are routinely found in DD lesions.

[b] All isolations are shown for comparison to PCR results. Abbreviations: IF, isolation failed; NIA, no isolation attempted. If isolation was successful the isolated strains are listed.

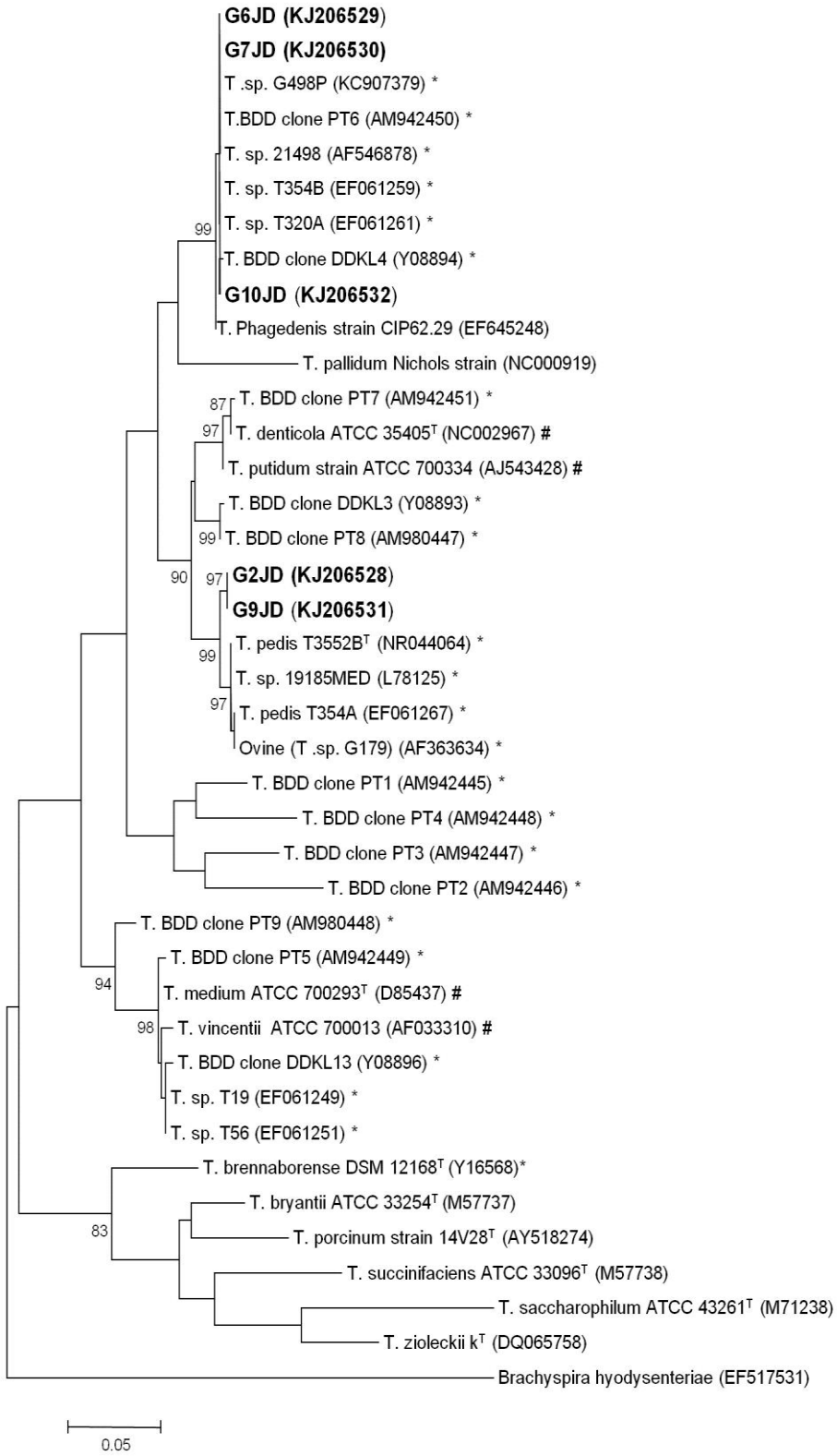
3.3.2.3 Culture of spirochaetes and phylogenetic analysis of spirochaete isolates

Spirochaetes were successfully isolated from 5/10 (50%) of cultured lesion samples (Table 3.5). The isolates G6JD, G7JD and G10JD (Genbank accession codes: KJ206529, KJ206530, KJ206532, respectively) were identified as belonging to the *T. phagedenis*- like spirochaetes and shared 100% 16S rRNA gene sequence identity with the *T. phagedenis*- like DD spirochaete strain T320A (Genbank accession: EF061261), previously isolated from a dairy cow DD lesion in the UK (Evans *et al.* 2008). Isolates G2JD and G9JD (Genbank accession codes: KJ206528, KJ206531, respectively) belonged to the *T. pedis*

spirochaetes and shared 99% 16S rRNA gene sequence identity with *T. pedis* T3552B (NR 044064) previously isolated from a dairy cow DD lesion in the UK (Evans *et al.* 2008).

All successfully cultured isolates clustered with their respective closest spirochaete relatives upon phylogenetic analysis (Figure 3.8). Goat lesion isolates G6JD, G7JD and G10JD clustered closely with the *T. phagedenis*- like spirochaetes, as would be expected. Lesion isolates G2JD and G9JD clustered with the *T. pedis* spirochaetes; however, they formed a separate clade resulting from five nucleotide substitutions in the 16S rRNA gene; A69G, T73C, A219G, T405C, A440G (locations relative to those for *Escherichia coli* 16S rRNA gene sequence (Ehresmann *et al.* 1975)).

Figure 3.8: Phylogeny of goat DD treponemes. A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~1,000 aligned bases showing the relationship between the strains isolated here (shown in bold) from goat foot lesions and other DD associated and commensal treponeme 16S rRNA gene sequences. Bootstrap confidence levels are shown as percentages of nodes, and only values above 70% are shown. = previously reported 16S rRNA gene sequences from BDD lesions; # = previously reported 16S rRNA gene sequences from human oral periodontal infections.*


G6JD (KJ206529)
G7JD (KJ206530)
T .sp. G498P (KC907379) *
T.BDD clone PT6 (AM942450) *
T. sp. 21498 (AF546878) *
T. sp. T354B (EF061259) *
T. sp. T320A (EF061261) *
99
T. BDD clone DDKL4 (Y08894) *
G10JD (KJ206532)
T. Phagedenis strain CIP62.29 (EF645248)
T. pallidum Nichols strain (NC000919)
87
T. BDD clone PT7 (AM942451) *
97
T. denticola ATCC 35405T (NC002967) #
T. putidum strain ATCC 700334 (AJ543428) #
T. BDD clone DDKL3 (Y08893) *
99
T. BDD clone PT8 (AM980447) *
90
97
G2JD (KJ206528)
G9JD (KJ206531)
T. pedis T3552BT (NR044064) *
99
T. sp. 19185MED (L78125) *
97
T. pedis T354A (EF061267) *
Ovine (T .sp. G179) (AF363634) *
T. BDD clone PT1 (AM942445) *
T. BDD clone PT4 (AM942448) *
T. BDD clone PT3 (AM942447) *
T. BDD clone PT2 (AM942446) *
T. BDD clone PT9 (AM980448) *
T. BDD clone PT5 (AM942449) *
94
T. medium ATCC 700293T (D85437) #
98
T. vincentii ATCC 700013 (AF033310) #
T. BDD clone DDKL13 (Y08896) *
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. brennaborense DSM 12168T (Y16568)*
T. bryantii ATCC 33254T (M57737)
83
T. porcinum strain 14V28T (AY518274)
T. succinifaciens ATCC 33096T (M57738)
T. saccharophilum ATCC 43261T (M71238)
T. zioleckii kT (DQ065758)
Brachyspira hyodysenteriae (EF517531)
0.05

3.4 Discussion

Since the first CODD report in 1997 (Harwood *et al.* 1997) it has become apparent that there is an infective component and that the specific treponemes closely associated with BDD in dairy cattle (Stamm *et al.* 2002; Evans *et al.* 2008, 2009b; Klitgaard *et al.* 2008) are likely involved in CODD, and may be a primary initiating agent (Dhawi *et al.* 2005; Angell *et al.* 2014). However, the available microbiological data produced thus far has been limited and not sufficient for proving or disproving a causative association. The current study is a comprehensive attempt to consider the link between BDD treponemes and CODD and to address the role of other bacteria frequently detected in infectious lameness issues in sheep.

What is clear from the study is that the DD treponemes (individually and frequently collectively) are present in all CODD lesions, whereas in contrast, they are totally absent in samples from healthy sheep foot tissue. This is very strong data supportive of a primary infective aetiology for CODD by these organisms. Interestingly, our data also shows that other bacteria frequently associated with other sheep foot infections may also be commonly detected in CODD lesions, though with a far less striking frequency than BDD treponemes when comparing CODD lesions and healthy feet.

Thus, a key question is whether BDD treponemes are the primary or secondary infections leading to the development of CODD lesions. What is clear is that they are present in all CODD lesions and this adds weight to the hypothesis that they are primary aetiological agents. However, they may also be secondary infections to other, possibly non-infective, lesions in sheep feet. It has become apparent that the BDD treponemes must be considered as promiscuous and opportunistic infective agents as it has been clearly demonstrated that they can invade other (non-infective) lesions in cattle feet, such as white line disease and sole ulcers and clinically manifest as new serious infectious diseases which are very difficult to treat (Evans *et al.* 2011a). Previously, only a 70% association of treponemes with CODD lesions was

found (Moore *et al.* 2005), much lower than the 100% reported in this study (albeit by different methodologies) which is in accordance with the 100% association shown in dairy cattle BDD lesions (Evans *et al.* 2009b). Additionally, the previous study (Moore *et al.* 2005) also detected *Treponema* DNA in 38% of healthy foot tissue samples. It should be noted that the previous study (Moore *et al.* 2005) used a genus specific treponeme PCR assay, which is not specific for the DD- associated *Treponema* phylogroups, but targets all *Treponema* species. Therefore, this previous study gave no indication to whether the *Treponema* species detected were pathogenic DD- associated *Treponema* species found commonly in dairy cattle BDD lesions, or simply commensal treponemes found in the environment or a ruminant's GI tract (Evans *et al.* 2012). As seen in our present study, using the general treponeme PCR assay, treponemes were detected in 68% of healthy foot tissue samples; however, all of these healthy tissue samples were negative when tested for the three DD- associated *Treponema* phylogroups.

In dairy cattle BDD samples, it was shown that *T. medium*- like, *T. phagedenis*-like and *T. pedis* spirochaetes were individually present in 96.1%, 98%, and 76.5% of DD lesions respectively (Evans *et al.* 2009b). These results are similar to the findings in this CODD study, in terms of the percentage detection of the three *Treponema* phylogroups, with 67%, 85% and 71% of CODD lesions positive for *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively. In both cattle and sheep DD lesions, *T. phagedenis*-like spirochaetes seem to be the most commonly detected spirochaetes. However, *T. medium*- like spirochaetes appear to be less prevalent in CODD lesions compared with BDD lesions. Importantly, one or more of the BDD treponemes are found in all cattle (Evans *et al.* 2009b) and sheep CODD lesions using the phylogroup specific nested PCR assays.

Treponemes are anaerobic, highly fastidious bacteria, and notoriously difficult to grow in culture (Norris *et al.* 2002). Despite this, treponemes were isolated from a significant number of CODD lesions in this study, supporting the

evidence from the aforementioned molecular data and further highlighting DD treponeme abundance in these lesions. In agreement with previous BDD studies, the *T. phagedenis*- like spirochaetes were the most commonly isolated treponeme group from BDD and CODD lesions (Evans *et al.* 2009b). This is also consistent with our PCR detection findings of an increased prevalence of this phylogroup in CODD lesions.

The phylogeny data clearly shows that treponemes isolated from BDD and CODD fall into one of three well-defined groups and are completely different from those identified in GI tract samples (Evans *et al.* 2012). Unsurprisingly, one of the CODD isolates (G3S4S) belonging to the *T. pedis* spirochaetes group, shared 100% 16S rRNA gene sequence identity to the previous *T. pedis* spirochaete isolated from a CODD lesion (Dermirkan *et al.* 2001). More interestingly, *T. pedis* spirochaete isolate G3ST1 shared 100% 16S rRNA gene sequence identity with *T. pedis* T3552B (Genbank accession: NR044064) which was isolated from a dairy cow DD lesion (Evans *et al.* 2008), suggesting these treponemes cannot be distinguished between host species. These results provide further evidence towards a shared aetiopathogenesis and for treponeme transmission between different host species. Further studies are needed to delineate whether there are specific changes to the three DD treponeme phylogroups associated with adaptation to different hosts and which underpin specific transmission cycles.

The CODD lesions were often associated with *F. necrophorum*, which was present in 71% of CODD lesions, versus a 9% prevalence in healthy foot tissue samples. This is consistent with previous data (Moore *et al.* 2005) where *F. necrophorum* was detected in a very low number of healthy sheep foot samples compared with CODD lesions. In that study, *F. necrophorum subsp. necrophorum* was present in 86% of CODD lesions compared with 46% of normal feet, and *F. necrophorum subsp. funduliforme* was present in 28% of CODD lesions compared with 0% in healthy foot samples (Moore *et al.* 2005).

In the current study, *D. nodosus*, interestingly, was present in a similar number (59%) of CODD lesions as *F. necrophorum*. However, *D. nodosus* had a much higher detection rate in healthy tissues (39%) than *F. necrophorum* (9%). *D. nodosus* has previously been detected in CODD lesions with a prevalence of 74% compared to 31% in healthy foot tissues (Moore *et al.* 2005). These findings support our data and suggest that *D. nodosus* does not have a primary infective role in CODD lesions, but may have a secondary role.

In sheep footrot, it is generally considered that *D. nodosus* plays a primary role and *F. necrophorum* a secondary role in lesion development (Beveridge 1941; Kennan *et al.* 2001; Witcomb *et al.* 2014). The current study shows that *F. necrophorum*, has a strong association with CODD (9% prevalence in healthy tissues versus a 71% prevalence in CODD lesions), though not as strong as the BDD treponemes. However, it is possible that in CODD, like in footrot, *F. necrophorum* may act as a secondary invader to the DD- associated treponeme infection, as it apparently does to *D. nodosus* in footrot. This role of *F. necrophorum* is supported by recent research which highlights *F. necrophorum* as a key but secondary invader in footrot (Witcomb *et al.* 2014). This finding is consistent with the existing understanding of the role of *Fusobacterium* spp. in other diseases. *Fusobacterium necrophorum* and other *Fusobacteria* are found in polymicrobial infections causing lesions and abscesses (Brook 2002; Hofstad 2006), and are considered to progress disease severity through relationships with other pathogens (Brook and Walker 1986; Tan *et al.* 1996).

This study included a large number of farms in order to investigate CODD lesions from a large geographical area. However, due to the obvious ethical limitations in obtaining healthy foot tissue samples, a study design including healthy animal foot tissue from animals on the same farms from which CODD foot samples were obtained was not possible in all cases. This could introduce a weakness in the sampling strategy used as it could be the case that sheep sampled for CODD lesions, and therefore from a farm with CODD present, could have bacteria present on their feet due to environmental contamination.

However, the inclusion of healthy foot tissue from a small number of animals which also had a CODD lesion on another one of their feet (therefore healthy tissue obtained from a CODD positive farm) limits this bias in sampling strategy. Additionally, many lesions and healthy tissues were used in this study to allow for reasonable analysis on results to be possible, however more lesional samples would allow for more significance to be placed on results. For these reasons all associations between bacterial species and tissue samples have to be interpreted with this in mind, and the possibility of other unknown external influencing factors.

Interestingly, the CODD lesions obtained from the sheep that also had their remaining healthy foot tissues sampled, were all positive for at least one of the DD- associated *Treponema* phylogroups; however, all the remaining healthy feet from each animal tested negative for DD- associated treponemes. When looking at the presence of *D. nodosus* and *F. necrophorum* in these animals, no consistent finding was observed. For example, *D. nodosus* and *F. necrophorum* were found to be present in the CODD lesion obtained from sheep 101; however, the remaining healthy feet of the animal were all negative for *D. nodosus* and all positive for *F. necrophorum.* By contrast, the CODD lesion from sheep 102 tested negative for both *D. nodosus* and *F. necrophorum*, but 1/3 and 2/3 of the remaining healthy feet from the animal were positive for *D. nodosus* and *F. necrophorum,* respectively. This provides more evidence towards these two bacterial species playing a lesser role in CODD lesions than the specific DD associated treponemal bacteria which are only present in the lesions and not healthy foot tissue.

The fastidious nature of treponemes means it is extremely hard to successfully isolate them from tissue samples. However, DD- associated treponemes were isolated from a large number of CODD lesions, and included all three DD-associated *Treponema* phylogroups. This, together with PCR data showing that all CODD lesions contained at least one or more DD- associated treponemes, supports the hypothesis that treponeme species, specifically the ones

associated with BDD, are likely to be important aetiological agents in CODD lesions. Our data also indicates that in many CODD lesions, *F. necrophorum* may contribute to lesion pathogenesis.

The goat foot disease documented here was extremely severe with no early lesions identified; although in the majority of cases it appeared that the lesions originated at the coronary band, as with CODD in sheep (Davies *et al.* 1999; Winter 2008). The lesions in eight of the goats examined (Figures 3.5 and 3.6), where there was separation of hoof horn at the level of the coronary band accompanied by extensive under-running of the hoof horn capsule, bear a marked similarity to the published lesion descriptions of CODD in sheep (Davies *et al.* 1999; Winter 2008) and our observations of CODD lesions in this study. In all of these studies, CODD is described as an initial ulcerative lesion at the coronary band followed by extensive under-running of the horn, with subsequent loss of the hoof capsule. The lesions observed in the seven other goats examined differed by their severity, appearing to have progressed to the sole of the foot. These were characterised by extensive horn loss on the sole of the foot differing from footrot in that the underlying exposed tissue appearing haemorrhagic and granulomatous. These resembled CODD but were appeared to show a further progressed clinical picture.

The strong association between lesion description and incidence and the presence of DD- associated treponemes in the goat lesions, and their absence in healthy goat foot tissue, would suggest the involvement of treponemes in the disease process, as considered for DD in dairy cattle, beef cattle and sheep (Evans *et al.* 2008; Sayers *et al.* 2009, Chapter 4; Sullivan *et al.* 2013, 2015a). The detection rate of the DD- associated treponemes found in the goat lesions is similar to that reported in cattle DD lesions and sheep CODD lesions (Evans *et al.* 2009; Moore *et al.* 2005; this study). Additionally, all CODD lesions and goat lesions were positive for at least one of the DD- associated *Treponema* phylogroups as is found in DD lesions in dairy cattle (Evans *et al.* 2008).

The isolation from the goat lesions of three treponemes which are 100% identical to the *T. phagedenis*- like spirochaetes consistently found within dairy cattle DD lesions (Evans *et al.* 2008; 2009b), also suggests a role for these pathogenic bacteria within the goat lesions. The isolation of two treponemes which are part of the *T. pedis* spirochaetal group, but show small nucleotide changes, could suggest there are host species specific variations within this treponeme phylogroup.

In this condition, as in CODD in sheep, it is not clear if the treponemes are the primary infection causing the lesion or whether they are secondary infections of an established lesion (of unknown aetiology) which change the nature of the infection and the clinical outcome. To fully understand the aetiology of this foot disease, early lesions would need to be investigated. However, the isolation of similar treponemes to those isolated from BDD and CODD lesions and the high detection rate of these DD- associated phylogroups in the lesions would support the hypothesis that this novel foot condition is highly associated with infection by DD treponemes. The prevalence and severity of the disease reported on just one UK goat dairy farm is very worrying and justifies further investigations in the future. Further studies are needed to identify effective means of prevention and/or treatment to reduce spread to other goat herds in the UK and elsewhere. This study suggests that DD may have emerged in yet another host species and vigilance is suggested in goat herds and in other domesticated species.

With the emergence of this CODD- like disease in dairy goats and recently a USA elk foot disease reported as both clinically and aetiologically similar to CODD (Clegg *et al.* 2015), the disease is clearly increasing in importance and geographical spread. It is very clear that CODD leads to severe welfare issues for the animal involved and leads to significant financial implications for farmers and hence is a food security issue. Thus, understanding the pathogenesis of CODD is key to developing the means of managing and preventing the spread of this debilitating disease. The phylogenetic analysis of

Treponema isolates from CODD and goat lesions here highlights the close clustering of these isolates with the previously isolated BDD lesion treponemes. This strong phylogenetic relationship further strengthens the case for CODD and BDD having a shared treponemal aetiopathogenesis and possibly includes this new goat foot disease. Interestingly, epidemiological data demonstrates an association between the presence of CODD in sheep with cattle on farms (Angell *et al.* 2014) and the study areas which allowed for identification of a CODD- like manifestation in USA elk were co-grazed by cattle and sheep (Clegg *et al.* 2015) and the goat farm investigated was previously inhabited by dairy cattle. Taken together, these observations suggest this disease is managing to transmit between host species effectively on shared farmland.

From this study, it would appear that DD treponemes are likely to be the primary infective agent in CODD lesions, and possibly a new form of foot disease in goats, although the promiscuous nature of the DD treponemes and their wide range of tissue tropisms must be considered when making such conclusions. Additionally, the data presented here further identifies that the three DD treponeme specific PCR assays can be used as a differential diagnostic tool for CODD and other DD manifestations from different geographic regions and hosts. Further molecular studies on *Treponema* isolates obtained from DD lesions of different animal species are necessary to compare their genetic relatedness and therefore help determine routes of transmission of this disease and delineate any host adaptation. Further studies should also focus on fully understanding the causality of CODD, as although treponemes are highly associated with the disease, it has not been proven they are the primary causative agent. As it has not been proven that treponemes are the primary, or only, causative agent of CODD, the results of this study, although convincing, do not prove causality. Inoculation of live healthy animal feet with treponemes and subsequent isolation of treponeme bacteria would be necessary to understand treponemes role in CODD further.

Chapter 4

The clinical manifestation and bacteriology of digital dermatitis in beef cattle.

4.1 Introduction

Bovine digital dermatitis causes severe lameness in dairy cattle worldwide. The disease in dairy cattle is recognised as being polytreponemal in aetiology (Klitgaard *et al.* 2008; Nordhoff *et al.* 2008; Evans *et al.* 2009b) with the three phylogroups; *T. medium*- like, *T. phagedenis*- like and *T. pedis* (Stamm *et al.* 2002; Evans *et al.* 2008, 2009a, 2009b) isolated from dairy cattle lesions in the UK and the US. The disease has now been reported in dairy cattle in nearly all countries where they are farmed. Additionally other species now suffer with the disease, including sheep (CODD) in the UK and Republic of Ireland (Harwood *et al.* 1997; Davies *et al.* 1999; Sayers *et al.* 2009; Chapter 3; Sullivan *et al.* 2015b) and there is now a possible caprine (goat) form in the UK (Chapter 3; Sullivan *et al.* 2015c) and also in a wildlife host, North American Elk from Washington State USA (Clegg *et al.* 2015). Thus, with such an apparent ability to spread, it might be expected that DD is present and possibly common in beef cattle too, particularly as many of the breeds used as dairy cattle are part of beef production farming. However, there have only been anecdotal reports about BDD in beef cattle and no clinical or bacteriological descriptions of any suspect lesions.

There is very little information on the causes of lameness in beef cattle possibly but due to their shorter lifespan or difficulties handling these animals. However, they do appear to experience lameness from a wide variety of causes shared with dairy cattle; laminitis-related claw lesions including haemorrhages in the white line and the sole, sole ulcers, white-line fissures, double soles and interdigital hyperplasia (Fjeldaas *et al.* 2007).

It is now widely accepted that BDD is a polymicrobial disease. Given this, the tendency of beef cattle to be different breeds, fed different diets and subjected to different housing regimes than dairy cattle, gives reason for further investigations into beef cattle BDD. Until recently there have been only anecdotal reports of BDD lesions occurring in beef cattle, with no definitive published data and no information on the clinical manifestations, bacteriology or prevalence of the disease.

Hence, there are several questions concerning our poor knowledge of digital dermatitis in beef cattle:

1. Do beef cattle have BDD lesions?
2. Are beef cattle BDD lesions clinically similar to those reported in dairy cattle and /or sheep?
3. Do beef cattle BDD lesions share the same bacterial composition as the disease in dairy cattle and, in particular, do they have a significant treponemal involvement?

These are important questions as the knowledge gained may enable effective intervention strategies to be considered if the disease is significant in clinical severity and prevalence and if the aetiopathogenesis can be understood.

The possible involvement of other organisms such as *D. nodosus* and *F. necrophorum* has been investigated in dairy cattle BDD lesions (Cruz *et al.* 2005; Capion *et al.* 2012; Rasmussen *et al.* 2012; Knappe-Poindecker *et al.* 2013). Previously, *D. nodosus* had been found in between 27% to 60% of dairy cattle BDD lesions (Capion *et al.* 2012; Rasmussen *et al.* 2013) and a recent

study detected the bacteria in all BDD lesions tested (albeit only eight samples were tested) (Knappe-Poindecker *et al.* 2013). *Fusobacterium necrophorum* has been detected in dairy cattle BDD lesions, with studies detecting the bacteria in up to 42% of BDD lesions (Cruz *et al.* 2005; Klitgaard *et al.* 2008) but it has been suggested to be a secondary invader (Klitgaard *et al.* 2008). Additionally, a recent investigation into the role of these bacterial species in CODD lesions in sheep detected *F. necrophorum* and *D. nodosus* in 71% and 59% of the lesions, respectively (Chapter 3; Sullivan *et al.* 2015b).

The accumulating evidence suggests that the aetiopathogenesis of DD in both sheep and cattle are the same. Thus it would be worthwhile, along with analysis of the role *Treponema* species in beef BDD lesions, to consider the potential involvement of these other lameness associated bacteria. Additionally, a comprehensive comparison of the bacterial species found in the DD lesions of dairy cattle, beef cattle and also sheep, would give a better understanding of the relationship between the aetiopathogenesis and transmission of DD in these animals, and other previously unaffected animal species where this disease has now emerged.

The current study aimed to further our understanding of the clinical manifestation and aetiology of BDD in beef cattle by producing an in depth report of the manifestation of the disease in beef cattle and surveying a large number of lesions and healthy foot tissue for detection and isolation of DD-associated treponemes and other lameness associated bacteria. Additionally, comparisons were made to compare the bacteriology of DD lesions in beef cattle, dairy cattle and sheep. I hypothesise that BDD in beef cattle will appear clinically similar to that found in dairy cattle and treponemes highly associated with BDD in dairy cattle will also be present in a statistically significant proportion of beef BDD lesions tested.

4.2 Methods

4.2.1 Clinical description of BDD in Beef cattle

Two Gloucestershire farms (UK) in December 2012 were attended to inspect beef cattle with suspected BDD lesions. Animals suspected of having BDD lesions were assessed by the attending veterinary surgeon (RB). Farm one was a beef rearing unit, with 120 beef cattle. The farm estimate was 25 animals per year (21%) required treatment for BDD. Farm two was a finishing unit and the farm estimate was 15/3000 animals were treated for BDD each year (0.5%). However, neither farm had true prevalence or incidence data, since at no time did all animals have their feet examined for typical lesions.

On each farm animals the suspected of suffering from BDD, seven on farm one and five on farm two, were examined and following confirmation of what appeared to be BDD on all animals, the foot was cleaned, lesions photographed and description of lesions noted.

4.2.2 Sample collection and Farm information

Beef cattle BDD lesions were sampled from four different farms between December 2012 and July 2014. These farms were located in Gloucestershire (Gloucestershire farm 1 and farm 2 as above), and North Wales (North Wales farm 1 and farm 2). From these farms, a total of 26 BDD lesion samples were obtained, of these 21 were surgical biopsies and five were swabs of lesions.

Additionally, eight surgical biopsies of beef cattle BDD lesions and 38 healthy beef cattle foot skin biopsies were collected from a fallen stock centre (March 2014- June 2014). The healthy foot tissue samples were obtained from beef cattle that did not have any evidence of BDD or any other foot lesions. These cattle were from a fallen stock centre which received animals from farms within Lancashire, Cheshire and South Cumbria. This gave a total of 34 beef cattle BDD lesion samples (surgical biopsies and swab samples) and 38 healthy foot tissue samples.

Briefly, Gloucestershire farm 1 was a beef-rearing unit, with around 120 beef cattle and farm 2 (Gloucestershire) was a finishing unit, finishing around 3000 animals each year. North Wales farm 1 was a beef-finishing unit, finishing around 800-1000 beef animals per year and North Wales farm 2 was a beef suckler herd of about 60 cattle.

On all farms from which BDD surgical biopsies were obtained, farmers had isolated lame animals suspected of suffering from BDD. These lame animals were then all inspected for BDD lesions. A cow was defined as having BDD if one or more feet had a clear lesion consistent with the clinical signs of BDD in dairy cattle (Cheli and Mortellaro 1974; Blowey and Sharp 1988).

Beef cattle and dairy cattle BDD lesion and swab samples were collected from animals as per Methods 2.1.1 and 2.1.5, respectively. Samples were transported and stored as described in Method 2.1.1. All samples obtained from the fallen stock centre were collected using the same methods as per BDD lesion sample collection from farms, excluding the use of anaesthesia.

In addition to the beef BDD samples collected in this study, the genomic DNA's from 43 dairy cattle BDD lesions, and six healthy dairy cattle foot tissue collected during 1996 to 2007 and previously investigated by this laboratory for the presence of *Treponema* species (Evans *et al.* 2009b) were included in this investigation. An additional four healthy dairy cattle feet sample controls were collected from the fallen stock centre (as above).

4.2.3 Culture of spirochaetes

Treponema culture was attempted on all samples from beef cattle BDD lesions (biopsies and swab samples), but was not attempted on healthy foot tissue samples. The culture technique used, subsequent bacterial DNA extraction, gene cleaning and sequencing was carried out as described in Methods 2.2, 2.3.1, 2.5.1 and 2.5.2, respectively.

4.2.4 Tissue and swab DNA extraction

All samples from beef cattle BDD lesions (biopsies and swab samples) and healthy foot tissue samples were used for DNA extraction. DNA extraction was performed as described in Method 2.3.2.

4.2.5 PCR assays

All beef cattle BDD lesion samples and healthy foot tissue samples were subjected to nested PCR assays specific for the three DD- associated treponeme groups, *T. medium*- like, *T. phagedenis*- like, *T. pedis* and the treponeme genus specific 16S rRNA PCR assay, as described in Methods 2.4.2 and 2.4.3, respectively. Additionally, all beef cattle BDD lesion samples and healthy foot tissue samples were subjected to the species-specific *D. nodosus* PCR assay and a species-specific *F. necrophorum* PCR assay (targeted the lktA gene), as described in Methods 2.4.4 and 2.4.5, respectively.

In addition to the beef BDD samples collected in this study, dairy cattle BDD lesions (n= 43) and healthy dairy foot skin samples (n= 6) previously collected (2003-2007), were also probed by PCR for the presence of *D. nodosus* and *F. necrophorum*, along with healthy dairy cattle foot skin tissues collected in this study (n= 4). This was designed to enable a comparison of *Treponema* species, *D. nodosus* and *F. necrophorum* detection rates between dairy and beef cattle BDD lesions.

4.2.6 Phylogenetic analysis of spirochaete isolates

To understand the relationship of the isolated spirochaetes with other treponemes, and in particular those previously isolated from dairy cattle BDD lesions and sheep CODD lesions, a phylogenetic tree of 16S rRNA gene sequences from each isolate was produced according to Method 2.6. The most appropriate evolution model was predicted using "model test" as implemented in the Topali programme (Milne *et al.* 2009). The final model for nucleotide substitutions chosen was the TrN model (Tamura and Nei 1993), used to infer

a bootstrapped maximum likelihood tree (bootstrapping was performed 10,000 times).

4.2.7 Nucleotide Accession numbers

The 16S rRNA gene GenBank accession numbers determined as part of this study are KP750190- KP750179 and KP859539- KP859539.

4.2.8 Statistical Analysis

A chi-square test (with Yates correction) was used to identify associations between the presence of each bacteria; *T. medium*- like, *T. phagedenis*- like, *T. pedis*, *F. necrophorum*, *D. nodosus*, with presence or absence of BDD lesions in beef cattle. Chi-square tests (with Yates correction) were also used to identify any co-associations between the presence of each DD- associated *Treponema* phylogroup and *D. nodosus* and *F. necrophorum* in beef cattle BDD lesions. Additionally, the same statistical analysis was performed to identify any co-association between *D. nodosus* and *F. necrophorum* in healthy foot tissue samples. In all analyses, an associated probability (*P*-value) of < 0.05 was considered significant. The chi-square test is described in detail in Method 2.7.1.

4.2.9 Comparisons of bacterial presence in beef and dairy cattle BDD and sheep CODD lesions

The prevalence of *T. medium*- like, *T. phagedenis*- *like* and *T. pedis* DD spirochaetes, *D. nodosus* and *F. necrophorum* in beef cattle BDD lesions (this study), dairy cattle BDD lesions (Evans *et al.* 2009b; this study) and sheep CODD lesions (Chapter 3; Sullivan *et al.* 2015b) was compared. Additionally, the comparable information for healthy tissue samples from each set of animals was included.

4.3 Results

4.3.1. Clinical description of BDD in beef cattle

Typical BDD lesions presented as 30 – 60mm diameter circular areas of brown moist exudate, primarily in the region of the caudal interdigital cleft, at the junction of the skin with the soft perioplic horn of the heel. Lesion cleaning revealed an underlying raw proliferative area with a stippled appearance. This was intensely sensitive to simple digital pressure. Figure 4.1 shows a mild/early lesion and Figure 4.2 is of a more severe lesion undergoing proliferative change. Lesions were concurrent with what is generally seen in dairy cattle BDD lesions (Cheli and Mortellaro 1974; Blowey and Sharp 1988; Evans *et al.* 2008). In another case, lesions also occurred on the anterior coronary band (Figure 4.3); this can lead to disruption of hoof wall formation. Occasional lesions extended into the interdigital cleft, sometimes on the surface of interdigital skin, leading to interdigital hyperplasia, or extended dorsally to the accessory digits. In all cases, the primary clinical sign was lameness. On farms where lesions were described (Gloucestershire farms 1 and 2), simply lifting and cleaning the affected area, application of topical antibiotics held in place with a dressing for 2 – 3 days, in most cases resulted in uneventful recovery. Recovery periods were not recorded. In herd outbreaks, prevention and control on farm one was addressed by daily foot bathing in 5% formalin.

4.3.2 PCR assays

The results of the *Treponema* genus-specific and specific DD *Treponema* phylogroup PCR assays of beef cattle BDD lesions and healthy foot tissues are shown in Tables 4.1 and 4.2, respectively.

All BDD samples (biopsies and swabs combined) (n= 34) were found to be positive for general *Treponema* genus DNA. The phylogroup specific *Treponema* PCR's detected *T. medium*- like, *T. phagedenis*- *like* and *T. pedis* DD spirochaete DNA, in 27/34 (79%), 31/34 (91%) and 24/34 (71%) of beef BDD lesions, respectively. All BDD lesion samples tested were positive for at

least one or more of the DD- associated *Treponema* phylogroups, with 19/34 (56%) of the beef BDD lesion samples positive for all three DD- associated *Treponema* phylogroups.

The genus specific *Treponema* PCR assay found *Treponema* DNA in 24/28 (63%) of beef cattle healthy foot tissue samples. However, all healthy foot tissues were negative for the three DD- associated *Treponema* phylogroups. Results of the specific PCR assays for the detection of *D. nodosus* and *F. necrophorum* in beef cattle BDD lesions and healthy foot tissues are shown in Tables 4.1 and 4.2, respectively. The *D. nodosus* specific PCR assay detected *D. nodosus* DNA in 23/34 (68%) of beef BDD lesion samples. Of the healthy foot tissues sampled in this study, *D. nodosus* DNA was detected in 10/38 (26%) of healthy foot tissue samples. The *F. necrophorum* PCR assay detected *F. necrophorum* DNA in 15/34 (44%) of BDD lesion samples and 12/38 (32%) of healthy foot tissue samples.

Figure 4.1: A mild digital dermatitis lesion on a beef cow foot, in the typical location, the bulb of a hind heel.

Figure 4.2: A more severe lesion undergoing proliferative change on a beef cow foot. The lesion appears on the bulb of the heel and is ulcerative and granulomatous in appearance.

Figure 4.3: A lesions occurring on the anterior coronary band of the beef cows foot.

Table 4.1: PCR detection of treponemes, D. nodosus and F. necrophorum in beef cattle BDD lesion biopsies.

Sample	Biopsy date (mo/yr)	Details (location sample obtained)	Treponeme isolated[b]	Result					
				Specific PCR for group[c]:			*Treponema* PCR	*F. necroph-orum*	*D. nodosus*
				1	2	3			
1	12/12	Gloucestershire F1	1A	+	+	-	+	-	+
2	12/12	Gloucestershire F1	2C, 2D	+	+	+	+	+	-
3	12/12	Gloucestershire F1	3E, 3C14	+	+	+	+	-	+
4	12/12	Gloucestershire F2	10C	+	+	+	+	-	+
5	12/12	Gloucestershire F2	11A	+	+	+	+	-	+
6	12/12	Gloucestershire F2	12C37	+	+	+	+	-	+
7	12/12	Gloucestershire F2	IF	+	+	+	+	+	+
8	12/12	Gloucestershire F2	IF	+	+	+	+	+	-
9	12/12	Gloucestershire F2	IF	-	+	+	+	-	-
10	03/13	North Wales F1	2L7, 2LC	+	+	+	+	+	+
11	03/13	North Wales F1	6LD	+	+	+	+	+	+
12	03/13	North Wales F1	9L	-	+	-	+	-	-
13	03/13	North Wales F1	IF	+	-	+	+	+	+
14	04/13	Gloucestershire F1	IF	+	+	+	+	+	+
15	04/13	Gloucestershire F1	L5	+	+	-	+	-	+
16	04/13	Gloucestershire F1	L6	+	+	-	+	+	+
17	04/13	Gloucestershire F1	IF	+	+	+	+	+	-
18	04/13	Gloucestershire F1	IF	+	+	-	+	+	+
19	01/14	Gloucestershire F1	IF	-	+	-	+	+	-
20	01/14	Gloucestershire F1	L13	-	+	-	+	-	+
21	01/14	Gloucestershire F1	IF	+	+	-	+	+	+
22	01/14	Gloucestershire F1	IF	+	+	+	+	-	+
23	01/14	Gloucestershire F1	IF	+	-	+	+	+	+
24	03/14	FSC	L7	+	-	+	+	-	+
25	03/14	FSC	L11	-	+	-	+	+	-
26	05/14	FSC	IF	+	+	+	+	-	-
27	05/14	FSC	IF	+	+	+	+	-	+
28	05/14	FSC	IF	-	+	-	+	-	-
29	05/14	FSC	IF	+	+	+	+	-	+
30	07/14	FSC	IF	-	+	+	+	+	+
31	07/14	FSC	IF	+	+	+	+	-	-
32	07/14	North Wales F2	L10	+	+	+	+	-	-
33	07/14	North Wales F2	L8	+	+	+	+	-	+
34	07/14	North Wales F2	L12	+	+	+	+	-	+

[a] Abbreviations: F1, Farm 1; F2, Farm 2; FSC, Fallen stock centre.
[b] All isolations are shown for comparison to PCR results. Abbreviations: IF, isolation failed. If isolation was successful the isolated strains are listed.
[c] Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis- like* and *T. pedis* spirochaetes, respectively which are routinely found in dairy cattle BDD lesions.

Table 4.2: PCR detection of treponemes, D. nodosus and F. necrophorum in healthy beef cattle foot tissue biopsies.

Sample	Biopsy date (mo/yr)	Result					
		Specific PCR for group[a]:			*Treponema* PCR	*F. necropho-rum*	*D. nodosus*
		1	2	3			
1	03/14	-	-	-	-	-	-
2	03/14	-	-	-	-	-	-
3	03/14	-	-	-	+	-	-
4	03/14	-	-	-	+	+	-
5	03/14	-	-	-	-	-	-
6	03/14	-	-	-	-	-	-
7	03/14	-	-	-	-	+	-
8	03/14	-	-	-	+	+	+
9	03/14	-	-	-	+	-	-
10	03/14	-	-	-	+	-	-
11	03/14	-	-	-	+	-	-
12	03/14	-	-	-	-	+	-
13	03/14	-	-	-	-	-	-
14	04/14	-	-	-	+	-	-
15	04/14	-	-	-	+	-	+
16	04/14	-	-	-	+	-	+
17	04/14	-	-	-	+	-	+
18	04/14	-	-	-	+	-	+
19	04/14	-	-	-	-	+	-
20	04/14	-	-	-	+	-	-
21	04/14	-	-	-	-	+	+
22	04/14	-	-	-	+	-	-
23	04/14	-	-	-	+	-	+
24	04/14	-	-	-	+	-	-
25	04/14	-	-	-	+	+	-
26	04/14	-	-	-	-	-	-
27	04/14	-	-	-	+	+	-
28	04/14	-	-	-	+	-	-
29	04/14	-	-	-	-	-	-
30	04/14	-	-	-	+	+	-
31	04/14	-	-	-	+	-	-
32	04/14	-	-	-	-	-	-
33	04/14	-	-	-	+	+	-
34	05/14	-	-	-	+	+	-
35	05/14	-	-	-	-	-	+
36	05/14	-	-	-	+	-	-
37	05/14	-	-	-	-	+	+
38	05/14	-	-	-	+	-	+

[a]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis- like* and *T. pedis* spirochaetes, respectively which are routinely found in dairy cattle BDD lesions.

Of the dairy cattle BDD lesion samples 24/43 (56%) and 14/43 (33%) were positive for *D. nodosus* and *F. necrophorum* DNA, respectively. *Fusobacterium necrophorum* and *D. nodosus* was detected in 2/10 (20%) and 2/10 (20%) of healthy dairy cattle foot tissue, respectively.

4.3.3 Statistical Analysis

Chi-square analysis indicated that the proportion of samples positive for the three DD- associated *Treponema* phylogroups, *T. medium*- like, *T. phagedenis*- *like* and *T. pedis* DD spirochaetes, was significantly higher in beef cattle BDD lesion samples than in healthy foot tissue samples (all $P < 0.0001$).

Chi-square analysis indicated that the proportion of beef cattle BDD samples positive for *D. nodosus* was significantly higher in BDD lesions than in healthy beef cattle foot tissue samples ($P = 0.0010$). However, the chi-square test indicated that the proportion of beef cattle BDD samples positive for *F. necrophorum* was not significantly higher in BDD lesions than in healthy beef cattle foot tissue samples ($P = 0.3935$).

Statistical analysis indicated that the proportion of dairy cattle BDD samples positive for *D. nodosus* was not significantly higher in BDD lesions than in healthy dairy cattle foot tissue samples ($P = 0.0911$), and the proportion of dairy cattle BDD samples positive for *F. necrophorum* was also not significantly higher in BDD lesions than in healthy dairy cattle foot tissue samples ($P = 0.6915$).

All *P* values for co-associations (Chi-square analysis) between bacterial species in beef BDD lesions are listed in Table 4.3. In beef BDD lesions, there was a statistically significant co-association between the presence of *T. medium*- like and *T. pedis* DD spirochaetes ($P = 0.0231$). However, there were no statistically significant co-associations identified between any other bacterial species in BDD lesions.

The chi-square test revealed there was no statistically significant co-association between the two non-treponemal bacteria, *D. nodosus* and *F. necrophorum* in healthy foot tissue. It was not possible to perform statistical analysis to identify co-association between the treponemal bacterial in healthy foot tissue as no DD *Treponema* DNA was detected in healthy foot tissues.

Table 4.3: Associations between bacteria present in beef cattle BDD lesions (Chi-squared analysis with P values).

		***Treponema* group[a]:**			***D. nodosus***	***F. necrophorum***
***Treponema* group[a]:**		**1**	**2**	**3**		
	1	-	-	-	-	-
	2	0.8604	-	-	-	-
	3	0.0231*	0.6119	-	-	-
D. nodosus		0.1797	0.0893	0.9612	-	-
F.necrophorum		0.9399	0.8298	0.9467	0.7176	-

[a]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis- like* and *T. pedis* spirochaetes, respectively.
**P* value shows statistical significance.

4.3.4 Culture of spirochaetes and phylogenetic analysis of spirochaete isolates

As part of this study, twenty spirochaetes were successfully isolated from 17/34 (50%) of beef BDD lesion samples (Table 4.1). In some BDD lesion samples, multiple isolates were obtained.

Ten of these isolates (50%) were identified as belonging to the *T. phagedenis-like* spirochaete group, with all sharing 100% 16S rRNA gene sequence identity with the *T. phagedenis- like* DD spirochaete strain T320A (Genbank accession: EF061261), previously isolated from a UK dairy cow BDD lesion (Evans *et al.* 2008).

Four isolates (19%) belonged to the *T. medium*- like spirochaetes. One isolate (3C14) shared 100% 16S rRNA gene sequence identity with *T. medium*- like DD spirochaete strain T19 (Genbank accession: EF061249) previously isolated from a dairy cow BDD lesion in the UK (Evans *et al.* 2008). The remaining three *T. medium*- like spirochaete isolates shared a higher sequence identity (99%) with the *T. medium*- like strain T136E (Genbank accession: FJ204242), also previously isolated from a dairy cow BDD lesion in the UK (Evans *et al.* 2009b), which differ from *T. medium*- like DD spirochaete strain T19 (Genbank accession: EF061249), by a single nucleotide substitution.

Five isolates (25%) belonged to the *T. pedis* spirochaetes. All *T. pedis* isolates shared 100% 16S rRNA gene sequence identity with *T. pedis* T3552B

(Genbank accession: EF061268) previously isolated from a dairy cow BDD lesion in the UK (Evans *et al.* 2008).

Interestingly, one isolate (2L7), on the basis of 16S rRNA gene sequence analysis, shared less than 97% sequence similarity with all currently recognized treponeme species. The phylogroup that isolate 2L7 shared the highest sequence identity to was the *T. medium*- like spirochaetes, sharing 96% 16S rRNA sequence identity. When including clones from relevant metagenomic studies, this novel treponeme shared the highest sequence identity, 97.7%, with *Treponema* clone PT9 (Genbank accession: AM980448) previously identified by 16S rRNA gene sequencing of bacteria from BDD lesions in Denmark (Klitgaard *et al.* 2008). On the basis of the proposal of a new species requiring a maximum sequence identity limit to it's nearest taxonomically defined relatives of 97% 16S rRNA gene similarity (Stackebrandt and Goebel 1994), it may be possible to designate this isolate as a novel species in the near future after additional polyphasic phenotyping and genotyping.

Upon phylogenetic tree analysis, 18/19 beef BDD treponeme isolates separated into three distinct phylogroups corresponding exactly to the three *Treponema* phylogroups commonly isolated from dairy cattle BDD lesions (Figure 4.4). The isolate 2L7, which upon sequence analysis was found to not belong to any of the three commonly isolated DD treponeme phylogroups on the basis of 16S rRNA sequence identity, unsurprisingly formed a separate subgroup with its closest relative *T.* sp. PT9 (Genbank accession: AM980448). However, isolate 2L7 still remained within what can be considered the large cluster of DD *Treponema* and did not cluster with the commensal *Treponema* species.

4.3.5 Comparisons of bacterial presence in beef and dairy cattle BDD and sheep CODD lesions

The results from the data produced in this study and previous data from the historical samples used for comparison are listed in Table 4.4. All beef, dairy and sheep animals with DD were positive for at least one of the three DD

associated *Treponema* phylogroups, with a breakdown of the percentage detection rates of each of the groups present in the table. The *T. medium*- like spirochaetes were present in 79%, 98% and 67% of beef, dairy and sheep DD lesions, respectively. The *T. phagedenis- like* and *T. pedis* spirochaetes were present in 91%, 98%, 85% and 71%, 79% and 71% of beef, dairy and sheep DD lesions, respectively. All three DD- associated *Treponema* phylogroup specific PCR assays did not amplify any DNA in beef cattle, dairy cattle or sheep healthy foot tissues.

The other two lameness associated bacteria investigated, *D. nodosus and F. necrophorum*, were present in 68%, 56%, 59% and 44%, 33%, 71% of beef, dairy and sheep DD lesions, respectively. *Dichelobacter nodosus* and *F. necrophorum*, were present in 26%, 20%, 39% and 33%, 20%, 9% of beef, dairy and sheep healthy foot tissues, respectively.

Figure 4.4: Phylogenetic tree showing the relationship between the treponeme strains isolated here from beef cattle BDD lesions (shown in bold) and other DD associated and commensal treponeme 16S rRNA gene sequences. A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~1,200 aligned bases. Bootstrapped 10,000 times, and only bootstrap values above 70% are shown for clarity. = previously reported 16S rRNA gene sequences from BDD lesions.*

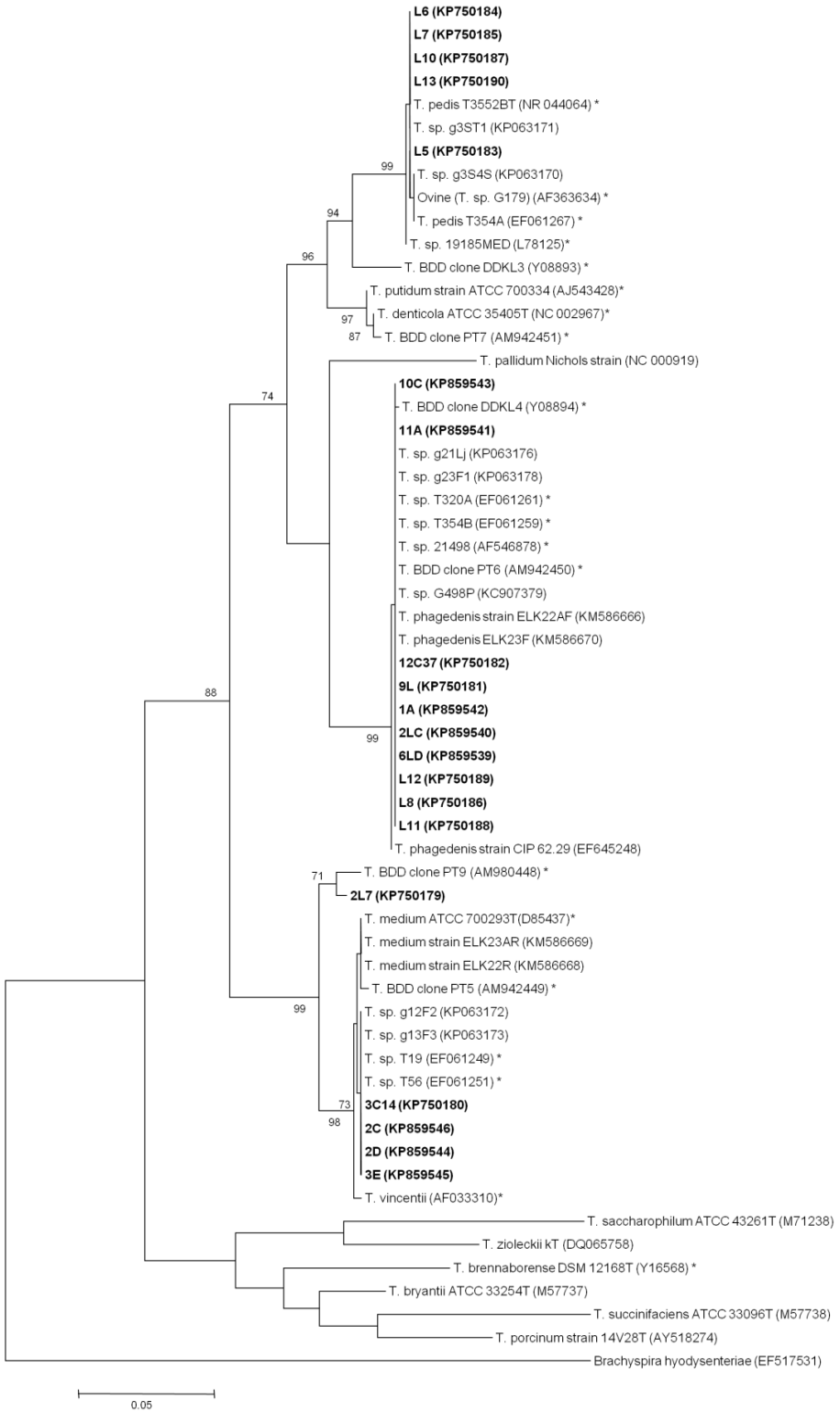

L6 (KP750184)
L7 (KP750185)
L10 (KP750187)
L13 (KP750190)
T. pedis T3552BT (NR 044064) *
T. sp. g3ST1 (KP063171)
L5 (KP750183)
T. sp. g3S4S (KP063170)
Ovine (T. sp. G179) (AF363634) *
T. pedis T354A (EF061267) *
T. sp. 19185MED (L78125)*
T. BDD clone DDKL3 (Y08893) *
T. putidum strain ATCC 700334 (AJ543428)*
T. denticola ATCC 35405T (NC 002967)*
T. BDD clone PT7 (AM942451) *
T. pallidum Nichols strain (NC 000919)
10C (KP859543)
T. BDD clone DDKL4 (Y08894) *
11A (KP859541)
T. sp. g21Lj (KP063176)
T. sp. g23F1 (KP063178)
T. sp. T320A (EF061261) *
T. sp. T354B (EF061259) *
T. sp. 21498 (AF546878) *
T. BDD clone PT6 (AM942450) *
T. sp. G498P (KC907379)
T. phagedenis strain ELK22AF (KM586666)
T. phagedenis ELK23F (KM586670)
12C37 (KP750182)
9L (KP750181)
1A (KP859542)
2LC (KP859540)
6LD (KP859539)
L12 (KP750189)
L8 (KP750186)
L11 (KP750188)
T. phagedenis strain CIP 62.29 (EF645248)
T. BDD clone PT9 (AM980448) *
2L7 (KP750179)
T. medium ATCC 700293T(D85437)*
T. medium strain ELK23AR (KM586669)
T. medium strain ELK22R (KM586668)
T. BDD clone PT5 (AM942449) *
T. sp. g12F2 (KP063172)
T. sp. g13F3 (KP063173)
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
3C14 (KP750180)
2C (KP859546)
2D (KP859544)
3E (KP859545)
T. vincentii (AF033310)*
T. saccharophilum ATCC 43261T (M71238)
T. zioleckii kT (DQ065758)
T. brennaborense DSM 12168T (Y16568) *
T. bryantii ATCC 33254T (M57737)
T. succinifaciens ATCC 33096T (M57738)
T. porcinum strain 14V28T (AY518274)
Brachyspira hyodysenteriae (EF517531)
99
94
96
97
87
74
99
88
71
99
73
98
0.05

Table 4.4: A comparison of PCR detection rates of Treponema species, D. nodosus and F. necrophorum in beef cattle, dairy cattle and sheep DD lesions and healthy foot tissues.

Animal	**DD status**	***Treponema* group[a]**			***D. nodosus***	***F. necrophorum***
		1	**2**	**3**		
Beef cattle	BDD+	27/34 **(79%)**	31/34 **(91%)**	24/34 **(71%)**	23/34 **(68%)**	15/38 **(44%)**
	BDD-	0/38 **(0%)**	0/38 **(0%)**	0/38 **(0%)**	10/38 **(26%)**	12/38 **(32%)**
Dairy cattle	BDD+	42/43 **(98%)**	42/43 **(98%)**	34/43 **(79%)**	24/43 **(56%)**	14/43 **(33%)**
	BDD-	0/10 **(0%)**	0/10 **(0%)**	0/10 **(0%)**	2/10 **(20%)**	2/10 **(20%)**
Sheep	CODD+	39/58 **(67%)**	49/58 **(85%)**	41/58 **(71%)**	34/58 **(59%)**	41/58 **(71%)**
	CODD-	0/56 **(0%)**	0/56 **(0%)**	0/56 **(0%)**	22/56 **(39%)**	5/56 **(9%)**

[a]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis- like* and *T. pedis* spirochaetes, respectively.

4.4 Discussion

In almost all countries where dairy cattle are farmed BDD has been reported. However, there has been a lack of reports and scientific data on not only the manifestation of the disease in beef cattle, but also the bacteriology of beef cattle BDD lesions. Until recently the only study of BDD in beef cattle came from the abattoir studies of Brown *et al.* (2000) in southeast USA. Dairy cattle and beef cattle are obviously bred for very different purposes and are therefore under completely different selection pressures and exposed to various environmental differences. As a result, the aetiology and presentation of BDD

in beef cattle cannot be presumed to be the same as what has been reported in an intensively studied parallel disease in dairy cattle.

The clinical presentation of BDD in beef cattle appears to be identical to lesions found in dairy cattle BDD. Lesions were mostly found on the heel bulb, with an underlying raw proliferative area which appeared erosive and granular with a stippled appearance. Therefore, feet had clear lesions consistent with the clinical signs of BDD in dairy cattle, including the size, location and appearance of lesions (Cheli and Mortellaro, 1974; Blowey and Sharp 1988). Some cases appeared to have progressed to more severe lesions that are commonly seen in dairy cattle BDD, which is possibly attributed to the fact that beef cattle are often less easily observed, restrained and treated than dairy cows where, through milking practices, cattle can be more closely observed.

What is evident from the PCR assay data is that, similarly to dairy cattle, DD treponemes previously detected in dairy cattle BDD lesions are also strongly associated with beef cattle lesions. All beef BDD lesions were positive for at least one of the three DD- associated *Treponema* phylogroups, and most convincingly, no healthy beef cattle foot tissues contained DD- associated *Treponema* phylogroup DNA. Over half (19/34) 56%, of the lesions harboured all three DD- associated *Treponema* phylogroups. Additionally, all three *Treponema* phylogroups were individually detected in between 71% and 91% of lesions, and were unsurprisingly present in a statistically significant proportion of BDD lesions compared to healthy foot tissues. These data, showing the high prevalence of treponemes in the lesions, particularly their presence in all beef BDD lesions and complete absence in healthy beef foot tissues, provides evidence supporting the hypothesis that treponemes may be the primary aetiopathogenic agents in dairy cattle lesions but also may be in beef cattle BDD lesions.

Whilst this study clearly demonstrated a strong association between BDD lesions and specific *Treponema* species, analysis of the data for the other two

lameness associated bacteria, *D. nodosus* and *F. necrophorum*, did not reveal such a close link with disease. *Dichelobacter nodosus* has previously been found in 27% to 100% of dairy cattle BDD lesions (Capion *et al.* 2012; Knappe-Poindecker *et al.* 2013; Rasmussen *et al.* 2013) and *F. necrophorum* detected in between 22% and 44% of dairy cattle BDD lesions (Cruz *et al.* 2005; Klitgaard *et al.* 2008). However, due to their generally lower prevalence in the dairy BDD lesions compared with treponemes, and their frequent (though lesser) presence in healthy foot tissues, a secondary role for these bacteria has been considered more likely. The present study provides further evidence to support this theory, as although *D. nodosus* and *F. necrophorum* were present in 68% and 44% of beef cattle BDD lesions respectively, *D. nodosus* was also present in 26% of healthy beef foot tissues and *F. necrophorum* in 33%. However, *D. nodosus* was found to be in a statistically significant proportion of BDD lesions compared with healthy foot tissues, indicating that this bacteria may play a role in the aetiopathogenesis of the lesions of some animals. In a parallel analysis, dairy BDD lesions investigated for the presence of *D. nodosus* and *F. necrophorum* produced similar results and conclusions. *Dichelobacter nodosus* was present in 56% of dairy BDD lesions compared with 20% of healthy foot tissues, and *F. necrophorum* present in 33% of dairy BDD lesions versus 20% of healthy dairy foot tissues. Both of these bacteria were not found to be in a strong and specific association with dairy BDD lesions compared with dairy healthy foot tissue. However as with all the results in this study care has to be taken when interpreting the data due to small sample sizes and possible other influencing factors. Associations could be due to true link between these bacteria and lesions, but could also be due to other unknown factors.

The bacteriological data, taken as a whole for both beef and dairy cattle BDD lesions, clearly indicates that treponemal bacteria probably play a primary causative role in both beef and dairy cattle BDD lesions. However, due to the large proportion of lesions containing *D. nodosus* and PCR assay data failing to provide a timeline of bacterial invasion into tissue, it does have to be

considered that *D. nodosus* may play a role in the disease process of BDD in cattle.

Fusobacterium necrophorum appears to have a low association with BDD lesions in both beef and dairy cattle, however in sheep CODD lesions, *F. necrophorum* appears to be significantly disease associated. *Fusobacterium necrophorum* was present in 71% of lesions and in healthy sheep foot tissues was found to be present in only 9% of tissues (Chapter 3; Sullivan *et al.* 2015b). This is interesting as it appears that although both of these lameness associated bacteria are present in a proportion of both cattle and sheep BDD lesions, *F. necrophorum* appears to be sheep associated and *D. nodosus* cattle associated. However, it could be interpreted that the data presented here clearly suggests that *Treponema* are primary invaders in CODD and that *D. nodosus* and *F. necrophorum* are likely to be secondary invaders of established lesions.

When comparing the prevalence of the individual *Treponema* phylogroups between beef cattle, dairy cattle DD and sheep CODD lesions, little differences were found. From the samples analysed in this study, and previously collected data (Evans *et al.* 2009b; Chapter 3; Sullivan *et al.* 2015b), it can be seen that very similar proportions of DD lesions across all three animals were found to contain *T. phagedenis- like*, *T. pedis* and *T. medium*- like DD spirochaetes, ranges 85% - 98%, 71% - 79%, 67- 98% respectively (Table 4.4). Consistently across all three animal groups *T. phagedenis- like* spirochaetes are the most commonly detected treponeme phylogroup, and therefore possibly the most promiscuous or pathogenic of the three commonly isolated DD *Treponema* phylogroups.

The fastidious nature of treponemes makes it extremely difficult to successfully isolate them from tissue and swab samples. However, in this study twenty treponemes were isolated from beef BDD lesions, and included all three DD- associated *Treponema* phylogroups. To the surprise of the authors, one of the treponemes cultured (2L7), although closely related to the *T. medium*- like DD spirochaete group (96%), is actually a genetically distinct treponeme, not

belonging to any of the three DD *Treponema* phylogroups commonly isolated from dairy cattle BDD lesions. This novel treponeme still clustered closely with the pathogenic DD treponemes, and was distinctly separate from the commensal treponemes upon phylogenetic analysis. This, together with it's isolation from an active BDD lesion, provides evidence towards a role as a pathogenic treponeme. Although extensive work has been carried out on dairy cattle BDD lesions it has not yet been isolated from their lesions. However, it was closely related (97.7%) to *T.* sp. PT9 (Genbank accession: AM980448) which was detected from cattle BDD lesions in Denmark (Klitgaard *et al.* 2008). On the basis of 16S rRNA gene sequence similarity, it could be the case that this previously detected treponeme, *Treponema* clone PT9 (Genbank accession: AM980448) and the *Treponema* isolate 2L7 from this study represent a new taxa. The other possibility is that the treponeme isolated in this study forms a separate taxa to this previously detected treponeme. Further phenotypic studies are required before it can be determined whether they represent the same or novel taxa. Additionally, whether this treponeme is a new phylogroup of treponemes involved in all species DD lesions but has yet to be isolated from other lesions remains unknown. It does however suggest that other treponemal species may be playing a role in DD lesions than those we frequently isolate.

Beef cattle BDD lesions, according to anecdotal reports from vets/farmers, have increased in prevalence in recent years. Prevalence estimates of BDD in dairy cattle herds range from between 20 and 30 per cent (Brown *et al.* 2000; Cramer *et al.* 2008; Barker *et al.* 2009) and a more recent study found lesions in 62% (460/742) of dairy cattle studied (Nielsen *et al.* 2012). However, the only available data (Brown *et al.* 2000; this chapter; Sullivan *et al.* 2013, 2015a) appears to suggest a lower within-herd prevalence rate in beef cattle (0.5-21%), than is commonly found in dairy cattle. It is interesting to speculate whether the so far reported farm prevalence rates of beef cattle BDD are either an underestimate of the true prevalence of BDD in these animals, or that these beef animals are truly contracting the disease less than dairy cattle.

Now with possible forms of DD in other species now being reported, with the same *Treponema* phylogroups detected in the lesions, this disease is clearly increasing in importance, geographical spread and host range. Therefore understanding the aetiopathogenesis of DD is key to developing means of managing and preventing the spread of this disease. It is now clear from this study that the DD treponemes detected and isolated from dairy cattle and sheep DD lesions are also present in all beef BDD lesions and absent in healthy feet tissue samples. This supports treponeme bacteria as the primary infective aetiological agent. However, this also raises concern over the transmission of treponemes between species on farms, especially for sheep and beef cattle which are commonly kept on the same premises.

The similarities in the clinical manifestations, treponeme isolates and prevalence's of treponeme bacteria in beef cattle, dairy cattle and sheep DD lesions, importantly that all lesions contain at least one of the commonly associated DD *Treponema* phylogroups, provides more reason for more vigilance to be taken in terms of cross-species transmission. These treponemes seem to be highly infectious, and are now causing disease in previously unaffected host species, including wildlife. Therefore, understanding the aetiology of DD in farm animals is imperative to limit disease spread and aid future treatments and control measures. This study provided vital, and the first reported, clinical and bacteriological information on beef cattle BDD lesions.

Chapter 5

Investigating the presence of *Treponema* spp. in the ruminant gastrointestinal tract - a metagenomics approach

5.1 Introduction

Whilst it is fairly clear that specific treponemes are the primary infectious agent in BDD and CODD, the infection reservoir and route(s) of transmission are not well defined. Evans *et al.* (2009b) potentially identified a route of transmission into foot skin via hair follicles. However, it is not known if there are other routes or whether there are, apart from the foot lesions, other sites of DD-treponeme infection in cattle or sheep. A PCR approach, using primers to specifically detect DD- associated treponemes failed to find many positive tissues, although there was preliminary molecular data showing DD treponemes in gingival tissues and at the recto-anal junction in BDD cows. An investigation is needed is to determine whether treponemes are present in GI tract tissues and whether they are related to BDD/CODD and can be influenced by external factors such as nutrition.

Fortunately, recent developments in molecular genomics means that such an approach is realistic and, conveniently, can use other researchers published datasets and interrogate them with specific questions. This approach is called "metagenomics" and is based on global sequencing of all organisms in a given

population and enables comparisons of the ranges of organisms between populations. In a farm environment, this is a really powerful tool as it enables really complex infective environments to be analysed for microbial populations without relying upon the need to culture and isolate individual organisms. This is a huge step forward in our analytical ability of microbial populations and can be put to considerable practical usage, particularly in veterinary science. Thus, quite complex biological questions may be asked of the many metagenomic databases which are often freely accessible via the internet. Similarly to other chapters within this thesis particular attention will be paid to the 16S rRNA gene. However, in the case of this chapter focus will be on what is known as the 16S microbiome. Microbiomes are the collective genetic material of the microorganisms that reside in an environmental niche, in the case of the 16S microbiome, the collective 16S genetic material from the variety of organisms present in the niche. As this chapter will focus solely on the 16S rRNA gene, although a "metagenomic approach" will be taken, it may be said the 16S microbiome within the niches was investigated.

In terms of the roles of treponemes in BDD and CODD, databases may be interrogated to provide answers to several questions, such as: What can cause increases in treponeme numbers and variety in the farm environment and within animal tissues? Where are the DD- associated treponemes harboured? There has actually been preliminary evidence for the presence of DD treponemes in the bovine GI tract by Evans *et al.* (2012).

Cattle and sheep are ruminants so their digestive system is very different from humans, dogs and horses; the key difference being the presence of four stomach compartments, the first of which is called the rumen. The rumen contains bacteria and a range of other microbes that have the task of digesting the cellulose in the plants they eat. The cow regurgitates, chews and then swallows the food several times in a process known as "ruminating" .The combination of the microbes in the rumen and the cow chewing and re-chewing food enables food to break down sufficiently for the nutrients to be absorbed by the animal. Different diets are used depending on the time or year

or personal preference. Forage feeds are fed such as the mainstays of grass, and in addition, crops like stubble, turnips, kale, forage rape, forage rye, forage peas, can all be grazed. Most of these crops are also stored or ensiled for winter feeding, where they are joined by a vast range of conserved forages such as grass silage, maize silage, wholecrop cereal silage, alkalage, hay and straw. High-concentrate diets such as cottonseed hulls, soybean hulls, corn gluten feed, brewer's grain, distillers dried grains, and rice meal are used because they can increase production (Fernando *et al.* 2010). They are also used in high-yielding dairy cattle during mid to late lactation to help them to restore their body condition (Vazquez-Añon *et al.* 1997).

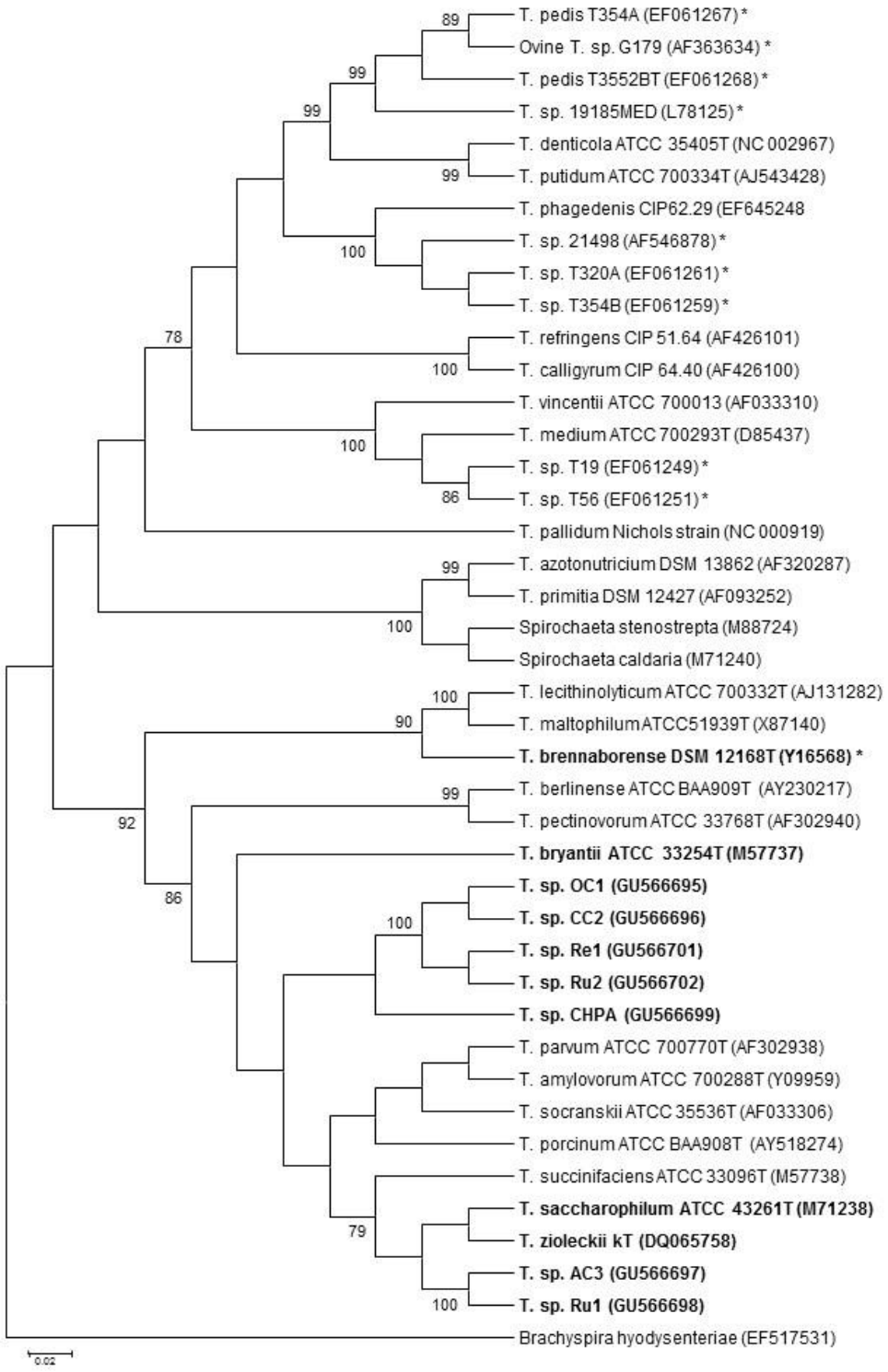
Ruminant species contain GI tract *Treponema* species which are non-pathogenic and live in the rumen as commensals (Paster and Canale-Parola, 1985; Evans *et al.* 2011b). If these treponemes are able to survive in the GI tract, it seems highly plausible that pathogenic species of treponemes may also reside in parts of the GI tract. This would even provide a possible transmission route for the treponemes via passage through the GI tract and then through the rectum and passed on to faeces which are then spread in to the environment. This would also provide an explanation for the association between low levels of hygiene on farm and DD.

Although these are commensal treponemes in the rumen and faeces, it is interesting to analyse any increases/decreases in these commensal species in response to environmental factors, such as diet, as it is accepted that a variation in the diet and the microenvironments of the bovine GI tract can lead to alterations in the diversity and composition of many bacterial communities within the GI tract (Kong *et al.* 2010; Chen *et al.* 2011; Kim *et al.* 2011). The treponemes that have been found in ruminant GI tracts appear to be very clearly genotypically and phenotypically different to pathogenic DD treponemes (Figure 5.1).

This investigation aims to use previously collected data to survey the effects of diet on the frequency and abundance of species of treponemes in the GI tract of cattle and sheep. If certain conditions increase the levels of commensal

Treponema in the GI tract, it may be possible this affects the rumen conditions enabling pathogenic treponemes (if present) to also increase, or cause them to be shed from the GI tract into the farm environment. Additionally, this study will survey previously collected metagenomic databases of cattle and sheep GI tract bacterial communities for the actual presence of DD treponemes.

Figure 5.1: Phylogenetic tree of treponemes from the bovine GI tract (shown in bold) based on alignment of 16S rRNA gene sequences for comparison with other isolated treponemes. Comparisons are over 1,320 aligned bases. Bootstrap confidence levels are shown as percentages of nodes, and only values above 80% are shown. The model for nucleotide substitutions chosen was TrN model (Tamura and Nei 1993). GenBank accession numbers are shown next to each strain. = previously reported 16S rRNA gene sequences from BDD lesions.*


T. pedis T354A (EF061267) *
Ovine T. sp. G179 (AF363634) *
T. pedis T3552BT (EF061268) *
T. sp. 19185MED (L78125) *
T. denticola ATCC 35405T (NC 002967)
T. putidum ATCC 700334T (AJ543428)
T. phagedenis CIP62.29 (EF645248
T. sp. 21498 (AF546878) *
T. sp. T320A (EF061261) *
T. sp. T354B (EF061259) *
T. refringens CIP 51.64 (AF426101)
T. calligyrum CIP 64.40 (AF426100)
T. vincentii ATCC 700013 (AF033310)
T. medium ATCC 700293T (D85437)
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. pallidum Nichols strain (NC 000919)
T. azotonutricium DSM 13862 (AF320287)
T. primitia DSM 12427 (AF093252)
Spirochaeta stenostrepta (M88724)
Spirochaeta caldaria (M71240)
T. lecithinolyticum ATCC 700332T (AJ131282)
T. maltophilum ATCC51939T (X87140)
T. brennaborense DSM 12168T (Y16568) *
T. berlinense ATCC BAA909T (AY230217)
T. pectinovorum ATCC 33768T (AF302940)
T. bryantii ATCC 33254T (M57737)
T. sp. OC1 (GU566695)
T. sp. CC2 (GU566696)
T. sp. Re1 (GU566701)
T. sp. Ru2 (GU566702)
T. sp. CHPA (GU566699)
T. parvum ATCC 700770T (AF302938)
T. amylovorum ATCC 700288T (Y09959)
T. socranskii ATCC 35536T (AF033306)
T. porcinum ATCC BAA908T (AY518274)
T. succinifaciens ATCC 33096T (M57738)
T. saccharophilum ATCC 43261T (M71238)
T. zioleckii kT (DQ065758)
T. sp. AC3 (GU566697)
T. sp. Ru1 (GU566698)
Brachyspira hyodysenteriae (EF517531)
0.02

5.2 Methods

5.2.1 Finding appropriate bacterial community databases

A literature search was performed using the National Center for Biotechnology Information (NCBI) Pubmed website (NCBI 2013b) and relevant databases were identified. These were generated from analyses of the bacterial content of the cow (dairy, beef or hybrids) and sheep rumen, faeces and manure. Also, data was available from studies which had looked more specifically at the bacterial diversity within the rumen epithelium.

The metagenomic databases gave the 16S rRNA gene sequence for bacteria found in the studied material. The reason for identifying species by the 16S rRNA gene is that it is used as the standard for classification and identification of microbes, because it is present in most microbes and is adequately conserved for PCR and sequencing from different species of bacteria.

Some of these studies had compared the rumen bacteria/epithelial bacteria before and after a change in diet. These studies investigated either the whole 16S rRNA bacteria content of the tissue investigated in the GI tract (metagenomic databases produced), or investigated the frequency of specific bacteria using oligonucleotide primers (e.g. specific for treponeme species).

5.2.2 Source of metagenomic data for cow and sheep rumen contents, tissues and faeces

All metagenomic studies used in this present study to investigate the presence of treponemes in the GI tract, or the effect of diet on the bacteria in the GI tract are listed in Table 5.1. Some studies may appear more than once in the table as they studied more than one location ie. both faeces and rumen content were investigated.

Table 5.1: Detailed information of previous studies used in this investigation to look for the presence of DD treponemes in the GI tract.

Area studied	**Reference**	**Animal (s) / area sampled[a]**	**Location of study**	**Spirochaetes present**	**Treponemes present[b]**	**Change in diet/other experimental change?**
Faeces	Hook *et al.* 2011	Dairy cattle- 4 (P)	Canada	-	-	-
	Patton *et al.* 2009	Dairy cattle- 19 (IN)	Minnesota	+	+ (9) *Treponema Candidatus T. suis* AM284386 99%	-
	Ozutsumi *et al.* 2005	Dairy cattle- 3 (IN)	Japan	-	-	-
	Durso *et al.* 2010	Beef cattle- 6 (IN)	California	-	-	-
Manure	Hanajima *et al.* 2011	Dairy farm manure- 1 (IN)	Japan	+	-	Slurry aerated at different intensities
	Ravva *et al.* 2011	Dairy farm manure- 2 (IN)	California	+	+ (7) *Treponema Candidatus T. suis* AM284386 99%	-
Rumen contents	Kong *et al.* 2010	Dairy cattle- 4 (P)	Canada	-	-	Diet comparison- different forages
	Tajima et a., 2001	Dairy cattle – 8 (P)	Japan	+	+ *Treponema bryantii* (M57737) 100% (FU)	low vs. High concentrate diet
	McGarvey *et al.* 2010	Dairy cattle- 12 (IN)	California	-	-	Diet comparison- monensin absent and present
	Kim *et al.* 2011	Dairy cattle- 4 (IN)	Czech Republic	+	+ (4) *Treponema bryantii* (M57737) >97%	Diet comparison- Forage vs. Forage and concentrates
	Hook *et al.* 2011	Dairy cattle- 4 (P)	Canada	-	-	-
	Karnati *et al.* 2007	Dairy cattle- 8 (IN)	Ohio	-	-	Diet comparison- different supplemental methionine
	Tajima *et al.* 1999	Dairy cattle- 2 (P)	Japan	+	+ (2) *Treponema bryantii* (M57737) >97%. (1)- Unknown	-
	Tajima *et al.* 2000	Dairy cattle- 8 (P)	Japan	-	-	low vs. High concentrate diet
	Whitford *et al.* 1998	Dairy cattle- 5 (P)	Canada	-	-	-
	Lee *et al.* 2012	Dairy cattle- 3 Beef cattle- 6 (P)	Korea	+	+ (18) *Treponema bryantii* (M57737) >97%	-
	Uyeno *et al.* 2007	Dairy cattle- 4 (P)	Japan	-	-	-
	Ozutsumi *et al.* 2005	Dairy cattle- 7 (P)	Japan	+	+ (3) *Treponema bryantii* (M57737)	Protozoa effect- faunated cattle vs. Unfaunated.

					98%, (1) *Treponema Candidatus T. suis* AM284386 94%	
	Wang *et al.* 2005	Dairy cattle- (FU)	China	+	+ (1) *Treponema bryantii* (M57737) 89%	-
	Tajima *et al.* 2007	Dairy cattle- 8 (P)	Japan	+	+ (3) *Treponema bryantii* (M57737) >97%	Temperature and humidity changes
	Li *et al.* 2012b	Dairy cattle- 4 (IN)	California	+	+ (48) *Treponema bryantii* (M57737) >97%. (18) *Treponema refringens* (AF426101) >90%. (5) *Treponema Candidatus T. suis* AM284386 90%.	Butyrate infusion into the rumen
	Perumbakkam and Craig 2011	Dairy cattle- 2 (P) Sheep- 3 (P)	Oregon	+	+ (1) *Treponema porcinum* (NR042942) 92%.	-
	Hernandez *et al.* 2008	Dairy cattle- 16 (P)	Australia	-	-	-
	Stevenson and Weimer 2007	Dairy cattle- 2 (IN)	Wisconsin-Madison	-	-	-
	Ohene-Adjei *et al.* 2008	Dairy cattle (FU)	Canada	+	+ (8) *Treponema bryantii* (M57737) >92%	-
	Pitta *et al.* 2010	Beef cattle- 14 (P)	Texas	+	+ (FU)	low vs. High concentrate diet
	Fernando *et al.* 2010	Beef cattle- 8 (P)	Kansas state	+	+ (5) *Treponema bryantii* (M57737) >97%. (29) *Treponema Candidatus T. suis* AM284386 >91%. (1) *Treponema zioleckii st. kT* (DQ065758) 98%.	low vs. High concentrate diet
	Brulc *et al.* 2008	Beef cattle- 3 (P)	California	+	-	-
	Leng *et al.* 2012	Beef cattle- 4 (P)	China	-	-	-
	An *et al.* 2005	Beef cattle- (FU)	China	+	+ (1) *Treponema sp. Ru1* (GU566698) 98%.	-
	Bekele *et al.* 2011	Sheep- 3 (IN)	Japan	+	+ (76) *Treponema bryantii* (M57737) >97%. (1) *Treponema zioleckii* st. kT (DQ065758 >97%. (70) uncultured ruminal treponemes >97%. (166) Uncultured treponemes 88-97%.	Diet change- Alfalfa hay vs. Orchardgrass hay vs. Concentrate diet.
	Perumbakkam *et al.* 2011	Sheep- 4 (P)	Oregon	+	+ (4) *Treponema bryantii* strain NK4A124 (GU324416) >99%. (1) *Treponema bryantii* (M57737) 98%. *Treponema*	Diet- addition of 2,4,6-trinitrotoluene to diet.

					socranskii clone OE059 (JN713251) 92%. (1) *Treponema porcinum* strain 14V28 (NR042942) 88%.	
	Koike *et al.* 2003	Sheep- 2 (IN)	Japan	+	+ (2) *Treponema bryantii* strain NK4A124 (GU324416) 94%. (2) *Treponema bryantii* strain RUS-1 (NR104781) >93%.	Diet change- Alfala hay vs. Orchardgrass hay
	Kenters *et al.* 2011	Sheep-1 (IN)	New Zealand	+	+ (2) *Treponema bryantii* (M57737) >97%.	-
	Perumbakkam and Craig 2012	Sheep- 1 (IN)	Oregon	-	-	Addition of 1,3,5-Trinitro-1,3,5-triazacyclohexane (RDX) to rumen.
Rumen epithelial tissue	Li *et al.* 2012a	Beef cattle- 3 (IN)	Canada	+	+ (4) *Treponema bryantii* strain NK4A124 (GU324416) 88-98%. (5) *Treponema bryantii* strain RUS-1 (NR104781) 90-99%. (1) Treponema brennaborense DSM 12168 (CP002696) 89%. (1) *Treponema Candidatus T. suis* AM284386 93%. (5) *Treponema refringens* (AF426101) 93%.	-
	Sadet-Bourgeteau *et al.* 2010	Sheep- 8 (IN)	France	+	+ (1) *Treponema refringens* (AF426101) 90%.	low vs. High concentrate diet

[a]Animals studied indicates whether the samples were taken from a beef, dairy or sheep animal. The number indicates the amount of animals sampled. In brackets abbreviations, P; the study pooled samples from all animals studied, IN; all animals investigated had their samples individually sequenced rather than pooling the samples. In case of manure samples the number indicates how many farms the manure was taken from. FAU; frequency of animals unknown.
[b]Abbreviation FU; Frequency of bacteria unknown. If obtainable frequency is listed in brackets and closest cultured relative.

5.2.3 Analysing the databases for the presence of treponemes using BLAST

Identification of *Treponema* present in all these bacterial databases were determined using the Basic Local Alignment Search Tool (BLAST) which is available as part of the NCBI website (NCBI 2013a).

As part of the website, BLAST finds regions of local similarity between sequences of your choice. The program compares nucleotide or protein sequences to its sequence databases and calculates the statistical significance of matches. It allows comparison of whole databases of sequences with a

known species of interest in order to identify similar sequences in bacterial data. This software is available on the NCBI website.

Using BLAST, each database of sequences was uploaded and the alignment tool was used to compare the database to a representative GI *Treponema* species, *T. bryantii* strain RUS-1 (Genbank accession: M57737) and a representative DD treponeme *T. phagedenis* strain T320A (Genbank accession: EF061261) which were used as the "query sequences". The query sequence is the sequence which all the sequences in the database are compared to. The output is a list of alignment results between the query and the database subjects ranked based on length and significance. This allowed for identification of the most similar nucleotide sequences in the gene databases related to *T. bryantii* strain RUS-1 and *T. phagedenis* strain T320A. Any sequence which was listed as having above 70% query coverage (the length of query sequence that matches the subject sequence), and had a minimum identity of 82% (the highest percent identity the set of aligned segments to the same subject sequence) was saved. This enabled the exclusion of all other bacteria in the databases that were not spirochaetal bacteria. If all sequences in a database had below 70% query coverage (i.e. all were short sequences) just the maximum identity was used.

This was carried out for all databases, including databases which looked specifically at the effect of diet on the GI tract bacterial content.

5.2.4 Determining the relatedness of spirochaetes found in the GI tract of cattle and sheep to DD treponemes

The 16S rRNA gene sequence relatedness of spirochaetes found in the databases to the DD treponemes and ruminal treponemes was determined using BioEdit (version 7.0.9.0, Hall 1999)**.** Using Bioedit enabled a more accurate estimate of relatedness between the 16S rRNA gene sequences, which would help in determining the exact amount of shared sequence identity of the spirochaete sequences with the known treponemes.

Each database was opened in Bioedit and all sequences removed excluding the highest scoring sequence alignments to the two *Treponema* species found in BLAST. For further investigation of the relevant 16S rRNA sequences, Bioedit was used to determine the range of *Treponema* species in the databases and in particular, DD- associated *Treponema* or treponemes highly similar to DD-associated *Treponema* (putative DD *Treponema*). The 16S rRNA sequences were aligned using multiple sequence alignment program, ClustalW 1.81 (Thompson *et al.* 1994). The alignments were trimmed to the shortest sequence to allow effective comparisons of the sequences.

Nucleotide sequence identity matrices were produced for the aligned and trimmed sequence alignments in Bioedit. This gave a comparison between each sequence compared with every other sequence in the alignment, with sequence identity scores listed for each set of sequences between 0.00 (zero identity) and 1.00 (complete identity). The sequence identity matrices were used to determine the relatedness of the spirochaetal bacteria from the databases to the DD treponeme and the GI tract treponeme.

In previous phylogenetic analyses of the spirochaetes, it was found that there were two distinct large subgroups of treponemes (Paster *et al.* 1991; Evans *et al. 2*011b). Furthermore in terms of bovine treponeme sequences it is known that the DD treponemes are closely related to one of those subgroups and GI tract treponemes to the other (Evans *et al.* 2011b). Paster *et al.* (1991) found that interspecies similarity within the group in which DD treponemes are located was 89.9%, interspecies similarity within the GI tract treponeme group was 86.2% and interspecies similarity between the two groups was 84.2% (0.842). The *Treponema* group has an average interspecies similarity of 81.9% (0.819) (Paster *et al.* 1991).

Using this work it was determined that sequences with a similarity of 82% (0.82) or more when compared to the DD or GI tract treponeme were treponemes. Sequences that had a similarity of 90% (0.9%) or more when compared to the DD treponeme were considered to be within that subgroup and those that had a similarity of 86.2% (0.862) when compared to the GI tract

treponeme were considered to be within the commensal GI tract subgroup (Paster *et al.* 1991). To be considered as the same species to which they were being compared to, the sequences had to have a similarity of 97% (0.97) or greater (Goebel and Stackebrandt 1994).

Any spirochaetal bacteria found to have over 97% (0.97) sequence identity to the ruminal treponeme (and therefore part of the same species) were not further analysed as these are identified as non DD- associated treponemes.

Spirochaetal bacteria with over 97% sequence identity to any of the DD-associated treponemes and any spirochaete found to have a higher sequence identity to the DD- associated treponemes than to the ruminal treponemes, was selected for subsequent phylogenetic tree analysis.

5.2.5 Phylogenetic tree analysis

As there have been many treponemes isolated from DD lesions, to give a better picture of how closely related the treponeme sequences found in metagenomic databases were to DD- associated treponemes, phylogenetic analyses was performed (Method 2.6) including several DD and GI tract treponeme isolates.

Reference strains were added to the file be aligned using the ClustalW Multiple Alignment. The spirochaetes were the DD- associated treponemes; *T. medium*-like strains T19 (EF061249) and T56 (EF061251), *T. phagdenis*- like strains T320A (EF061261) and T354B (EF061259) and *T. pedis* strains T3552B^{T} (EF061268) T354A (EF061267) which are representatives from each of the three unique treponeme groups previously isolated from BDD lesions (Evans *et al.* 2008). Additionally, sixteen other treponemes previously isolated from cattle BDD lesions in five studies (Choi *et al.* 1997; Walker *et al.* 1999; Trott *et al.* 2003; Evans *et al.* 2008; Klitgaard *et al.* 2008) and a sheep treponeme isolate from a sheep CODD lesion was added (Demirkan *et al.* 2001).

The rumen treponeme, *T. bryantii* strain RUS-1 ATCC 33254T (M57737) (Stanton *et al.* 1991) was included. Fifteen other reference treponeme strains from the rumen were included from six studies (Paster *et al.* 1991; Tajima *et*

al. 1999, 2007; Yang *et al.* 2010; Van De Vossenberg and Joblin 2002; Evans *et al.* 2011b). Seven treponeme strains isolated from bovine faeces were also included (Patton *et al.* 2009).

Treponema brennaborense DSM 12168^{T} (Y16568) (Schrank *et al.* 1999) was included in the analysis, as this treponeme was isolated from a BDD lesions but curiously clusters with the GI tract treponemes.

An out group was added, 16S rRNA sequence of *Brachyspira hyodysenteriae* strain AN1082/90 (EF517531) (Råsbäck *et al.* 2007b).

Each database in which treponemes of interest were found, were analysed separately, producing separate phylogenetic trees comparing each set of treponemes with GI tract treponemes and DD- associated treponemes.

After distinguishing which DD- associated treponeme isolates the sequences clustered with, further analysis was performed. This involved producing sequence identity matrices to compare all the treponemes found to their closest related known *Treponema* species (the treponemes the sequences clustered with upon phylogenetic analyses) and to each other.

5.2.6 Diet associated fluctuations in *Treponema* frequency and relative abundance in the GI tract

Although the previous analyses focused on looking specifically for DD-associated treponemes in all databases, the studies investigating the effect of diet on the bacterial content of the GI tract were then analysed to determine the frequency of general *Treponema* (all species) before and after the diet changes. As these databases had already had their metagenomic data analysed for treponemes (using BLAST), this simply involved determining the frequency of treponemes in each dataset (before and after diet change) regardless of the species of treponemes, which in some cases the study had already done. However there were some limitations, in some cases the studies focusing on diet associated bacterial shifts did not use whole genomic methods (metagenomics) but used specific 16S rRNA primers to look specifically for certain bacteria ie. *T. bryantii*.

5.3 Results

5.3.1 Literature search for ruminal 16S rRNA bacterial databases

Thirty seven suitable bacterial databases were found. All of which had gathered 16S rRNA sequence data from the rumen (contents/liquid fraction) (*n*= 29), rumen epithelial tissue (*n*= 2) or faeces (*n*= 4), and manure (*n*= 2).

Initial investigations of the databases determined the presence of any treponemes bacterial sequence present using BLAST. It revealed the presence of treponemes in 21 of the 37 datasets (57%). 17/29 (59%) of rumen content databases contained treponemes, 2/2 (100%) of the rumen epithelium databases, 1/4 (25%) of faeces databases and 2/2 (100%) manure databases.

5.3.2 Determining the relatedness of spirochaetes found in the GI tract of cattle and sheep to DD treponemes

The matrices produced in Bioedit revealed the relatedness of the treponemes found in the 21 databases to GI treponemes (using *T. bryantii* strain RUS-1 as a reference) and DD treponemes (using *T. phagedenis* strain T320A as a reference).

Most of the treponemes found were near identical on the basis of 16S rRNA gene sequence identity to either known commensal *Treponema* species or closely related to such species (>97% sequence identity to the GI tract treponemes within the matrix, or had a higher sequence identity to the GI tract treponeme than to the DD- associated pathogenic treponeme in the matrix). If after a BLAST analysis of the closely related GI treponemes proved them to not belong to a specific species of treponeme, it is likely that these treponemes were yet to be cultured, characterised and taxonomically appraised ruminal treponemes.

No databases were identified as containing known DD- associated treponemal phylogroups (>97% sequence identity to the DD treponeme in the sequence identity matrices).

Three of the databases contained unknown species of *Treponema* (*n*= 24), belonging neither to commensal *Treponema* nor DD- associated *Treponema* species, but had a higher 16S rRNA sequence identity to DD- associated treponemes. An example of the produced sequence identity matrices is shown in Table 5.2, which contains all five unknown treponemes from database 1, from the study by Li *et al.* (2012a), showing how the treponemes with a higher sequence identity to DD treponemes were distinguished. The treponemes which were found to have a higher 16S rRNA sequence identity to DD-associated treponemes were considered as putative DD treponeme clones, belonging to the DD treponeme large phylogenetic cluster.

Table 5.2: Matrix describing the 16s rRNA gene sequence similarity between sequences of interest in the database from the study by Li et al. (2012b). A sequence identity matrix resulting from 16S rRNA gene sequence analysis of sequences identified from database 1 compared to a BDD treponeme (T. sp. T320A) and a commensal GI tract treponeme (T. bryantii strain RUS-1*). Highlighted are the percentage sequence identities to the known treponemes, given as an proportion of 1. 'ID' indicates the sequences have identical 16S rRNA gene sequence identity. In yellow are values corresponding to relatedness of the unknown treponemes found with known DD- associated treponeme (T. sp. T320A) and commensal ruminal treponeme (T. bryantii strain RUS-1).*

	L406RT-1-G07	**L406RT-6-H06**	**L406RT-5-D08**	**L406RT-5-D09**	**L406RT-6-A08**	***T. sp. T320A***	***T. bryantii*** strain RUS-1
L406RT-1-G07	ID	0.994	0.994	0.994	0.994	0.867	0.771
L406RT-6-H06	0.994	ID	1	1	1	0.872	0.776
L406RT-5-D08	0.994	1	ID	1	1	0.872	0.776
L406RT-5-D09	0.994	1	1	ID	1	0.872	0.776
L406RT-6-A08	0.994	1	1	1	ID	0.872	0.776
T. sp. T320A	0.867	0.872	0.872	0.872	0.872	ID	0.818
***T. bryantii* strain RUS-1**	0.771	0.776	0.776	0.776	0.776	0.818	ID

Databases were taken from the following studies: Li *et al.* (2012a), (Sadet-Bourgeteau *et al.* (2010), and Li *et al.* (2012b) which are referred to as database 1, database 2 and database 3, respectively. Information on the databases and their corresponding studies is given in Table 5.3. Database 2 and 3 are both papers which were also used in the diet analysis. Database 1 did not change any environmental variables whilst determining the bacterial content of the rumen epithelium and contained seventeen treponemes of which five were putative DD treponeme clones with a higher sequence identity to DD treponemes. In database 2 there was one treponeme found (Uncultured rumen bacterium clone 13-P5 (AM884113)), and this single treponeme was again a putative DD treponeme clone. Database 3 contained 10977 treponemes. However, which of these sequences were found in the rumen before/after the infusion of butyrate is not provided, so this number is the amount of treponemes found from a combination of four dairy cows at 6 different time points from 0hr to 16hr after ruminal butyrate infusion. Eighteen of these were treponemes of unknown species related closely to DD treponemes. The remaining were showed either >97% 16S rRNA relatedness to *T. bryantii* strain RUS-1 (M57737) or were more closely related to commensal treponemes than DD- associated treponemes.

Table 5.3: Putative DD treponeme clones *closely related to DD- associated treponemes identified from ruminal bacterial sequence databases.*

Study	**Database**	**Animal tested**	**Location**	**Experime-ntal change?**	**Average bp length of sequences**	**Number of sequences analysed**	**Total *Treponema***	**Putative DD treponeme clones**
Li *et al.* 2012a	1	Four beef steers	Rumen epithelium	N/A	1500	2785	17 (all 1 animal)	5
Sadet-Bourgeteau *et al.* 2010	2	Sheep	Rumen epithelium	Alfalfa hay diet to high concentrate and back to hay	810	2010	1 (after high conc. diet then switch back to hay)	1
Li *et al.* 2012b	3	Four dairy cows	Rumen content	Ruminal butyrate infusion	600	75594	10977 (combined no. before and after infusion)	18

5.3.3 Phylogenetic analysis of the unknown putative DD treponeme clones

Upon phylogenetic analysis the previously identified ruminal treponemes formed their own cluster, as did the DD- associated treponemes. However, the putative DD treponeme clones (*n= 24)*, from all three databases) did not cluster with ruminal treponemes as would be expected from their origin. It was revealed that these putative DD rumen treponeme clones clustered with the DD- associated treponemes, in particular with *T.* sp. PT1 (AM942445), PT2 (AM942446) PT3 (AM942447) and PT4 (AM942448), previously isolated from BDD lesions (Klitgaard *et al.* 2008) and also *Treponema* sp. clone DDKL-12 (Y08895) and *Treponema* sp. clone DDKL-20 (Y08897), also isolated from BDD lesions (Choi *et al.* 1997).

Figure 5.2 shows the phylogenetic tree revealing the phylogenetic relationship between DD- associated *Treponema* and commensal GI *Treponema* 16S rRNA gene sequences with the sequences obtained from database 1. The same phylogenetic analysis containing sequences obtained from database 2 is seen in figure 5.3, and figure 5.4 corresponds to sequences obtained from database 5.

The putative DD treponeme clones found in database 1 and 2 clustered closely with the *T.* sp. PT1 (AM942445), PT3 (AM942447), PT4 (AM942448), DDKL-12 (Y08895) and DDKL-20 (Y08897). The *T.* sp. PT2 (AM942446) forms its own branch above the putative DD treponeme clones, but still is part of the cluster of treponemes, suggesting a close phylogenetic relationship.

The putative DD treponeme clone found in database 3, clone 13-P5 (AM884113), divided into two clusters. Twelve sit above the *Treponema* spp. isolated by Klittgaard *et al.* (2008) and Choi *et al.* (1997), and 6 sit below them. This suggests there may be more diversity in the putative DD treponeme clones found in database 3. However, even though all of the putative DD treponeme clones do not sit perfectly together, the same picture is seen as in the other

phylogenetic trees, whereby all of the putative DD treponeme clones and the above DD treponemes form one large cluster.

To determine exactly how similar the putative DD treponeme clones 16S rRNA sequences were to their closest related treponemes (*T.* sp. PT1 (AM942445), PT3 (AM942447), PT4 (AM942448), DDKL-12 (Y08895), DDKL-20 (Y08897) and *T.* sp. PT2 (AM942446)), further sequence identity matrices were produced including these additional reported BDD treponeme sequences.

The *Treponema* clones DDKL-12 (Y08895) and DDKL-20 (Y08897) upon aligning with some of the putative DD treponeme clone sequences were found to only align with a relatively small number of nucleotides (~300-400bp). This made four of the putative DD treponeme clone sequences incomparable to *T.* clone DDKL-12 (Y08895), these are indicated by a dash (-) in the table. The number of nucleotides aligned and compared in each comparison are next to each of the sequence identity figures (0-1) in brackets. If the number of nucleotides compared to the DD treponeme for all of the sequences was the same, the bp size is listed under the DD treponeme name. Table 5.4 shows the relevant regions of the matrices. Sequence names have been shortened to just clone numbers/name.

To compare all of the putative DD treponeme clones to each other a large matrix was produced (Figure 5.5). The matrix was colour coded to show high and low sequence identity scores between the treponemes.

No treponeme sequences were identical to each other; however the areas of green show there are groups of sequences which are very similar. The treponemes from database 1 all shared over 97% sequence identity to each other, indicating they are the same species. Four of the treponemes from this database shared over 99% sequence identity to each other, the only sequence that didn't, L406RT-6-D08, still shared over 97% sequence identity with all the other sequences in database 1.

The putative DD treponeme clones from database 3 appear to be divided into two groups, according to their similarity with each other. Seven sequences show a much higher sequence identity score to each other than to the other eleven sequences, and the eleven also show a high relation to each other but a much lower sequence identity to the remaining seven. However, none of them have over 97% sequence identity to each other and therefore are mostly likely not the same species of treponeme, but of similar species. The splitting of the treponemes into two groups coincides with the phylogenetic tree (Figure 5.4) produced from these sequences with DD- associated and GI tract treponemes. The database 3 treponemes seem to form two groups within the tree also, indicating there does seem to be two sets of treponemes found in database 3, which are closely related to each other within their group.

Table 5.4: 16s rRNA sequence identity of the putative DD treponeme clones from all 3 databases compared to their closest treponeme relatives. Putative DD treponeme clones are listed in the left column and DD treponemes run horizontal. The number after each putative DD treponeme clone refers to the database source. T. BBD is an abbreviation for Treponema clone isolated from BDD lesion. Sequence identities are given as a number between 0-1, followed by the size of sequence compared in brackets. (-) indicates the sequences were incomparable. Highlighted cells indicate the highest sequence identity score for each of the putative DD treponeme clones compared to the DD treponemes.

	T.BDD clone PT1[a]	**T.BDD clone PT2[a]**	**T.BDD clone PT3[a]**	**T.BDD clone PT4[a]**	**DDKL-12**	**DDKL- 20 (420)**
L406RT-1-G07 (1)	0.908(1325)	0.892(1325)	0.903(1325)	0.900(1325)	0.877(580)	0.873
L406RT-6-H06 (1)	0.907(1325)	0.891(1325)	0.906(1325)	0.902(1325)	0.879(580)	0.873
L406RT-5-D08 (1)	0.909(1325)	0.893(1325)	0.905(1325)	0.901(1325)	0.875(580)	0.871

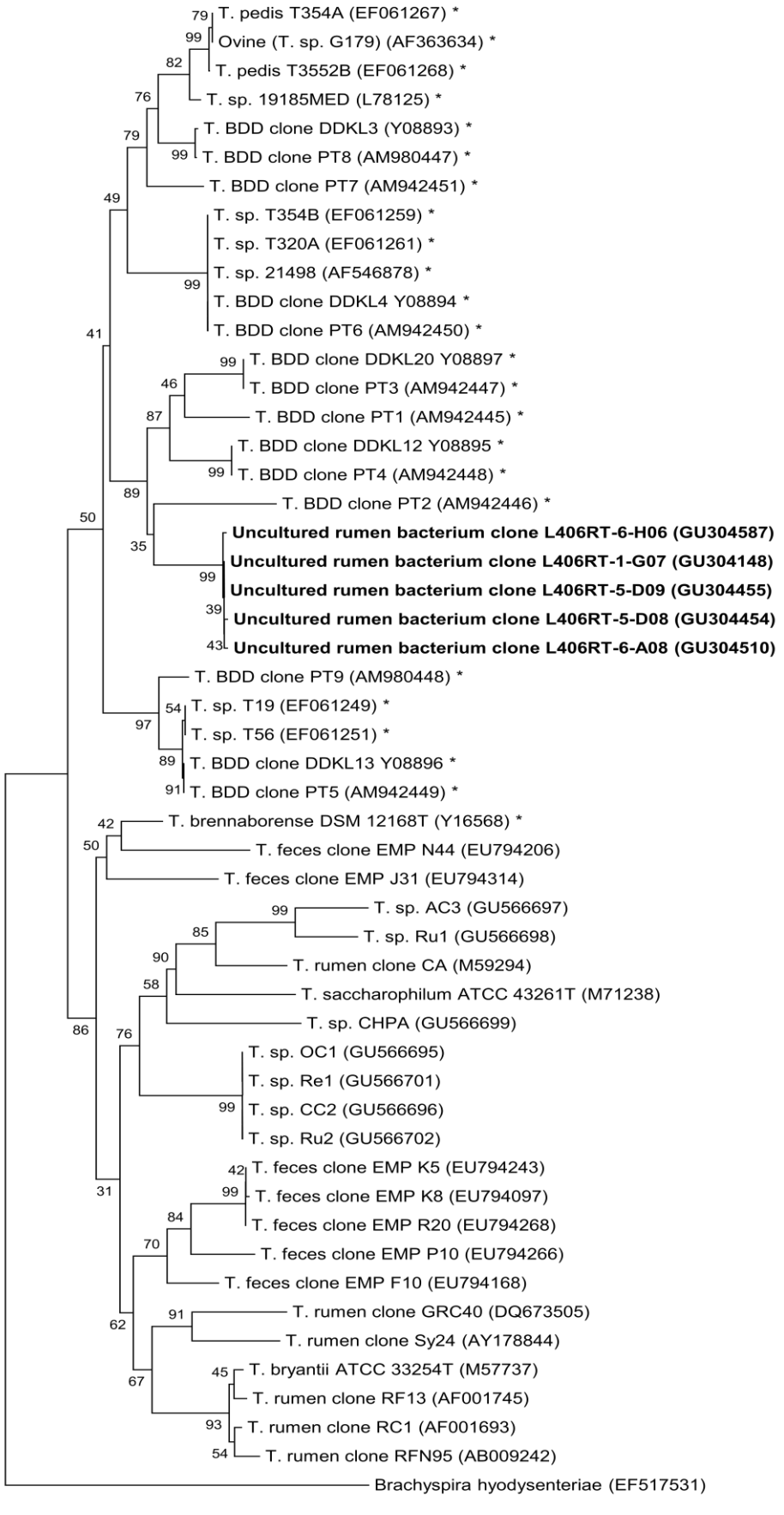
L406RT-5-D09 (1)	0.910(1325)	0.894(1325)	0.906(1325)	0.902(1325)	0.877(580)	0.873
L406RT-6-A08 (1)	0.909(1325)	0.893(1325)	0.905(1325)	0.901(1325)	0.875(580)	0.871
13-P5 (2)	0.851(753)	0.826(753)	0.838(753)	0.844(753)	0.877(580)	0.873
114896 (3)	0.837(602)	0.794(602)	0.842(602)	0.832(602)	-	0.804
213087 (3)	0.857(602)	0.821(602)	0.864(602)	0.857(602)	0.861(300)	0.848
294958 (3)	0.848(602)	0.812(602)	0.856(602)	0.847(602)	0.850(300)	0.819
340877 (3)	0.837(602)	0.805(602)	0.844(602)	0.836(602)	0.841(300)	0.815
512974 (3)	0.855(602)	0.821(602)	0.862(602)	0.855(602)	0.859(300)	0.844
668650 (3)	0.854(602)	0.818(602)	0.861(602)	0.852(602)	0.859(300)	0.835
109640 (3)	0.856(602)	0.836(602)	0.846(602)	0.854(602)	0.776(300)	0.834
133908 (3)	0.833(602)	0.81(602)	0.832(602)	0.837(602)	0.779(300)	0.811
143086 (3)	0.853(602)	0.826(602)	0.848(602)	0.857(602)	0.784(300)	0.831
165259 (3)	0.857(602)	0.835(602)	0.854(602)	0.859(602)	0.813(300)	0.841
179487 (3)	0.861(602)	0.837(602)	0.857(602)	0.863(602)	0.810(300)	0.845
201576 (3)	0.846(602)	0.819(602)	0.839(602)	0.846(602)	0.793(300)	0.818
255676 (3)	0.847(602)	0.82(602)	0.841(602)	0.849(602)	-	0.821
335428 (3)	0.851(602)	0.822(602)	0.843(602)	0.851(602)	-	0.822
344375 (3)	0.804(602)	0.783(602)	0.801(602)	0.806(602)	-	0.768
479469 (3)	0.845(602)	0.82(602)	0.841(602)	0.847(602)	0.797(300)	0.821
648831 (3)	0.84(602)	0.817(602)	0.833(602)	0.839(602)	0.794(300)	0.814
651572 (3)	0.849(602)	0.82(602)	0.846(602)	0.851(602)	0.780(300)	0.824

The areas of high sequence identity can be seen between the database 1 sequences and many of the database 3 sequences. From the sequences from database 3, eleven sequences received over 90% sequence identity scores with database 1 sequences, however the other seven received lower scores of 82.6 % and 87.5%. The database 3 group shares a higher sequence identity to the database 1 sequences than the other group from database 5.

The putative DD treponeme clone 13-P5 (AM884113) from database 2 was found to share 94.1% sequence identity to all of the database 1 treponemes.

However, it had a much lower sequence identity to the database 3 treponemes. Again, like with database 1, this database 2 treponeme was more similar to the one group of database 3 treponemes, than the other group of database 3 treponemes. Compared with the group of eight treponemes in database 3, the database 2 treponeme appeared to be very different in sequence identity (indicated by the red cells).

Figure 5.2: A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~1,300 aligned bases showing the relationship between the novel treponeme sequences found in database 1 (Li et al. 2012a) (shown in bold) and other DD associated and commensal treponeme 16S rRNA gene sequences. The final model for nucleotide substitutions was the general time reversal (GTR) model (Tavare 1986), used to infer a bootstrapped maximum likelihood tree; bootstrapping was performed 10,000 time. = previously reported 16S rRNA gene sequences from BDD lesions.*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Ovine (T. sp. G179) (AF363634) *
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T. BDD clone PT8 (AM980447) *
T. BDD clone PT7 (AM942451) *
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T. sp. T320A (EF061261) *
T. sp. 21498 (AF546878) *
T. BDD clone DDKL4 Y08894 *
T. BDD clone PT6 (AM942450) *
T. BDD clone DDKL20 Y08897 *
T. BDD clone PT3 (AM942447) *
T. BDD clone PT1 (AM942445) *
T. BDD clone DDKL12 Y08895 *
T. BDD clone PT4 (AM942448) *
T. BDD clone PT2 (AM942446) *
Uncultured rumen bacterium clone L406RT-6-H06 (GU304587)
Uncultured rumen bacterium clone L406RT-1-G07 (GU304148)
Uncultured rumen bacterium clone L406RT-5-D09 (GU304455)
Uncultured rumen bacterium clone L406RT-5-D08 (GU304454)
Uncultured rumen bacterium clone L406RT-6-A08 (GU304510)
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T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. BDD clone DDKL13 Y08896 *
T. BDD clone PT5 (AM942449) *
T. brennaborense DSM 12168T (Y16568) *
T. feces clone EMP N44 (EU794206)
T. feces clone EMP J31 (EU794314)
T. sp. AC3 (GU566697)
T. sp. Ru1 (GU566698)
T. rumen clone CA (M59294)
T. saccharophilum ATCC 43261T (M71238)
T. sp. CHPA (GU566699)
T. sp. OC1 (GU566695)
T. sp. Re1 (GU566701)
T. sp. CC2 (GU566696)
T. sp. Ru2 (GU566702)
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T. feces clone EMP F10 (EU794168)
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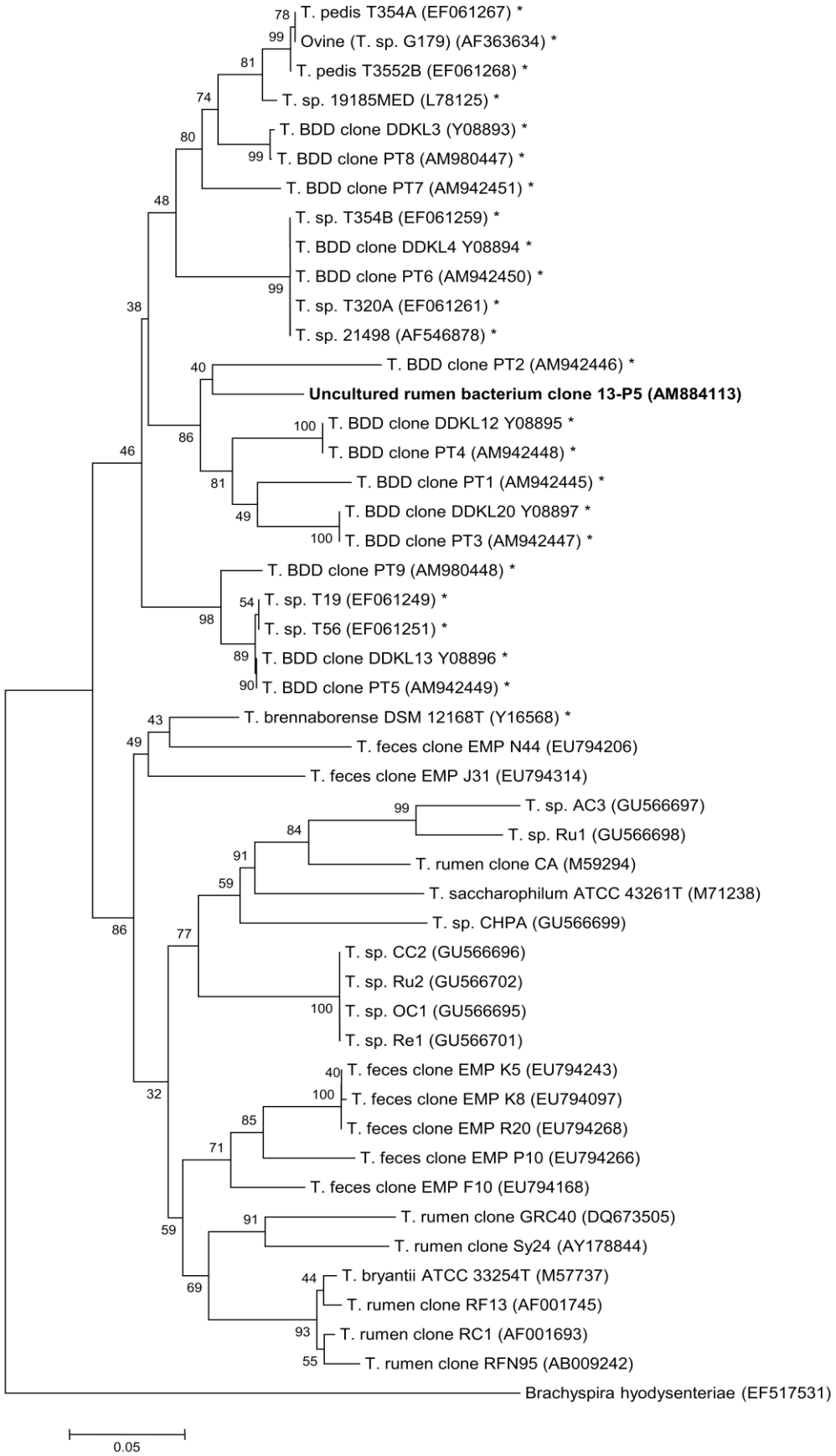
T. rumen clone Sy24 (AY178844)
T. bryantii ATCC 33254T (M57737)
T. rumen clone RF13 (AF001745)
T. rumen clone RC1 (AF001693)
T. rumen clone RFN95 (AB009242)
Brachyspira hyodysenteriae (EF517531)
0.05

Figure 5.3: A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~800 aligned bases showing the relationship between the novel treponeme sequences found in database 2 (Sadet-Bourgeteau et al. 2010) (shown in bold) and other DD associated and commensal treponeme 16S rRNA gene sequences. The final model for nucleotide substitutions was the TrN model (Tamura and Nei 1993), used to infer a bootstrapped maximum likelihood tree; bootstrapping was performed 10,000 time. = previously reported 16S rRNA gene sequences from BDD lesions.*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T. BDD clone DDKL3 (Y08893) *
T. BDD clone PT8 (AM980447) *
T. BDD clone PT7 (AM942451) *
T. sp. T354B (EF061259) *
T. BDD clone DDKL4 Y08894 *
T. BDD clone PT6 (AM942450) *
T. sp. T320A (EF061261) *
T. sp. 21498 (AF546878) *
T. BDD clone PT2 (AM942446) *
Uncultured rumen bacterium clone 13-P5 (AM884113)
T. BDD clone DDKL12 Y08895 *
T. BDD clone PT4 (AM942448) *
T. BDD clone PT1 (AM942445) *
T. BDD clone DDKL20 Y08897 *
T. BDD clone PT3 (AM942447) *
T. BDD clone PT9 (AM980448) *
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
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T. BDD clone PT5 (AM942449) *
T. brennaborense DSM 12168T (Y16568) *
T. feces clone EMP N44 (EU794206)
T. feces clone EMP J31 (EU794314)
T. sp. AC3 (GU566697)
T. sp. Ru1 (GU566698)
T. rumen clone CA (M59294)
T. saccharophilum ATCC 43261T (M71238)
T. sp. CHPA (GU566699)
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T. sp. Ru2 (GU566702)
T. sp. OC1 (GU566695)
T. sp. Re1 (GU566701)
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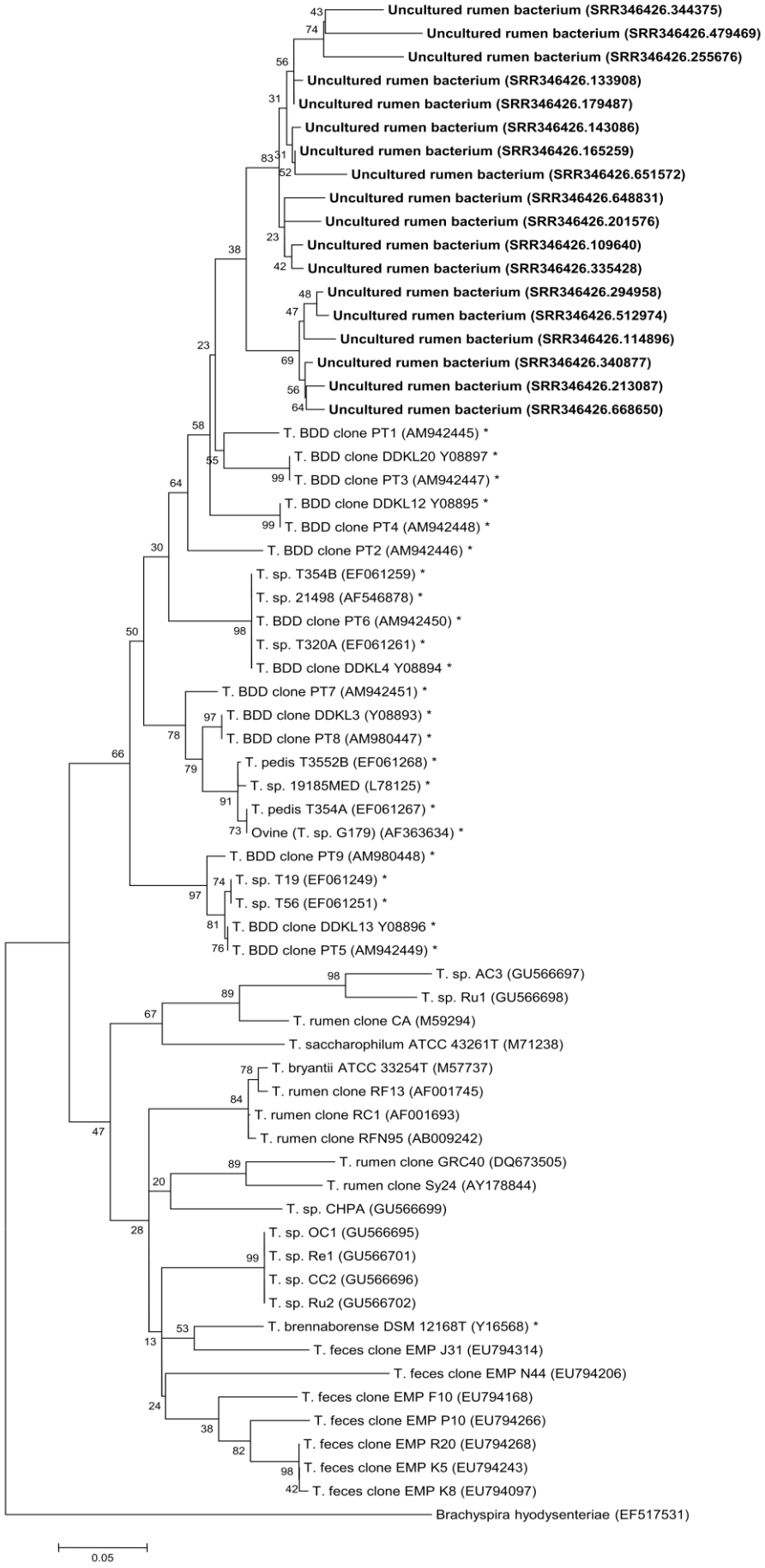
T. feces clone EMP K8 (EU794097)
T. feces clone EMP R20 (EU794268)
T. feces clone EMP P10 (EU794266)
T. feces clone EMP F10 (EU794168)
T. rumen clone GRC40 (DQ673505)
T. rumen clone Sy24 (AY178844)
T. bryantii ATCC 33254T (M57737)
T. rumen clone RF13 (AF001745)
T. rumen clone RC1 (AF001693)
T. rumen clone RFN95 (AB009242)
Brachyspira hyodysenteriae (EF517531)
0.05

Figure 5.4: A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~600 aligned bases showing the relationship between the novel treponeme sequences found in database 3 (Li et al. 2012b) (shown in bold) and other DD associated and commensal treponeme 16S rRNA gene sequences. The final model for nucleotide substitutions was the general time reversal (GTR) model (Tavare 1986), used to infer a bootstrapped maximum likelihood tree; bootstrapping was performed 10,000 times. = previously reported 16S rRNA gene sequences from BDD lesions.*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Uncultured rumen bacterium (SRR346426.179487)
Uncultured rumen bacterium (SRR346426.143086)
Uncultured rumen bacterium (SRR346426.165259)
Uncultured rumen bacterium (SRR346426.651572)
Uncultured rumen bacterium (SRR346426.648831)
Uncultured rumen bacterium (SRR346426.201576)
Uncultured rumen bacterium (SRR346426.109640)
Uncultured rumen bacterium (SRR346426.335428)
Uncultured rumen bacterium (SRR346426.294958)
Uncultured rumen bacterium (SRR346426.512974)
Uncultured rumen bacterium (SRR346426.114896)
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T. BDD clone PT1 (AM942445) *
T. BDD clone DDKL20 Y08897 *
T. BDD clone PT3 (AM942447) *
T. BDD clone DDKL12 Y08895 *
T. BDD clone PT4 (AM942448) *
T. BDD clone PT2 (AM942446) *
T. sp. T354B (EF061259) *
T. sp. 21498 (AF546878) *
T. BDD clone PT6 (AM942450) *
T. sp. T320A (EF061261) *
T. BDD clone DDKL4 Y08894 *
T. BDD clone PT7 (AM942451) *
T. BDD clone DDKL3 (Y08893) *
T. BDD clone PT8 (AM980447) *
T. pedis T3552B (EF061268) *
T. sp. 19185MED (L78125) *
T. pedis T354A (EF061267) *
Ovine (T. sp. G179) (AF363634) *
T. BDD clone PT9 (AM980448) *
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. BDD clone DDKL13 Y08896 *
T. BDD clone PT5 (AM942449) *
T. sp. AC3 (GU566697)
T. sp. Ru1 (GU566698)
T. rumen clone CA (M59294)
T. saccharophilum ATCC 43261T (M71238)
T. bryantii ATCC 33254T (M57737)
T. rumen clone RF13 (AF001745)
T. rumen clone RC1 (AF001693)
T. rumen clone RFN95 (AB009242)
T. rumen clone GRC40 (DQ673505)
T. rumen clone Sy24 (AY178844)
T. sp. CHPA (GU566699)
T. sp. OC1 (GU566695)
T. sp. Re1 (GU566701)
T. sp. CC2 (GU566696)
T. sp. Ru2 (GU566702)
T. brennaborense DSM 12168T (Y16568) *
T. feces clone EMP J31 (EU794314)
T. feces clone EMP N44 (EU794206)
T. feces clone EMP F10 (EU794168)
T. feces clone EMP P10 (EU794266)
T. feces clone EMP R20 (EU794268)
T. feces clone EMP K5 (EU794243)
T. feces clone EMP K8 (EU794097)
Brachyspira hyodysenteriae (EF517531)
0.05

Figure 5.5: Sequence identity matrix comparing all novel treponemes found in databases 1, 2 and 3. 0 indicates 0% sequence identity, 0.99 indicates 99% sequence identity and ID indicates identical sequences (100% identity). Areas of green show high sequence identity, through to red indicating low sequence identity.

	L406RT-1-G07(1)	L406RT-6-H06(1)	L406RT-5-D08(1)	L406RT-5-D09(1)	L406RT-6-A08(1)	13-P5(2)	114896(3)	213087(3)	294958(3)	340877(3)	512974(3)	668650(3)	109640(3)	133908(3)	143086(3)	165259(3)	179487(3)	201576(3)	255676(3)	335428(3)	344375(3)	479469(3)	648831(3)	651572(3)
L406RT-1-G07(1)	ID	0.994	0.972	0.997	0.996	0.941	0.826	0.838	0.833	0.827	0.837	0.833	0.938	0.918	0.924	0.929	0.94	0.91	0.924	0.9	0.87	0.928	0.936	0.924
L406RT-6-H06(1)	0.994	ID	0.972	0.996	0.996	0.941	0.831	0.843	0.838	0.833	0.842	0.839	0.943	0.924	0.929	0.934	0.945	0.915	0.929	0.91	0.875	0.933	0.941	0.929
L406RT-5-D08(1)	0.972	0.972	ID	0.974	0.974	0.941	0.831	0.843	0.838	0.833	0.842	0.839	0.943	0.924	0.929	0.934	0.945	0.915	0.929	0.91	0.875	0.933	0.941	0.929
L406RT-5-D09(1)	0.997	0.996	0.974	ID	0.999	0.942	0.831	0.843	0.838	0.833	0.842	0.839	0.943	0.924	0.929	0.934	0.945	0.915	0.929	0.91	0.875	0.933	0.941	0.929
L406RT-6-A08(1)	0.996	0.996	0.974	0.999	ID	0.941	0.831	0.843	0.838	0.833	0.842	0.839	0.943	0.924	0.929	0.934	0.945	0.915	0.929	0.91	0.875	0.933	0.941	0.929
13-P5(2)	0.941	0.941	0.941	0.942	0.941	ID	0.73	0.763	0.758	0.757	0.767	0.769	0.84	0.839	0.839	0.86	0.859	0.823	0.836	0.84	0.786	0.841	0.819	0.847
114896 (3)	0.826	0.831	0.831	0.831	0.831	0.73	ID	0.9	0.889	0.877	0.895	0.886	0.812	0.82	0.821	0.809	0.814	0.815	0.806	0.81	0.775	0.812	0.82	0.817
213087(3)	0.838	0.843	0.843	0.843	0.843	0.763	0.9	ID	0.935	0.952	0.969	0.945	0.845	0.848	0.854	0.846	0.85	0.847	0.836	0.85	0.804	0.845	0.835	0.846
294958(3)	0.833	0.838	0.838	0.838	0.838	0.758	0.889	0.935	ID	0.91	0.923	0.913	0.831	0.832	0.838	0.83	0.835	0.824	0.825	0.83	0.791	0.833	0.822	0.839
340877(3)	0.827	0.833	0.833	0.833	0.833	0.757	0.877	0.952	0.91	ID	0.936	0.967	0.818	0.828	0.838	0.822	0.83	0.825	0.813	0.84	0.783	0.82	0.817	0.825
512974(3)	0.837	0.842	0.842	0.842	0.842	0.767	0.895	0.969	0.923	0.936	ID	0.951	0.835	0.84	0.845	0.832	0.846	0.831	0.822	0.83	0.804	0.835	0.828	0.842
668650(3)	0.833	0.839	0.839	0.839	0.839	0.769	0.886	0.945	0.913	0.967	0.951	ID	0.822	0.83	0.836	0.823	0.833	0.819	0.815	0.83	0.79	0.828	0.823	0.83
109640(3)	0.938	0.943	0.943	0.943	0.943	0.84	0.812	0.845	0.831	0.818	0.835	0.822	ID	0.91	0.927	0.936	0.943	0.918	0.927	0.92	0.861	0.922	0.923	0.908
133908(3)	0.918	0.924	0.924	0.924	0.924	0.839	0.82	0.848	0.832	0.828	0.84	0.83	0.91	ID	0.91	0.92	0.921	0.905	0.917	0.92	0.871	0.906	0.891	0.933
143086(3)	0.924	0.929	0.929	0.929	0.929	0.839	0.821	0.854	0.838	0.838	0.845	0.836	0.927	0.91	ID	0.936	0.94	0.956	0.929	0.94	0.87	0.919	0.902	0.918
165259(3)	0.929	0.934	0.934	0.934	0.934	0.86	0.809	0.846	0.83	0.822	0.832	0.823	0.936	0.92	0.936	ID	0.945	0.929	0.95	0.93	0.885	0.963	0.912	0.931
179487(3)	0.94	0.945	0.945	0.945	0.945	0.859	0.814	0.85	0.835	0.83	0.846	0.833	0.943	0.921	0.94	0.945	ID	0.922	0.934	0.93	0.897	0.928	0.916	0.917
201576(3)	0.91	0.915	0.915	0.915	0.915	0.823	0.815	0.847	0.824	0.825	0.831	0.819	0.918	0.905	0.956	0.929	0.922	ID	0.927	0.94	0.855	0.911	0.901	0.905
255676(3)	0.924	0.929	0.929	0.929	0.929	0.836	0.806	0.836	0.825	0.813	0.822	0.815	0.927	0.917	0.929	0.95	0.934	0.927	ID	0.93	0.871	0.933	0.907	0.913
335428(3)	0.902	0.908	0.908	0.908	0.908	0.836	0.81	0.85	0.833	0.841	0.834	0.83	0.922	0.915	0.942	0.934	0.93	0.942	0.932	ID	0.867	0.914	0.899	0.919
344375(3)	0.87	0.875	0.875	0.875	0.875	0.786	0.775	0.804	0.791	0.783	0.804	0.79	0.861	0.871	0.87	0.885	0.897	0.855	0.871	0.87	ID	0.868	0.86	0.877
479469(3)	0.928	0.933	0.933	0.933	0.933	0.841	0.812	0.845	0.833	0.82	0.835	0.828	0.922	0.906	0.919	0.963	0.928	0.911	0.933	0.91	0.868	ID	0.901	0.914
648831(3)	0.936	0.941	0.941	0.941	0.941	0.819	0.82	0.835	0.822	0.817	0.828	0.823	0.923	0.891	0.902	0.912	0.916	0.901	0.907	0.9	0.86	0.901	ID	0.884
651572(3)	0.924	0.929	0.929	0.929	0.929	0.847	0.817	0.846	0.839	0.825	0.842	0.83	0.908	0.933	0.918	0.931	0.917	0.905	0.913	0.92	0.877	0.914	0.884	ID

5.3.4 Diet associated fluctuations in *Treponema* frequency and relative abundance in the GI tract

Table 5.5 shows a summary of the results of several research papers investigating the effect of diet changes on bacteria in the GI tract. The studies found, encompassed many different areas including Japan, the USA and Canada, however a study of this kind was not found to have been carried out in the UK.

The research found that ruminal bacterial communities are not always at fixed levels but vary between host, the individual, microenvironment (area of GI tract and liquid or attached fractions), and diet.

The studies investigated a change in bacteria (composition or frequency) in the GI tract usually by experimentally transitioning animals from forage such as hay or grass (forages) to high concentrate diets involving a supplement with grains, such as wheat, or by comparing different animals on these two diets.

In five of the investigations it was discovered that a high grain diet caused an increase in the number of spirochaetes and in one investigation, a change in the community composition of spirochaetes. Results showed that transition from high-forage to high-grain diets caused alterations in the population dynamics and usually caused an increase in the number of *Treponema* species present. However some of the studies did not allow all of their data to be accessible so exact increases in treponemes, in some cases, was impossible to know.

Table 5.5: A summary of investigations into the association of diet and Treponema fluctuations in the GI tract. [a]Animals studied indicates whether the samples were taken from a beef, dairy or sheep animal. The number indicates the amount of animals sampled. In brackets abbreviations, P; the study pooled samples from all animals studied, IN; all animals investigated had their samples individually sequenced rather than pooling the samples. [b]FU indicates the frequency change in treponemes was unobtainable from the data. If frequency was possible to determine then it is listed in brackets as a percentage increase or decrease.

Study	**Geographical location**	**Animals studied[a]**	**Specific region of GI tract sampled**	**Presence of treponemes**	**Diet**	**Change in Spirochaete number[b]**
Bekele *et al.* 2011	Japan	Sheep- 3 (IN)	Rumen	+	Alfalfa hay vs. Orchard grass hay Vs. Concentrate diet	**No change in total number.** (change in composition)
Chen *et al.* 2011	Canada	Beef cattle- 24 (IN)	Rumen epithelial tissue-associated	+	Hay to high concentrate diet (high grain)	**Increase** (treponemes only present on higher grain diet) FU
Tajima *et al.* 2001	Japan	Dairy cattle- 8 (P)	Rumen	+	Hay to high concentrate (grain)	**Decrease** in *T. bryantii* (85% decline)
Fernando *et al.* 2010	USA	Beef cattle- 8 (P)	Rumen	+	Hay to high concentrate (grain)	**Increase** (60%)
Kim *et al.* 2011	Czech Republic	Dairy cattle- 4(IN)	Rumen (liquid fraction and adherent fraction)	+	Forage Vs. Forage + concentrate	**Increase** (4 treponemes present only when on high conc diet)
Pitta *et al.* 2010	USA	Beef cattle- 14(P)	Rumen	+	Bermuda grass(forage) Vs. high concentrate (wheat)	**Increase** with wheat diet (29%)
Li *et al.* 2012b	California	Dairy cattle- 4(IN)	Rumen	+	Ruminal butyrate infusion	**Increase** in relative abundance (2.19%- 5.9%)
Sadet-Bourgeteau *et al.* 2010	France	Sheep- 8(IN)	Rumen epithelium	+	Alfalfa hay diet vs.high concentrate diet and back to alfalfa hay diet	**Increase** (only 1 treponeme present after high conc diet and then back to alfalfa).

5.4 Discussion

5.4.1 The presence of spirochaetes in the GI tract and their relatedness to DD treponemes

It is commonly known that ruminant's GI tracts contain *Treponema* species which are non-pathogenic. However, the presence of disease-causing treponemes in the GI tract would provide a possible transmission route for the treponemes, via passage through the GI tract to faeces, which are then spread in the farm environment. However, by comparing 16S rRNA sequences, in all of the 37 databases found to contain ruminal bacterial data, no DD- associated treponemes were found to be present.

The most interesting finding was the presence of putative DD treponeme clones in three of the databases. Twenty four sequences were found, none of which belonged to any known GI treponeme species or DD- associated treponeme species. However, they were found to be more closely related to, sharing higher sequence identity, the DD- associated treponemes, and upon phylogenetic analysis clustered with the DD- associated treponemes. Within the DD- associated treponeme cluster, all the treponemes are pathogenic, which would suggest that the putative DD treponeme clone sequences found in the GI tract may also have a similar biological role. If so, they may be at certain times or in certain circumstances, shed through the GI tract and into faeces, as the other bacteria within the rumen does. Therefore, if they are involved in DD this could be a possible transmission route. However, although they appear to be more highly related to pathogenic DD associated treponemes, from the phylogenetic analysis and matrix analyses, this link cannot yet be proven.

In particular, the putative DD treponeme clones clustered with the *T.* sp. PT1 (AM942445), PT2 (AM942446) PT3 (AM942447) and PT4 (AM942448), previously isolated from BDD lesions (Klitgaard *et al.* 2008). They showed a

high association with BDD lesions, as they were found in 93%, 68%, 88% and 43% of BDD lesions tested, respectively. The putative DD treponeme clones were found to share between 81%-91% sequence identity with either of the four PT treponemes. Database 1 treponemes showed the highest sequence identity scores and database 3 the lowest, which could indicate that the database 1 treponemes are more closely related to the DD- associated treponemes.

The *T.* sp. PT1 (AM942445), PT2 (AM942446) PT3 (AM942447) and PT4 (AM942448) were found to be closely related to *Treponema refringens* by Klitgaard *et al.* (2008). *Treponema refringens* is a common commensal species, found normally in the genitalia of humans and primates (Canale-Parola 1984). However, *Treponema* species that fall in this cluster are often found in high numbers in BDD lesions, demonstrated by Klitgaard *et al.* (2008). They also discovered that these *Treponema* species were present in the deepest parts of the epidermis and dermis of the lesions, suggesting that these species may play a key role in the invasive nature of the disease. Although none of the putative DD treponeme clones shared over 97% sequence identity to the PT clones, the average sequence identity between some of the DD-associated treponemes is around 92% (e.g. between *T. pedis* strain T354A (EF061267) and *T. phagedenis* strain CIP 62.29 (EF645248)), and therefore scores of 91% could just indicate a different species of pathogenic treponeme. Further investigations into possible links between the GI tract bacterial population and DD would benefit from possibly focusing on this group of treponemes.

The origin of these putative DD treponeme clones is also interesting. The treponemes found from database 1 (Li *et al.* 2012a) were all found in the rumen epithelium tissue of beef cattle. Seventeen treponemes were found altogether in this study's database, ten from the rumen content and seven from the rumen tissue. Interestingly, all of the treponemes found in the rumen contents belonged to either *T. bryantii*, or were more closely related to other ruminal treponemes than pathogenic DD treponemes. However, of the seven

treponemes found in the rumen tissue, five of them (71.4%) were putative DD treponeme clones which clustered with the DD- associated treponemes. Therefore, not only are the putative DD treponeme clones only appearing to be present in the rumen epithelium tissue and therefore possibly tissue associated, but 10/12 (83%) of the commensal treponemes were found in the rumen content and therefore appear to be content associated. To coincide with this theory, the putative DD treponeme clone found in database 2 (uncultured rumen bacterium clone 13-P5) this time from sheep, was also found in the rumen epithelium tissue. Contradictory to this finding, the putative DD treponeme clones in database 3 were found in the rumen content of the dairy cattle studied.

Although the evidence so far cannot tell us definitively whether or not the putative DD treponeme clone sequences are tissue associated, it would make sense according to their closest cultivable treponeme species. The *T.* sp. PT1 (AM942445), PT2 (AM942446) PT3 (AM942447) and PT4 (AM942448), which the putative DD treponeme clones appear to be closest related to, are also tissue associated treponemes, along with the other DD- associated treponemes. If the putative DD treponeme clones are in fact tissue associated, they may follow the same trend as the other treponemes within the phylogenetic cluster, in that they are pathogenic. Additionally, although there were only two rumen epithelium studies, both of them contained these putative DD treponeme clones (100%), and only one study of the 20 (5%) non-epithelium studies containing treponemes was found to contain putative DD treponeme clones.

The putative DD treponeme clones found in database 3 appeared to be less like the other putative DD treponeme clones (from database 1 and 2) and also split into two groups according to their sequence identity to each other and to the other putative DD treponeme clones. This may indicate that they serve different biological roles within the rumen, such as the ones which shared a high sequence identity with the DD treponemes were pathogenic treponemes and the other group of database 3 treponemes which showed less were not.

However, without more substantial microbiome data of different rumen locations and associated pathology, we cannot distinguish the role of these putative DD treponemes within the rumen. It is interesting though that the putative DD treponeme clones found in the rumen content database (database 3) versus the epithelium studies, were the treponemes considered least like the pathogenic *T.* sp. PT1 (AM942445), PT2 (AM942446) PT3 (AM942447) and PT4 (AM942448). Therefore these treponemes belonging to the large cluster containing mostly GI tract treponemes are more likely to be commensals, possibly because they are less tissue associated, living within the rumen content (rather than directly being identified on rumen tissue) of the animal.

The putative DD treponeme clone 13-P5 (AM884113) found in database 2 and the putative DD treponeme clones found in database 3 were both from studies which changed the diet of the animal. Both of these studies, database 2 (Sadet-Bourgeteau *et al.* 2010) and database 3 (Li *et al.* 2012b), were both studies described previously which were analysed to investigate the effect of diet on the levels of treponemes in the GI tract. The putative DD treponeme clone 13-P5 (AM884113) was found in the rumen epithelium of a sheep, after it was fed a hay diet for 4 weeks, a high concentrate diet for the next 8 weeks and then switched back to hay for the last 8 weeks of the study. It was found in the sheep rumen epithelium at the final 8 week stage of the trial. This is interesting as no putative DD treponeme clone sequences were found before the change in diet. Given this putative DD treponeme clone is present after the change this may suggest that the actual known DD treponemes themselves may be able to survive/increase in frequency after a transition to a high grain diet. Database 3 was taken from the dairy cattle diet study by Li *et al.* (2012b), however it was not possible to determine whether the putative DD treponeme clones were found before or after the dietary change (infusion of exogenous butyrate). However, as was seen in the results, they did find that the relative abundance of treponemes increased after the infusion which could suggest it is these putative DD treponeme clone sequences which increase in abundance after the dietary change.

Since this research was first carried out, several authors have detected DD-associated treponemes in the GI tract. Nascimento *et al.* (2015), detected at least one of the three cultivatable DD treponeme phylogroups in 60% of rumen fluid samples tested.

Zinicola *et al.* (2015), used a metagenomic approach and identified DD treponemes in rumen fluid and also faecal samples. Additionally another study has identified DD- associated treponemes in environmental samples such as manure slurry from dairy farms using high-throughput sequencing (Klitgaard *et al.* 2014). These findings add strength to the theory that DD- associated treponemes are present in several parts of the GI tract. Additionally this new information, particularly the presence of DD- associated treponemes in faeces, could provide a clear route of transmission for DD between animals, and possibly between farms. However, to solidify the argument for the role of the GI tract as an infection reservoir of DD- associated treponemes, two things need to be addressed. Are the treponemes alive in the GI tract? These studies detected the treponemes in the rumen and faeces but this gives no indication of whether the bacteria are alive and therefore transmissible. Secondly, is it just rumen content/liquid which harbours these disease associated treponemes? From this present study the tissues of the GI tract seem the likely areas for pathogenic treponeme inhabitancy.

5.4.2 Diet associated fluctuations in *Treponema* frequency and relative abundance in the GI tract

It is at the farmers discretion whether to feed additional concentrates to their animals, and how much. Concentrates are used as an additional energy supply for animals, especially in the winter months when grass growth is low.

A change in ruminant diet or nutritional additives resulted in a variation in the frequency and relative abundance of *Treponema* species populations within the ruminant GI tract. In particular a transition in diet from forage, such as hay or grass, to a high grain diet, usually by the addition of concentrate, appears to cause significant alterations in treponeme abundance or composition. The

composition of *Treponema* species populations, either relative to the abundance of other ruminal bacteria (Li *et al.* 2012), or relative to other clades of treponemes (Bekele *et al.* 2011) was affected by nutrition and most of the time the transition of the diet to a high concentrate diet induces an increase in the numbers of treponemes identified. This suggests that a high-grain diet could predispose cattle to high numbers of treponemes in the GI tract.

It was interesting to find that only one study (Tajima *et al.* 2001), found that treponemes within the rumen decreased when the transition to the high concentrate diet occurred. However, this study only looked at one species of *Treponema*, *T. bryantii* strain RUS-1 which although known as a GI treponeme, is not the only one present in the rumen. It is possible that although this species decreased, other species of treponemes increased in the rumen. The results from the study by Bekele *et al.* (2011), may provide evidence for this theory, as they found that total *Treponema* abundance in the rumen (sheep) was 1.05%, but *T. bryantii* strain RUS-1 only made up 0.02% of the treponemes found, the rest were uncultured *Treponema* species. Additionally, phylogenetic analysis of the *Treponema* 16S rRNA gene sequences taken from the Bekele *et al.* (2011) study, showed that the treponemes formed clades of similar phylogeny, and the clades were associated with different diets. In the phylogenetic tree, clade I was mainly comprised of uncultured clones associated with concentrate feeding (58.4% of the overall concentrate clones); while clade II predominantly consisted of cultured *Treponema* clones associated with hay feeding (87.3% of the overall hay clones). These findings suggest that closely related phylogroups of rumen *Treponema* associated with a given diet, may play a role in the degradation of that particular diet in the rumen. The not yet cultured treponemes seem to be the treponemes which increase due to a high concentrate diet. Therefore a possible explanation for Tajima *et al.* (2001) contradictory results, may be other uncultured species/phylogroups of treponemes are increased due to a change in diet, whilst cultured species such as *T. bryantii* strain RUS-1 may actually be reduced in frequency under these ruminal conditions.

The study by Li *et al.* (2012b), found that dietary infusion of butyrate significantly increased the relative abundance of *Treponema.* VFA's, including butyrate are known to increase in the rumen on a high concentrate diet (Siciliano-Jones and Murphy 1989; Penner *et al.* 2009). Therefore, there may be a link between VFA's being produced during a high concentrate diet, and treponeme frequency/species abundance during a high concentrate diet and with infusion of butyrate. During the transition from a low to a high concentrate diet, it has been found that the levels of VFA's increase (Penner *et al.* 2009). This could explain why treponemes in the rumen increased, both when on a high concentrate diet and also when butyrate (a VFA) was infused.

It could be possible that different species of treponemes are affected differently by ruminal conditions such as pH. Feeding of high-concentrate diets to cattle can lead to ruminal pH depression (Goad *et al.* 1998; Gozho *et al.* 2005). If treponemes increase in the rumen, as seen in the studies, when the animal is on a high concentrate diet and with an increase in VFA's, it may be that it is a result of a preference for a lower pH. However, the study which primarily investigated the levels of *T. bryantii* strain RUS-1, found a decrease in the number of this treponeme under these conditions. A possible explanation for this is that GI tract treponemes such as *T. bryantii* strain RUS-1 decrease with decreasing pH, but other gut treponemes actually increase, such as the not yet cultured treponemes found by Bekele *et al.* (2011).

Treponemes within the rumen/rumen epithelium do seem to be affected by nutritional changes, often involving the change to a high concentrate diet. The above data suggests that an increase in treponemes under these conditions may be a result of an adaption to a high concentrate diet, possibly linked to the high VFA's/low pH levels in the rumen during this diet.

Another reason for additional concentrates is to deal with production pressures, animals are required to eat more, and higher levels of concentrates, and there is sometimes a need to house cattle in order to feed them high-grain diets (Huxley and Green 2010). It has been found in many studies that animals are

at more risk of DD infection when animals are housed (Rodríguez-Lainz *et al.* 1999; Barker *et al.* 2009). Therefore, you could attempt to attribute the rise in BDD to a high concentrate diet which increases treponemes, possibly DD treponemes, and then they are shed into the environment exposing the animals to the bacteria. However, this is virtually impossible to ascertain as when the animals are housed they are also often exposed to factors which are major risk factors for BDD in dairy cattle; unhygienic environments (Rodriguez-Lainz *et al.* 1999), hard floors (Logue 2011), and solid grooved flooring (Barker *et al.* 2009).

The change in treponemes found in this study show that treponemes in the GI tract are affected by environmental changes, such as diet, and that certain diets may predispose animals to having higher levels on *Treponema* within the GI tract. Although we are yet to consistently find DD- associated treponemes within the GI tract, if they are present there, even at low levels, certain diets may also increase their frequency. Therefore, if DD treponemes are in the GI tract, and shed into the faeces, certain diets may be a risk factor for DD in ruminants.

From the results shown, it would appear that treponemes do increase in frequency in the GI tract when the ruminant has been switched to a high concentrate diet. The specific species of treponeme which increase is unknown, however Tajima *et al.* (2001) found the common commensal species *T. bryantii* strain RUS-1 decreased on the high concentrate diet. Therefore it is possible that the conditions in the rumen on a high concentrate diet, favour other species such as pathogenic treponemes, indicated by the finding of putative DD treponeme clones in studies changing the diet of the animals. An investigation into the levels of specific treponeme species such as the ones found in this study (putative DD treponeme clones) under the different diets may be useful to determine their role in the rumen.

Along with further investigation into these putative DD treponeme clones, a further look into the GI tract tissues in particular, rather than content may be

useful. The bacterial populations present in the rumen epithelium tissue have not been studied in as much depth as ruminal content bacterial populations and as a result less is known about the phylogroups and dynamics present. Recent advances in culture-independent methods of identification such as 16S rRNA gene sequence analysis allow less biased investigation of the epimural bacterial population, as many of the bacteria, such treponemes, are very difficult to culture. The identification by Evans *et al.* (2012) of DD *Treponema* species in GI tract epithelial samples indicates that this may be an area of the GI tract where DD treponemes are most likely to be found. However, with new research detecting these treponemes in ruminal fluid and faeces, perhaps their presence in GI tract tissues occurs prior to this, with a migration into rumen content/faeces facilitating transmission into the environment.

Although no DD- associated treponemes were found to be present in the rumen bacterial sequences, the presence of putative DD treponeme clones could suggest a possible carriage site for the DD causing bacteria. Interestingly, the DD treponeme- like sequences were mainly found in the rumen epithelium and in studies which changed the diet of the ruminants. Preliminary evidence has suggested that the DD *Treponema* phylogroups may occasionally be present in the recto-anal junction. So together these results could suggest the GI tract as a possible means of DD *Treponema* carriage and transmission, but a concentration into the tissues of the GI tract in particular would appear to be the next step to discovering the transmission route of DD in both cattle and sheep.

Chapter 6

The carriage of digital dermatitis *Treponema* spp. in the gastrointestinal tract - a molecular approach

6.1 Introduction

First reported in dairy cattle in 1974 (Cheli and Mortellaro 1974), BDD has now been affecting farms worldwide for over 40 years. Furthermore, over these decades the disease has been reported in sheep (Harwood *et al.* 1997; Davies *et al.* 1999) and more recently beef cattle (Chapter 4; Sullivan *et al.* 2013, 2015a).

The *Treponema* phylogroups commonly isolated from dairy cow BDD lesions have now been detected and isolated in beef cattle BDD lesions (Chapter 4; Sullivan *et al.* 2015a) and CODD lesions (Sayers *et al.* 2009; Chapter 3; Sullivan *et al.* 2015b), goats in the UK (Chapter 3; Sullivan *et al.* 2015c) and North American Elk from Washington State USA (Clegg *et al.* 2015). Due to the promiscuous nature of these treponemes and their growing host range, it is important to identify possible infection reservoirs of these bacteria and then potential routes and means of transmission.

Although the disease has been reported for many years little is known about the routes of transmission or infection reservoirs of DD. There has been much

debate about the GI tract's role as a reservoir of infection of DD treponemes, with the bovine gingival and rectal tissues, rumen fluid and faeces identified as potential infection reservoirs. A study by Evans and *et al.* (2012) detected DD treponemes in the oral cavity and the rectum of dairy cattle and more recent work detected DD treponeme phylogroups in rumen fluid, faecal samples and slurry (Klitgaard *et al.* 2014; Nascimento *et al.* 2015; Zinicola *et al.* 2015). Although these studies identified cattle fluids that contain DD treponemes, it could be considered that the DD treponemes are tissue associated (Chapter 5) and therefore the tissues of the GI tract may be the active site of treponeme infection.

To date, isolations of DD treponemes from the bovine GI tract have failed. Furthermore, the sheep GI tract has not been analysed to see if it could be a reservoir of infection for CODD and/or BDD. Also, the tendency of beef cattle to be different breeds, fed different diets and subjected to different housing regimes than dairy cattle, is justification for further investigations into treponeme presence in both sheep and beef cattle GI tracts. This current study aimed to allow further understanding of the infection reservoirs of DD by surveying a number of beef cattle and sheep gingival and rectal tissues and faeces, from both DD symptomatic and asymptomatic animals, for the detection and isolation of DD- associated treponemes. For this study, I hypothesis that a small proportion of DD symptomatic and asymptomatic animals will be PCR positive for DD- associated treponemes in their rectal and gingival tissues.

6.2 Materials and Methods

6.2.1 Farm information and animal tissue and faeces collection

A total of fourty sheep and fourty beef animals were sampled for gingival and rectal tissues. Twelve of the fourty sheep which had rectal and gingival tissues investigated were CODD symptomatic and the remaining twenty eight were asymptomatic. Twelve of the fourty beef animals which had rectal and gingival

tissues investigated were BDD symptomatic with the remaining twenty eight asymptomatic. Beef cattle were defined as BDD symptomatic if one or more feet had a clear lesion consistent with the clinical signs of BDD (Cheli and Mortellaro 1974; Blowey and Sharp 1988). Briefly, lesions presented as circular areas of brown/grey moist exudate (30–60 mm diameter) in the region of the caudal interdigital cleft at the junction of the skin and horn of the heel with an underlying raw proliferative area. Sheep were defined CODD symptomatic if one or more feet had a clear lesion consistent with CODD clinical signs (Winter 2004a). These signs, although varied, include an ulcerative/granulomatous lesion at the coronary band which may extend under the hoof wall and in some cases lead to avulsion of the entire hoof capsule.

Twelve of the sheep gingival and rectal tissues (from twelve sheep) were collected from four farms in Wales (UK), farms 1-4, with the remaining twenty eight rectal and gingival tissues collected from a fallen stock centre which receives animals from farms within Lancashire, Cheshire and South Cumbria (England, UK). Eleven beef rectal and gingival samples were obtained from two farms, one located in Gloucester, England (farm 5) and one located in Wales (farm 6). These beef rectal (n= 11) and gingival (n= 11) samples were collected using a swabbing technique rather than a tissue biopsy. The remaining 29/40 beef rectal and 29/40 gingival tissues were obtained from the fallen stock centre, as above. All GI tissue/swab samples were collected between January 2013 and September 2014.

Gingival and rectal tissues were collected using sterile scalpels to extract 3 cm^2 biopsies post-mortem (from animals that had died naturally or had been euthanized by the owner). Rectal tissue and gingival tissues biopsies were collected from animals as per Methods 2.1.2 and 2.1.3, respectively. In the cases where a swabbing technique was used this was done as per Method 2.1.5. Samples were transported and stored as described in Method 2.1.1.

Faecal samples were collected from sheep (n= 79) and beef cattle (n= 41). All faecal samples were fresh, taken immediately after defecation. All faecal samples were collected according to Method 2.1.4. Sheep faeces samples were

collected from two farms, *n*= 55 from farm 1 (sampled in this study for GI tissues) and *n*= 25 from a farm in Wales (farm 7). When faecal samples were collected, DD status was subsequently determined. Of a total of 79 sheep faecal samples, 29 were obtained from CODD symptomatic sheep, and the remaining 50 were from CODD asymptomatic animals. Beef faeces were collected as per sheep faeces, from two other farms located in Wales, UK, namely farm 6 (also investigated for sheep GI tissues) and another farm located in Wales, UK, (farm 8) where 15/41 faecal samples were from BDD symptomatic beef animals and 26/41 were from BDD asymptomatic beef animals. Faecal samples were collected from January 2013 to July 2014.

All farms from which sheep rectal, gingival and faecal samples were obtained were CODD endemic farms with the exception of farm 3 which was CODD negative. All farms from which beef cattle rectal, gingival and faecal samples were obtained were BDD endemic farms.

6.2.2 Culture of spirochaetes

Bacterial isolation, specifically for treponemes, was attempted on all samples; rectal tissue samples (*n*= 40), (*n*= 40) gingival tissue samples (*n*= 40), (*n*= 40) and faeces (*n*= 41), (*n*= 79) from beef cattle and sheep, respectively.

The culture technique used, subsequent bacterial DNA extraction, gene cleaning and sequencing was carried out as described in Methods 2.2, 2.3.1, 2.5.1 and 2.5.2, respectively.

6.2.3 Tissue, swabs and faeces DNA extraction

For PCR analysis, tissues/swabs were thawed and DNA extracted using a DNeasy blood and tissue kit (Qiagen, United Kingdom), according to the manufacturer's instructions. Faecal samples were thawed and DNA extracted using a QIAamp DNA Stool Mini Kit (Qiagen, United Kingdom), according to the manufacturer's instructions. All genomic DNA was stored at -20°C.

6.2.4 Genus and phylogroup specific treponeme PCR assays

All samples (*n*= 280) were subjected to nested PCR assays specific for the three DD- associated treponeme groups, *T. medium*- like, *T. phagedenis*- like, *T. pedis* and the treponeme genus specific 16S rRNA gene PCR assay, as described in Methods 2.4.2 and 2.4.3, respectively.

6.2.5 Phylogenetic analysis of spirochaete isolates

To understand the relationship of the isolated spirochaetes with other treponemes, and in particular those previously isolated from DD lesions and ruminant GI tract's (considered commensals), a phylogenetic tree was produced according to Method 2.6. The most appropriate evolution model was predicted using "model test" as implemented in the Topali programme (Milne *et al.* 2009). The final model for nucleotide substitutions chosen was the TrN model (Tamura and Nei 1993), used to infer a bootstrapped maximum likelihood tree (bootstrapping was performed 10,000 times).

6.2.6 Nucleotide sequence accession numbers

The 16S rRNA gene GenBank accession numbers determined as part of this study are KR052445- KR052467.

6.2.7 Statistical analysis

Fisher exact tests were used to investigate associations between the presence of DD- associated *Treponema* phylogroups in the GI tract of beef cattle and sheep and the DD status of the animal (Method 2.7.2). Additionally, a chi-square test (with Yates correction) (Method 2.7.1) was used to identify associations between the DD status of animals and the isolation of *Treponema* spp. from faecal samples. The statistical test used was determined by the software used, based on values for statistical analyses. In all analyses, an associated probability (*P*-value) of < 0.05 was considered significant.

6.2.8 DD treponeme detection rates in beef cattle, sheep and dairy cattle gastrointestinal tract tissues

A comparison of PCR detection rates of *Treponema* DD phylogroups in the GI tissues of dairy cattle, beef cattle and sheep was carried out using the data produced in this study and previously generated dairy cattle data (Evans *et al.* 2012). The data was compared by frequency of animals which have been found to contain a DD treponeme phylogroup (or multiple phylogroups) in their GI tract tissue/s.

6.3 Results

6.3.1 *Treponema* genus and phylogroup specific PCR survey of gastrointestinal tissues

The results of the specific DD *Treponema* phylogroup and *Treponema* genus-specific PCR assays of sheep rectal and gingival tissues and of beef rectal and gingival tissues are shown in Tables 6.1 and 6.2, respectively.

Treponema DNA (as determined using the *Treponema* genus PCR assay) was present in 36/40 (85%) and 20/40 (50%) of sheep rectal samples and gingival samples, respectively. Phylogroup specific PCR assays for *T. medium*- like, *T. phagedenis*- like and *T. pedis* DD spirochaetes, showed that no *T. medium*- like DNA was present in any sheep rectal (n= 40) or gingival tissues (n= 40); however, 1/40 sheep rectal tissues were positive for *T. phagedenis*- like DD spirochaetes and 2/40 sheep rectal tissues were positive for *T. pedis* DD spirochaetes. All three positive rectal tissues were obtained from CODD symptomatic sheep (animals 3, 4 and 5). Neither *T. medium*- like nor *T. phagedenis*-like DD spirochaetes were detected in any of the sheep gingival tissues; however, *T. pedis* DD spirochaetes were present in 1/40 of the sheep gingival tissues. This *T. pedis* infected gingival tissue was obtained from a CODD symptomatic sheep which also had *T. pedis* DD spirochaete DNA present in it's rectal tissue (animal 3).

Treponema DNA (identified using the *Treponema* genus PCR assay) was present in 25/40 (63%) and 17/40 (43%) of beef cattle rectal samples and gingival samples, respectively. The phylogroup specific PCR's for *T. medium*-like, *T. phagedenis*- like and *T. pedis* DD spirochaetes, amplified no DNA from beef cattle rectal tissues. However, 4/40 gingival tissues were positive for *T. phagedenis*- like DD spirochaetes (animals 28, 34, 38, 39). No *T. medium*- like or *T. pedis* DD spirochaete DNA was amplified in beef cattle gingival tissues.

Table 6.1: PCR detection and isolation of treponemes in sheep rectal and gingival tissues.

Animal	Collection date (mo/yr)	Location[a]	DD status[b]	Result									
				Rectal tissue					Gingival tissue				
				Specific PCR for group[c]:			*Treponema* PCR	Isolation[d]	Specific PCR for group[c]:			*Treponema* PCR	Isolation[d]
				1	2	3			1	2	3		
1	06/13	F1	+	-	-	-	+	IF	-	-	-	+	IF
2	06/13	F1	+	-	-	-	+	IF	-	-	-	-	IF
3	06/13	F1	+	-	-	+	+	IF	-	-	+	+	IF
4	06/13	F1	+	-	-	+	+	IF	-	-	-	-	IF
5	08/13	F2	+	-	+	-	+	SR5R	-	-	-	+	IF
6	09/13	F3	-	-	-	-	+	IF	-	-	-	-	IF
7	09/13	F3	-	-	-	-	+	IF	-	-	-	-	IF
8	09/13	F3	-	-	-	-	+	IF	-	-	-	-	IF
9	09/13	F3	-	-	-	-	+	IF	-	-	-	-	IF
10	12/13	F4	+	-	-	-	+	IF	-	-	-	-	IF
11	12/13	F4	+	-	-	-	+	IF	-	-	-	-	IF
12	12/13	F4	+	-	-	-	+	IF	-	-	-	-	IF
13	01/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
14	01/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
15	03/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
16	03/14	FSC	-	-	-	-	-	IF	-	-	-	+	IF
17	03/14	FSC	-	-	-	-	-	IF	-	-	-	+	IF
18	03/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
19	03/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
20	03/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
21	03/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
22	03/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
23	03/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
24	03/14	FSC	+	-	-	-	+	IF	-	-	-	+	IF
25	03/14	FSC	+	-	-	-	+	IF	-	-	-	-	IF
26	03/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
27	03/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF

28	03/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
29	04/14	FSC	-	-	-	-	-	IF	-	-	-	+	IF
30	04/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
31	04/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
32	04/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
33	04/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
34	04/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
35	04/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
36	04/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
37	04/14	FSC	+	-	-	-	-	IF	-	-	-	+	IF
38	04/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
39	05/14	FSC	+	-	-	-	+	IF	-	-	-	+	IF
40	05/14	FSC	+	-	-	-	+	IF	-	-	-	+	IF

[a] Abbreviations: F, Farm with corresponding number; FSC, Fallen stock centre.
[b]Abbreviations: DD, digital dermatitis.
[c]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*-like and *T. pedis* spirochaetes, respectively which are routinely found in bovine DD lesions.
[d]All isolations are shown for comparison to PCR results. Abbreviations: IF, isolation failed. Successful isolations have strains listed.

Table 6.2: PCR detection and isolation of treponemes in beef cattle rectal and gingival tissues.

Animal	**Collection date (mo/yr)**	**Location[a]**	**DD status[b]**	**Result**									
				Rectal tissue					**Gingival tissue**				
				Specific PCR for group[c]:			***Treponema* PCR**	**Isolation[d]**	**Specific PCR for group[c]:**			***Treponema* PCR**	**Isolation[d]**
				1	**2**	**3**			**1**	**2**	**3**		
1	01/13	F1	+	-	-	-	+	IF	-	-	-	-	IF
2	01/13	F1	+	-	-	-	+	IF	-	-	-	-	IF
3	01/13	F1	+	-	-	-	+	IF	-	-	-	-	IF
4	04/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
5	04/14	FSC	+	-	-	-	-	IF	-	-	-	+	IF
6	04/14	FSC	+	-	-	-	-	IF	-	-	-	-	IF
7	05/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
8	05/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
9	05/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
10	05/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
11	05/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
12	05/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
13	05/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
14	05/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
15	06/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
16	06/14	FSC	+	-	-	-	+	IF	-	-	-	-	IF
17	06/14	FSC	+	-	-	-	-	IF	-	-	-	+	IF
18	06/14	FSC	-	-	-	-	-	IF	-	-	-	+	IF
19	06/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
20	06/14	FSC	-	-	-	-	-	IF	-	+	-	+	IF
21	06/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
22	06/14	FSC	-	-	-	-	+	IF	-	-	-	-	IF
23	07/14	F6	+	-	-	-	+	IF	-	-	-	+	IF
24	07/14	F6	+	-	-	-	+	IF	-	-	-	-	IF
25	07/14	F6	-	-	-	-	+	IF	-	-	-	+	IF
26	07/14	F6	+	-	-	-	+	IF	-	-	-	-	IF
27	07/14	F6	+	-	-	-	+	IF	-	-	-	+	IF
28	07/14	F6	-	-	-	-	+	IF	-	-	-	+	IF
29	07/14	F6	+	-	-	-	+	IF	-	-	-	-	IF

30	07/14	F6	+	-	-	-	+	IF	-	-	-	-	IF
31	08/14	FSC	-	-	-	-	-	IF	-	-	-	+	IF
32	08/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
33	08/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
34	08/14	FSC	-	-	-	-	+	IF	-	+	-	+	IF
35	08/14	FSC	-	-	-	-	+	IF	-	-	-	+	IF
36	08/14	FSC	-	-	-	-	-	IF	-	-	-	+	IF
37	08/14	FSC	-	-	-	-	-	IF	-	-	-	-	IF
38	08/14	FSC	-	-	-	-	+	IF	-	+	-	+	IF
39	08/14	FSC	+	-	-	-	+	IF	-	+	-	+	IF
40	09/14	FSC	+	-	-	-	+	IF	-	-	-	-	IF

[a] Abbreviations: F, Farm with corresponding number; FSC, Fallen stock centre.
[b]Abbreviations: DD, digital dermatitis.
[c]Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*-like and *T. pedis* spirochaetes, respectively which are routinely found in bovine DD lesions.
[d]All isolations are shown for comparison to PCR results. Abbreviations: IF, isolation failed. Successful isolations have strains listed.

The presence of one or more DD *Treponema* phylogroups in the GI tract of sheep and beef cattle was analysed according to the season from which the sample was collected (Table 6.3). Seasons were defined as; Winter (December- February); Spring (March- May); Summer (June- August); Autumn (September- November). As shown in Table 6.3, only GI tract tissues collected in summer were positive for DD *Treponema* phylogroup DNA. This was true for both sheep and beef cattle GI tract tissues.

Table 6.3: A comparison of PCR detection rates of Treponema DD phylogroups in the GI tissues of beef cattle and sheep in different seasons. Gingival and rectal tissue results have been combined to give a value and percentage of GI tract tissues positive for each species at the different times of year.

Season	Sheep GI tract tissues[a]	Beef cattle GI tract tissues[a]
Winter (December- February)	0/5 **(0%)**	0/3 **(0%)**
Spring (March- May)	0/28 **(0%)**	0/11 **(0%)**
Summer (June- August)	3/5 **(60%)**	4/25 **(16%)**
Autumn (September- November)	0/4 **(0%)**	0/1 **(0%)**

[a] GI tract tissues refers to rectal and oral cavity tissues combined.

6.3.2 *Treponema* genus and phylogroup specific PCR survey of faecal samples

The genus and phylogroup specific treponeme PCR assays and treponeme isolation results for sheep and beef faecal samples are shown in Tables 6.4 and 6.5, respectively.

Using the genus specific PCR assay, *Treponema* DNA was identified as present in 73/79 (92%) and 39/41 (95%) of sheep and beef cattle faeces samples, respectively.

All sheep (*n*= 79) and beef cattle faeces (*n*= 41) were negative for *T. medium*-like, *T. phagedenis*-like and *T. pedis* DD spirochaete DNA as determined using the respective PCR assays.

Table 6.4: PCR detection and isolation of treponemes in sheep faecal samples.

Sample (s)	Collection date (mo/yr)	Location sample obtained[a]	DD status[b]	Treponeme isolated[c]	Result			
					Specific PCR for group[d]:			*Treponema* PCR
					1	2	3	
1- 4	01/13	F1	-	IF	-	-	-	+
5	01/13	F1	+	IF	-	-	-	+
6- 10	01/13	F1	-	IF	-	-	-	+
11	01/13	F1	-	IF	-	-	-	-
12- 19	01/13	F1	-	IF	-	-	-	+
20	01/13	F1	+	SF20	-	-	-	+
21	01/13	F1	+	SF21a, SF21b	-	-	-	+
22, 23	01/13	F1	-	IF	-	-	-	+
24	01/13	F1	+	SF24a, SF24b	-	-	-	+
25	01/13	F1	-	IF	-	-	-	+
26	01/13	F1	+	SF26a, SF26b	-	-	-	+
27, 28	01/13	F1	-	IF	-	-	-	-
29	01/13	F1	+	SF29	-	-	-	+
30	01/13	F1	-	IF	-	-	-	-
31	01/13	F1	-	IF	-	-	-	+
32	01/13	F1	+	SF32a, SF32b	-	-	-	+
33	01/13	F1	+	IF	-	-	-	+
34	01/13	F1	+	IF	-	-	-	-
35- 37	01/14	F1	-	IF	-	-	-	+
38	01/14	F1	-	IF	-	-	-	-
39, 40	01/14	F1	-	IF	-	-	-	+
41	01/14	F1	+	IF	-	-	-	+
42, 43	01/14	F1	-	IF	-	-	-	+
44	01/13	F1	+	IF	-	-	-	+
45, 46	01/13	F7	-	IF	-	-	-	+
47	01/13	F7	-	SF47	-	-	-	+
48- 50	01/13	F7	-	IF	-	-	-	+
51	01/13	F7	+	SF51	-	-	-	+
52	01/13	F7	+	IF	-	-	-	+
53	01/13	F7	+	SF53	-	-	-	+

54, 55	01/13	F7	+	IF	-	-	-	+
56	01/13	F7	+	SF56	-	-	-	+
57	01/13	F7	+	IF	-	-	-	+
58	01/13	F7	-	IF	-	-	-	+
59	01/13	F7	+	SF59	-	-	-	+
60	02/13	F7	+	SF60	-	-	-	+
61	02/13	F7	-	IF	-	-	-	+
62	02/13	F7	-	SF62	-	-	-	+
63	02/13	F7	-	IF	-	-	-	+
64	02/13	F7	-	SF64	-	-	-	+
65	02/13	F7	+	IF	-	-	-	+
66	02/13	F7	-	SF66	-	-	-	+
67	02/13	F7	+	IF	-	-	-	+
68	02/13	F7	+	SF68	-	-	-	+
69	02/13	F7	+	SF69	-	-	-	+
70, 71	03/13	F1	+	IF	-	-	-	+
72	03/13	F1	-	IF	-	-	-	+
73	03/13	F1	+	IF	-	-	-	+
74	03/13	F1	-	SF74	-	-	-	+
75	03/13	F1	-	IF	-	-	-	+
76	03/13	F1	+	IF	-	-	-	+
77-79	03/13	F1	-	IF	-	-	-	+

[a] Abbreviation: F, Farm with corresponding number.
[b] Abbreviation: DD, digital dermatitis.
[c] All isolations are shown for comparison to PCR results. Abbreviations: IF, isolations failed. If isolation was successful the isolated strains are listed.
[d] Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively.

Table 6.5: PCR detection and isolation of treponemes in beef cattle faecal samples.

Sample (s)	**Collection date (mo/yr)**	**Location sample obtained[a]**	**DD status[b]**	**Treponeme isolated[c]**	**Result**			
					Specific PCR for group[d]:			***Treponema* PCR**
					1	**2**	**3**	
1- 2	03/13	F6	+	IF	-	-	-	+
3- 7	03/13	F6	-	IF	-	-	-	+
8	03/13	F6	+	IF	-	-	-	+
9- 13	07/14	F8	-	IF	-	-	-	+
14, 15	07/14	F8	+	IF	-	-	-	+
16	07/14	F8	-	IF	-	-	-	-
17, 18	07/14	F8	-	IF	-	-	-	+
19	07/14	F8	+	IF	-	-	-	+
20	07/14	F8	-	IF	-	-	-	+
21, 22	07/14	F8	+	IF	-	-	-	+
23- 25	07/14	F8	-	IF	-	-	-	+
26	07/14	F8	-	IF	-	-	-	-
27- 29	07/14	F8	-	IF	-	-	-	+
30, 31	07/14	F8	+	IF	-	-	-	+
32, 33	07/14	F8	-	IF	-	-	-	+
34	07/14	F8	+	IF	-	-	-	+
35- 37	07/14	F8	-	IF	-	-	-	+
38- 41	07/14	F8	+	IF	-	-	-	+

[a] Abbreviation: F, Farm with corresponding number.
[b] Abbreviation: DD, digital dermatitis.
[c] All isolations are shown for comparison to PCR results. Abbreviations: IF, isolations failed. If isolation was successful the isolated strains are listed.
[d] Groups 1, 2 and 3 are *T. medium*- like, *T. phagedenis*- like and *T. pedis* spirochaetes, respectively.

6.3.3 Isolation and phylogenetic analysis of spirochaetes

Isolation of spirochaetes was attempted from all rectal, gingival and faecal samples. All isolation attempts were unsuccessful from beef cattle rectal and gingival tissues. Isolation attempts were also unsuccessful from sheep gingival tissues. However, a spirochaete was isolated from one of the 40 sheep rectal tissues subjected to cultivation attempts (animal 5). This isolate, SR5R (Genbank accession: KR052467), isolated from animal 5, was identified as belonging to the *T. phagedenis*- like DD spirochaete group, sharing 100% 16S rRNA gene sequence identity with the *T. phagedenis*- like DD spirochaete strain T320A (Genbank accession: EF061261), previously isolated from a UK dairy cow BDD lesion (Evans *et al.* 2008).

All treponeme isolation attempts from beef cattle faecal samples were unsuccessful. However, twenty two spirochaetes were successfully isolated from 18/79 (23%) of sheep faecal samples. Multiple spirochaetes were isolated from some sheep faecal samples. All twenty two isolates shared over 99% 16S rRNA gene sequence identity with *Treponema* sp. CHPA (Genbank accession: GU566699), previously isolated from the GI tract contents of a DD positive dairy cow (Evans *et al.* 2012). Four of the 22 isolates shared 100% 16S rRNA gene sequence identity with *T.* sp. CHPA (Genbank accession: GU566699). Of the *T.* sp. CHPA isolates obtained from sheep faeces, 17/22 (77%), were isolated from the faeces of CODD symptomatic sheep. Sequence analysis revealed that the twenty two isolates could be separated into four groups based on 16S rRNA gene sequences. Within each group, isolates shared between 99.7% and 100% 16S rRNA gene sequence identity to each other. These groups appeared to relate to the farm from which the animal which had produced the faeces had originated (either farm 1 or farm 7). One of the groups of isolates consisted of eight treponemes, all of which came from animals from

farm 1. The second group of isolates consisted of ten treponemes, of which eight originated from farm 7 (remaining two from farm 1) and a third group of two isolates both came from animals from farm 7. The last group consisted of two treponemes, one of which was from the faeces of a sheep from farm 1 and the other from the faeces of a sheep from farm 7.

There was a marked difference in phylogenetic relationship between the sheep rectal tissue and sheep faecal isolates. Rectal tissue isolate SR5R, clustered with the *T. phagedenis*- like DD spirochaete group, within the larger group of the DD pathogenic treponemes (top half of Figure 6.1) whilst the 22 isolates obtained from sheep faeces samples clustered with the commensal treponemes (lower half of Figure 6.1), and in particular *T.* sp. CHPA (Genbank accession: GU566699).

6.3.4 Statistical analysis

From the results of the statistical analyses no significant association was found between the presence of DD- associated *Treponema* spp. presence in the GI tract and the DD status of the animal for sheep or beef cattle. However, interestingly there was a statistically significance association between DD status and the isolation of *T. sp.* CHPA identified ($P = 0.041$). This is based on isolation results and therefore care has to be taken when interpreting this *P* value.

6.3.5 DD treponeme detection in beef cattle, sheep and dairy cattle GI tract tissues

The summary of results for DD treponeme detection rates in beef cattle, sheep and dairy cattle GI tract tissues is shown in Table 6.6. Of the beef cattle, dairy cattle (Evans *et al.* 2012) and sheep sampled, 10%, 7.1% and 2.5% had DD treponeme phylogroup DNA present in their gingival tissues, respectively. In terms of rectal tissues, 0%, 11.1% and 7.5% were positive for DD treponeme phylogroup DNA in beef cattle, dairy cattle and sheep, respectively.

Figure 6.1: A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~1,000 aligned bases. The tree shows the relationship between the strains isolated here (shown in bold) from ruminant faeces and GI tissue and other DD associated and commensal treponeme 16S rRNA gene sequences. Bootstrapping was performed 10,000 times, and for clarity only bootstrap values above 70% are shown. = previously reported 16S rRNA gene sequences from BDD lesions.*

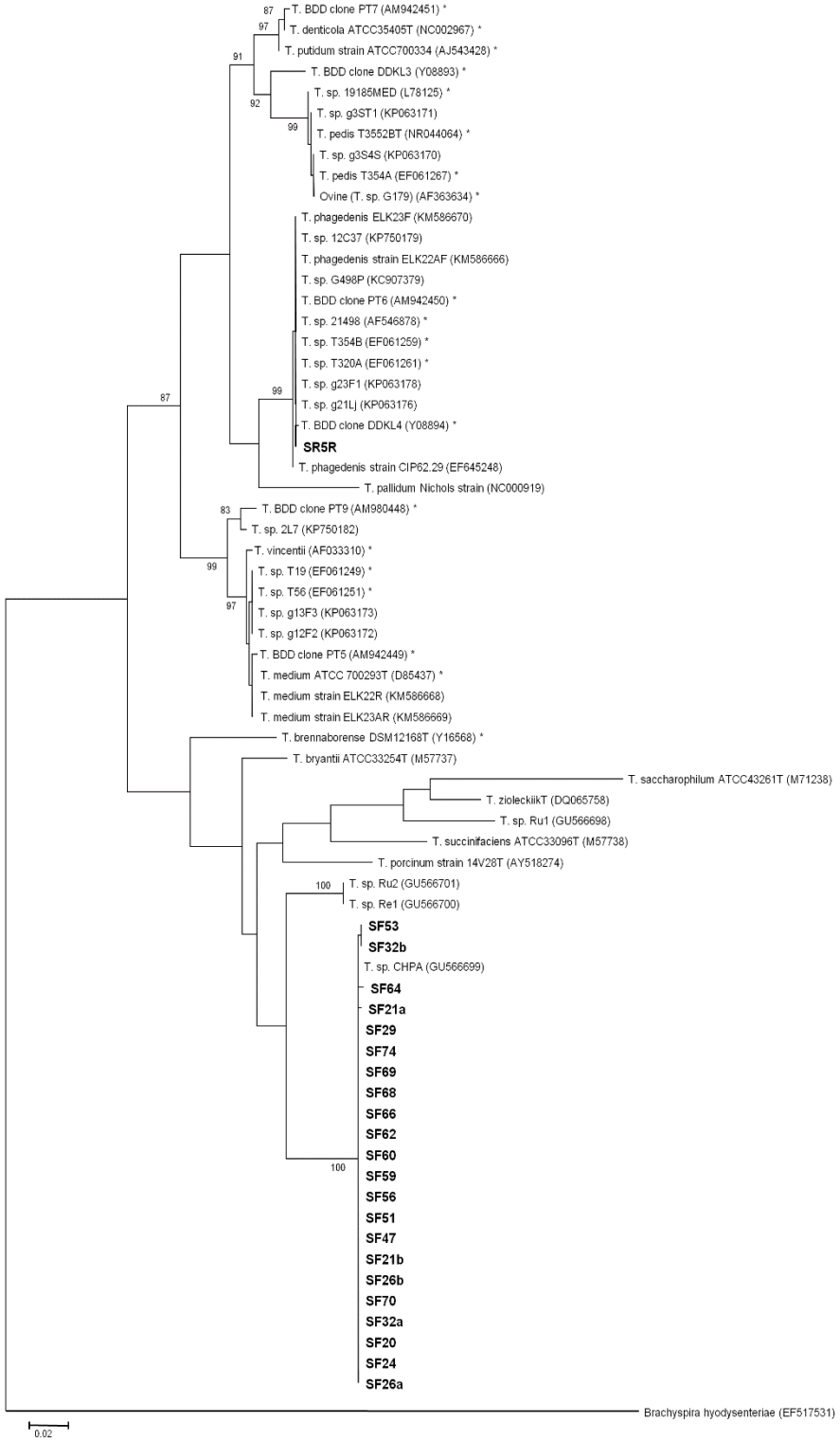

T. BDD clone PT7 (AM942451) *
T. denticola ATCC35405T (NC002967) *
T. putidum strain ATCC700334 (AJ543428) *
T. BDD clone DDKL3 (Y08893) *
T. sp. 19185MED (L78125) *
T. sp. g3ST1 (KP063171)
T. pedis T3552BT (NR044064) *
T. sp. g3S4S (KP063170)
T. pedis T354A (EF061267) *
Ovine (T. sp. G179) (AF363634) *
T. phagedenis ELK23F (KM586670)
T. sp. 12C37 (KP750179)
T. phagedenis strain ELK22AF (KM586666)
T. sp. G498P (KC907379)
T. BDD clone PT6 (AM942450) *
T. sp. 21498 (AF546878) *
T. sp. T354B (EF061259) *
T. sp. T320A (EF061261) *
T. sp. g23F1 (KP063178)
T. sp. g21Lj (KP063176)
T. BDD clone DDKL4 (Y08894) *
SR5R
T. phagedenis strain CIP62.29 (EF645248)
T. pallidum Nichols strain (NC000919)
T. BDD clone PT9 (AM980448) *
T. sp. 2L7 (KP750182)
T. vincentii (AF033310) *
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. sp. g13F3 (KP063173)
T. sp. g12F2 (KP063172)
T. BDD clone PT5 (AM942449) *
T. medium ATCC 700293T (D85437) *
T. medium strain ELK22R (KM586668)
T. medium strain ELK23AR (KM586669)
T. brennaborense DSM12168T (Y16568) *
T. bryantii ATCC33254T (M57737)
T. saccharophilum ATCC43261T (M71238)
T. zioleckiikT (DQ065758)
T. sp. Ru1 (GU566698)
T. succinifaciens ATCC33096T (M57738)
T. porcinum strain 14V28T (AY518274)
T. sp. Ru2 (GU566701)
T. sp. Re1 (GU566700)
SF53
SF32b
T. sp. CHPA (GU566699)
SF64
SF21a
SF29
SF74
SF69
SF68
SF66
SF62
SF60
SF59
SF56
SF51
SF47
SF21b
SF26b
SF70
SF32a
SF20
SF24
SF26a
Brachyspira hyodysenteriae (EF517531)
0.02

Table 6.6: A comparison of PCR detection rates of Treponema DD phylogroups in the GI tissues of dairy cattle, beef cattle and sheep.

Animal[a]	Rectal anal junction tissue[b]	Gingival tissue[c]
Dairy cattle (Evans *et al.* 2012)	3/27 **(11.1%)**	1/14 **(7.1%)**
Beef cattle (this study)	0/40 **(0%)**	4/40 **(10%)**
Sheep (this study)	3/40 **(7.5%)**	1/40 **(2.5%)**

[a] Animal GI tissues originated from with reference to corresponding study.
[b] Only rectal anal junction tissue positives have been used for the comparison and not rectal wall results.
[c] GI tissue results from Evans *et al.* (2012) have been corrected to give a figure for detection rate per tissue per animal, in cases where tissues from the same animal were sampled multiple times this has only been counted as 1 positive.

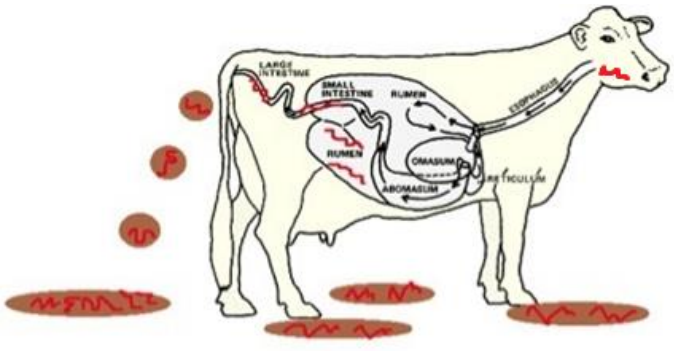
6.4 Discussion

Previous studies of BDD in dairy cattle and beef cattle, and more recently CODD in sheep, show that in all DD lesions one or more of the three cultivable DD treponeme phylogroups are present (Klitgaard *et al.* 2008; Nordhoff *et al.* 2008; Evans *et al.* 2009b; Chapter 3, 4; Sullivan *et al.* 2013, 2015a, 2015b). However, although these phylogroups are consistently detected and isolated from cattle and sheep lesions, studies have failed to isolate these pathogenic treponemes from potential transmission vectors and infection reservoirs. Therefore, there is still no clear evidence of DD treponemes surviving outside of the foot itself.

Recently, work has focused on the GI tract as a possible reservoir of DD treponemes in dairy cows. Figure 6.2 shows a schematic of how the GI tract may be an infection reservoir of DD treponemes, and how subsequently they may be shed into the environment to be spread to other animals. Evans *et al.* (2012) identified DD treponemes in two GI tract regions of dairy cattle, the oral cavity and rectal tissue, suggesting these areas as potential treponeme reservoirs. However, that study failed to isolate live DD treponemes from these tissues. Various other studies have now detected one or more of the commonly associated cultivatable DD treponeme phylogroups. Nascimento *et al.* (2015), detected at least one of the three cultivatable DD treponeme phylogroups in

60% of rumen fluid samples tested, and using a metagenomic approach, Zinicola *et al.* (2015), identified DD treponemes in rumen fluid and faecal samples. Additionally, Klitgaard *et al.* (2014) identified DD- associated treponemes in environmental samples such as manure slurry from dairy farms using high-throughput sequencing. This present study has however, for the first time, isolated one of the DD *Treponema* phylogroups from a ruminant rectal tissue sample. Importantly, this DD treponeme was isolated from a CODD positive sheep, a host species of which the GI tract has not previously been investigated as a reservoir for DD treponemes. This isolation of a DD treponeme from a deep tissue sample from the rectum of a DD infected animal is the first evidence of live and therefore transmissible DD treponemes within host tissues other than the foot.

Figure 6.2: Schematic to show how the GI tract may be an infection reservoir of DD treponemes, and how subsequently they may be shed into the environment to be spread to other animals. Red lines indicate treponemes in the rumen/rumen tissue/fluid, passing from the rumen into the intestines and out into the environment via the animals faeces.



Although DD treponemes are detectable in ruminant GI tract tissues, they appear to only be present there in a small percentage of animals when using the PCR approaches described in this study. Three sheep had DD *Treponema* phylogroup DNA (7.5%) in their rectal tissue, one of which had *T. pedis* DD

treponeme phylogroup DNA in both its gingival and rectal tissue. Although all beef cattle rectal tissues were negative for DD *Treponema* phylogroup DNA, four (10%) of beef animals had *T. phagedenis*- like DD spirochaete DNA present in their gingival tissue. The number of animals with DD treponeme DNA in their oral and rectal tissue is similar to what Evans *et al.* (2012) found in dairy cattle GI tract tissues. In terms of gingival tissues, the percentage of dairy cattle and beef cattle which contained *Treponema* DD phylogroup DNA in this tissue was extremely similar, 7.1% and 10% respectively. However, the percentage of sheep which contained *Treponema* DD phylogroup DNA in their gingival tissue was lower; 2.5% of animals tested. Conversely, the percentage of dairy cattle and sheep which contained *Treponema* DD phylogroup DNA in their rectal tissue was similar, 11.1% and 7.5% respectively. By contrast, no *Treponema* DD phylogroup DNA was detected in beef rectal tissues. What is clear is that the percentage of animals with DD treponemes present in these GI tract tissues appears small; however, it is possible that only a small proportion of animals may carry (in the GI tract) and shed these bacteria into faeces, and this could be all that was needed to effectively spread these bacteria in the farm environment. This is particularly true for the closely related spirochaetal bacteria, *Brachyspira hyodysenteriae*, the causative agent of swine dysentery in pigs, in which there is often only a small number of carrier animals which are able to sustain infection in affected farm (Songer and Harris 1978; Duff *et al.* 2014).

Interestingly, animals which were found to contain DD treponeme phylogroup DNA in either their oral cavity tissue or rectal tissue, were not necessarily DD positive animals. Only one of the four beef animals which contained DD treponeme phylogroup DNA in their gingival tissue were BDD symptomatic. It might be predicted that it would be cows with BDD lesions which had DD treponeme DNA in their GI tract (and therefore possibly capable of shedding the bacteria), but it appears from these results that this is not necessarily the case. Similarly, Evans *et al.* (2012) showed that although gingival tissues were only positive for DD treponeme carriage in cattle that were BDD positive, rectal tissue DD treponeme carriage showed no indication of an association

with symptomatic BDD. Additionally, the one sheep which upon PCR analysis was found to contain DD treponeme phylogroup DNA (*T. pedis*) in its gingival tissue and its rectal tissue, also had the same DD- associated *Treponema* phylogroup, *T. pedis*, present in its active CODD lesion (investigated previously; Chapter 3, animal 21; Sullivan *et al.* 2015b). This has been observed in a previous study whereby a DD symptomatic dairy cow contained *T. phagedenis*- like DNA in it's gingival, rumen and rectal tissues as well as in an active DD lesion (Evans *et al.* 2012).

From the PCR survey of GI tract tissues, it is interesting that there is a possible association between season and the carriage of DD treponemes in the GI tract. No DD- associated treponeme DNA was amplified from GI tract samples, for either sheep or beef, in any other season apart from summer. Reasons for this are unknown and contradict previous similar data on dairy cows (Evans *et al.* 2012) where the winter housing season was positively associated with DD treponeme carriage in the GI tract. This finding may be due to different management practices between the different host species. Alternatively, it may suggest that there are episodes of shedding on farms, as the majority of positive sheep GI tract tissues were collected from one farm on one day. Similarly, the majority of the positive beef GI tract tissues were collected from the fallen stock centre on the same day (although unknown, it is therefore possible they originated from the same farm). Worth noting is that this was a convenience sample and therefore other influencing factors may have been present and so not too much emphasis can be placed on the seasonal association.

Although a small proportion of samples were found to be positive for DD-associated treponeme bacteria, care has to be taken when interpreting these results. It is a possibility, like with any test results there may have been false negatives, or in fact the positive results gained were false positives. The PCR assays used have been validated by the sequencing of PCR products, however only a portion of samples are checked by sequencing. Additionally, the exact sensitivity and specificity of these PCR assays used to detect DD- associated bacteria, are unknown. Therefore although positive results are often validated

by sequencing, this does not account for all positive results and does not rule out a proportion of negative results actually being false negatives. Additionally cross reactivity with other treponeme species (non-pathogenic species) is also a possibility, and the degree of this is unknown as specificity of the assays is unknown. However the stringent design of the primers used in the PCR assays aims to increase ensure high specificity of the assays.

In comparison to previous work detecting the commonly isolated DD treponeme phylogroups in dairy cattle faeces (Klitgaard *et al.* 2014; Zinicola *et al.* 2015), it is interesting that we were unable to detect these same bacteria in either sheep (n= 79) or beef cattle (n= 41) faecal samples. This may imply that these bacteria are too low in abundance in faecal samples for detection using the DNA extraction and PCR assay techniques we have commonly used for detection of these bacteria in DD affected feet. Alternatively, like the GI tract tissues, there may only be a small number of animals that shed the bacteria into their faeces and so only an extremely large number of faeces would be sufficient to detect the treponemal bacteria. It may also be a technical failure of the PCR assays we have developed to detect the target treponemes from these specific samples.

It is possible to interpret the data in a different manner and suggest that the low numbers of positive GI tract tissues are simply due to contamination with DD treponemes from the farm environment or from other parts of the cows. However, most of the samples used in this study were deep tissue biopsies, including the sheep rectal samples the DD treponeme was isolated from, therefore it is unlikely due to contamination of the tissue. Furthermore, good asceptic technique was used during sample collection.

There was an extremely high isolation rate of spirochaetes from sheep faeces which was entirely due to repeated isolation of *T.* sp. CHPA, previously isolated from the faeces of a dairy cow (Evans *et al.* 2012) from multiple samples. However, what is striking is the percentage of these isolates that were obtained from CODD symptomatic sheep. A significant proportion, 17/22 (77%) of these spirochaete isolates were isolated from CODD symptomatic

sheep. It is interesting to speculate whether these animals are in fact shedding various species of treponemes into the environment (including DD treponeme species), but due to the issues involved in treponeme culture biasing towards the growth of predominant identifiable treponemes, we were only able to isolate this apparently commensal species of treponeme. However, this data is based on isolation results and so is difficult to interpret. Hence, to confirm and investigate this association, PCR analysis would need to specifically target this treponeme species. The reason for such a high isolation rate of CHPA treponemes from sheep faeces and yet no successful isolations from beef faeces is unknown, but may be due to differences in diet between beef animals and sheep and therefore faeces constituents. Additionally, despite there being a wide diversity of treponemes isolated/identified in the rumen (Paster and Canale-Parola 1982) it is interesting that, in the current study, the same phylogroup of rumen treponemes was consistently isolated from sheep samples. Interestingly *T.* sp. CHPA, when previously isolated, was from dairy cow faeces in the same geographical area. This suggests that this is not only a prominent, and previously not reported, ruminal commensal species in sheep but also a commensal shared by ruminants in this geographical region. Further studies are needed to characterise and taxonomically appraise *T.* sp. CHPA, towards proposition as a new species given that it shares less than 97% 16S rRNA gene sequence similarity with taxonomically defined closest relatives suggesting it is a novel species (Stackebrandt and Goebel 1994).

Upon further genetic analysis of the treponemes isolated from the sheep faecal samples, it was apparent that there were four groups which were highly similar (sharing >99.9% sequence identity) based on 16S rRNA gene sequence analysis, with said groupings corresponding to the farm the faecal samples were collected from. This apparent ruminant treponeme 16S rRNA gene diversity associated with specific farms is something that has not been reported in the DD treponemes with both dairy cattle BDD, beef cattle BDD and sheep CODD treponemes from different farms/geographical areas showing little or no 16S rRNA gene diversity (Evans *et al.* 2008; Chapter 3, 4; Sullivan *et al.* 2015a, 2015b).

The morphology and structure of treponemes, particularly their flagella, allows them to be highly motile bacteria, capable of rotational and translational movement (Radolf and Lukehart 2006; Evans *et al.* 2009a). It has previously been demonstrated, by antitreponemal immunohistochemical staining, that treponemes are capable of breaching the skin barrier via hair follicles and causing infection down into deep layers of surrounding tissues (Evans *et al.* 2009a). The ability of treponemes to access such breaches in skin barriers and bury deep into tissues makes it little surprise to find them in deep tissue biopsies of gingival and rectal tissue. However, given this and their detection in GI tract fluids, it could suggest these treponemes are more adaptable bacteria than previously thought. Their role as a pathogen in the feet of ruminants is well known, however their exact role whilst in these GI tissues is currently undetermined.

The detection of DD- associated treponeme phylogroup DNA in both sheep and beef cattle GI tract tissues, even in low numbers, indicates that the GI tract may be an important infection reservoir of DD treponemes in multiple DD suffering host species. The isolation of live pathogenic treponemes from the rectal tissue of a CODD positive sheep highlights for the first time that these bacteria can be live, and possibly transmissible if shed, in at least this GI tract tissue. The presence of pathogenic DD- associated treponemes in both the gingival and rectal tissue of one animal would suggest that certain animals may carry DD treponemes throughout their GI tract. The presence of DD treponemes in the GI tract of a small proportion of animals may indicate that only a small number of animals are able to shed these bacteria, and may be key to disease transmission on farms. The role of *T.* sp. CHPA needs further investigation to fully understand any specific contribution to the GI tract microbiome and to clarify the identified association with DD symptomatic animals.

Chapter 7

The presence of digital dermatitis *Treponema* spp. on cattle and sheep hoof trimming equipment

7.1 Introduction

Digital dermatitis was first reported in dairy cattle many years ago (Cheli and Mortellaro 1974) and is now an important disease in sheep in the UK (Harwood *et al.* 1997) and has been very recently confirmed in beef cattle (Chapter 4; Sullivan *et al.* 2013, 2015a). The primary causative agents of DD have failed to be isolated from outside of the animal and therefore transmission routes of the disease are unknown. This includes the three commonly isolated *Treponema* phylogroups from dairy cattle lesions in the UK and USA (Stamm *et al.* 2002; Evans *et al.* 2008), *T. medium*- like, *T. phagedenis*- like and *T. pedis* (Evans *et al.* 2008; 2009a).

Treatments, such as footbathing (Laven and Proven 2000; Laven and Hunt; 2002) and antibiotic treatments (Manske *et al.* 2002; Nishikawa and Taguchi 2008; Berry *et al.* 2010), have shown some clinical benefits. However, no single effective treatment has yet been identified making it imperative to gain more information on the transmission routes of DD to enable intervention measures to limit the spread of the disease.

There has been much debate about the role of the GI tract as a reservoir of infection of DD treponemes, with the bovine gingival and rectal tissues, rumen fluid and faeces identified as potential infection reservoirs. A study by Evans and *et al.* (2012) detected DD treponemes in the oral cavity and the rectum of dairy cattle and more recent work detected DD treponeme phylogroups in rumen fluid, faecal samples, slurry and the oral and rectal tissues of ruminants (Klitgaard *et al.* 2014; Nascimento *et al.* 2015; Zinicola *et al.* 2015; Chapter 6; Sullivan *et al.* 2015d). However, it was only recently that a DD treponeme was isolated from the GI tract of a CODD positive sheep. Although this suggests the GI tract is potentially an important infection reservoir for DD treponemes, the three *Treponema* phylogroups associated with DD are only consistently detected in the lesions themselves, and have yet to be isolated from outside of the host animal.

One item that regularly comes into direct contact with the feet of cattle and sheep is hoof trimming equipment. Regular hoof trimming can be an essential part of managing livestock. The frequency of which hoof trimming is carried out depends on the environment the ruminants live in and the diet they are fed, but currently trimming is recommended once or twice a year in cattle (Dairy Co 2013).

Several studies have shown a beneficial effect of hoof trimming on cattle lameness (Manske *et al.* 2002; Somers *et al.* 2005). However, a study conducted by Wells *et al.* (1999) raised the possibility of an association between animal hoof trimming and an increase in incidence of BDD. They found that the incidence of BDD increased with shorter hoof trimming intervals; if the primary hoof trimmer had worked on other farms the incidence of BDD significantly increased, and if the trimming equipment was not washed with water between cows, the percentage of herds with BDD significantly increased. Hoof trimming in sheep has also been implicated with negative effects in another foot disorder, footrot, with recent clinical trials indicating that trimming was counterproductive in the control of the disease (Kaler *et al.* 2010). It is possible that foot trimming exposes tissues on feet so that

opportunistic infections from feet lesions or the farm environment are more likely. Alternatively, the results of Wells *et al.* (1999), could be attributed to the DD *Treponema* phylogroups being present on the trimming equipment used to trim ruminant hooves, and being transmitted between animals by this route. For this to be possible, it would be important to determine whether treponemes are able to adhere to the metal equipment used to trim the hooves, and secondly if they can stay alive (and therefore transmissible) on the equipment.

This study aimed to investigate whether DD *Treponema* phylogroups could be detected on hoof trimming equipment after trimming the feet of ruminants suffering from DD and, therefore, be a possible route of transmission. Trimming equipment was investigated for the presence of the three previously isolated DD *Treponema* phylogroups, after being used to trim the hooves of DD symptomatic and asymptomatic cattle and sheep feet, and subsequently, after routine disinfection of the equipment.

7.2 Methods

7.2.1 Sample collection and Farm information

Samples were taken by the attending vet on the farm during routine hoof trimming and/or treatment of cattle (n= 24) and sheep (n= 13) hoof. Farms used in this study were from the Denbighshire, Monmouthshire and Gloucestershire areas, and were sampled on various dates between November 2012 and April 2013. Cattle farms A, B and D were dairy farms, farm C and E were beef farms (all in Gloucestershire), and farm F was a BDD negative dairy herd (no animals on the farm had BDD lesions) located in Monmouthshire. Dairy farms A, B and D consisted of 150–300 milking cows, farm F around 90 cows, cubicle-housed but partly running outside during the day in summer. All were largely closed (buying in animals rarely); however, the BDD negative farm, farm F, had been a closed herd for 15 years. Farm C was a beef rearing unit, with 120 beef cattle, and Farm E a finishing unit containing 3000 beef animals.

Sheep farm A was located in Gloucestershire, and sheep farm B in Denbighshire, both largely closed sheep farms consisting of around 200–450 ewes. Farm A housed sheep indoors, whereas B were mainly outdoors and indoors only for the lambing period.

The respective vets attending the farm went about their normal routine of assessing lame animals, diagnosing hoof disorders, routine/treatment trimming and any additional treatments. Sampled animals were chosen randomly from the ones already selected to be trimmed on the day of sampling. Cattle having their hooves trimmed were restrained in a crush, and sheep were either tipped by the vet/foot trimmer or placed into a small sheep tipper (Figure 7.1, 7.2). A cow was defined as having BDD if one or more feet had a clear lesion consistent with the clinical signs of BDD in dairy cattle (Cheli and Mortellaro 1974; Blowey and Sharp 1988) and sheep were defined as having CODD if one or more feet had a clear lesion consistent with the clinical signs of CODD (Winter 2004a). If animals had any other foot diseases present these were noted.

The foot was trimmed using a typical stainless steel hoof knife or scissor type trimmers (Figures 7.1, 7.2, 7.3). The trimming technique used was what would be considered a standard hoof trimming protocol as performed by the vet/hoof trimmer without intervention. However, this differed depending on whether the trimming was for treatment or routine hoof removal. Briefly, the excess hoof was trimmed parallel to the hair growth of the foot, heels paired to the same level as the soles of the toes and excess nail tissue around each toe removed and sole made flat. Instruments used were clean before use and hoof trimmers hands were either cleansed and disinfected or covered by fresh sterile gloves after each trim. The instruments were ensured clean by swabbing which was tested and unless the knife was negative the subsequent samples from the knife were not used in the analysis.

After the trimming of a hoof, the trimming instrument used was tested by swabbing, using a fixed protocol of a single swab passage across each side of

the blade, on both sides of the instrument. The swabs used were plain sterile cotton swabs with reattachable caps to avoid contamination after sampling (Copan Italia, Italy). The trimming instrument was then rinsed in a Department for Environment Food Rural Affairs (DEFRA)-approved iodine disinfectant (containing 2.5% (w/v) available iodine) for two to three seconds by immersing the instrument fully into the disinfectant by hand, and moving the instrument back and forth in the disinfectant. It was then wiped with a clean cloth to remove excess disinfectant and then retested with a fresh swab using the same technique. This swab which was used to test the knife after disinfection was also used as in "intermediate" result which enabled us to know whether the knife was clean when it next was used to trim a hoof. If this result was not negative and the knife was used again to trim the next animal's hoof the subsequent swabbing results of this sample were not used in the analysis. Care was taken by the trimmer to avoid the knife coming into contact with clothing, or any other possible cause of contamination. However this has to be considered as a possible contamination route to samples. Samples were collected or sent by post and on arrival at the laboratory were stored at −20°C.

Figure 7.1: A sheep undergoing hoof trimming. The hoof trimming equipment in this case is in the form of scissor type trimmer.

Figure 7.2: A sheep undergoing hoof trimming. The hoof trimming equipment in this case is in the form of scissor type trimmers which were used to trim some sheep feet.

Figure 7.3: A typical hoof trimming knife used to trim the hooves of both cattle and sheep. A plain cotton swab is shown to indicate what was used to sample the knives and where they were sampled.

7.2.2 Culture of spirochaetes

Bacterial isolation was attempted on eight swab samples. The swab samples used to attempt the culture of spirochaetes were randomly chosen from samples used to trim BDD symptomatic cattle and CODD symptomatic sheep. Four swabs were inoculated after sampling blades after trimming, two from sampling blades used to trim cattle hooves (cattle numbers 2 and 8) and two from blades used to trim sheep hooves (sheep numbers 2 and 4). The remaining four swabs that culture was attempted on were the corresponding second swabs used to sample the same instrument after it had been disinfected immediately after the initial trimming. The culture technique used, subsequent bacterial DNA extraction, gene cleaning and sequencing was carried out as described in Methods 2.2, 2.3.1, 2.5.1 and 2.5.2, respectively.

7.2.3 Swab DNA extraction

All swab samples were subjected to DNA extraction for PCR analysis. DNA extraction was carried out as described in Method 2.3.2.

7.2.4 PCR assays

All swab samples were subjected to nested PCR assays specific for the three DD- associated treponeme groups, *T. medium*- like, *T. phagedenis*- like, *T. pedis* and the treponeme genus specific 16S rRNA gene PCR assay, as described in Methods 2.3.2 and 2.3.3, respectively

7.2.5 Phylogenetic analysis of spirochaete isolates

To understand the relationship of any isolated spirochaetes with other treponemes, and in particular those previously isolated from dairy cattle BDD lesions and previous CODD lesions, a phylogenetic tree was produced according to Method 2.6. The most appropriate evolution model was predicted using “model test” in the Topali programme (Milne *et al.* 2009). The final model chosen for nucleotide substitutions was the TrN model (Tamua and Nei 1993). This was used to infer a bootstrapped maximum likelihood tree with bootstrapping performed 10,000 times.

7.2.6 Nucleotide Accession numbers

One 16S rRNA gene GenBank accession number was determined as part of this study which is: KF736097.

7.3 Results

7.3.1 PCR assays

The specific DD *Treponema* PCR assays and *Treponema* genus-wide PCR results are shown in Tables 7.1 and 7.2. Table 7.1 shows the treponemal detection data on blades used to trim the hooves of cattle, and Table 7.2 the same information for blades used to trim sheep hooves. Table 7.3 shows a summary and comparison of PCR detection rates of DD treponeme phylogroups on hoof trimming blades after trimming DD symptomatic and asymptomatic cattle, and again after subsequent disinfection of the blades. Table 7.4 provides a summary of the PCR detection rates of DD treponeme phylogroups on equipment after trimming and after subsequent disinfection.

Treponemal DNA was detected on the majority of foot trimming blades after trimming either sheep or cattle hooves. After trimming, blades were found to be positive for general *Treponema* DNA in 36/37 (97%) samples, 23/24 (96%) of cattle blades and 13/13 (100%) sheep blades. This was reduced to 13/37 (35%) after disinfection of the blade, 7/24 (29%) cattle, and 6/13 (46%) of sheep blades. The next question was whether these were the treponemes uniquely associated with DD lesions. After trimming, the phylogroup-specific PCR for *T medium*- like, *T phagedenis*-like and *T. pedis* DD spirochaetes, showed that they were present on 16/24 (67%), 15/24 (63%) and 10/24 (42%) of cattle blades, and 7/13 (54%), 6/13 (46%) and 10/13 (77%) of sheep blades, respectively. Combining cattle and sheep results, *T medium*- like, *T phagedenis*- like and *T. pedis* DD spirochaetes, had detection rates of 23/37 (62%), 21/37 (57%) and 20/37 (54%), respectively.

After disinfection, the detection rates of each of the three phylogroups reduced to, respectively, 5/24 (21%), 2/24 (8%) and 1/24 (4%) on cattle blades, and

4/13 (31%), 4/13 (31%) and 2/13 (15%) on sheep blades. Combined, the after disinfection detection rates for the DD treponemes were 9/37 (24%), 6/37 (16%) and 3/37 (8%), respectively (Table 7.4)

Of the blades used to trim DD symptomatic animals (*n*= 26), 25/26 were found to be positive for at least one of the DD *Treponema* phylogroups, 17/17 (100%) of cattle blades and 8/9 (89%) of sheep blades. This figure was reduced to 10/26 (38%) after disinfection of the blades, 7/17 (41%) of cattle blades and 3/9 (33%) of sheep blades. Trimming blades were also sometimes positive for DD treponemes after trimming DD- asymptomatic feet, cattle and sheep, though to a much lesser extent.

7.3.2 Culture of spirochaetes and phylogenetic analysis of spirochaete isolates

Following culture of a swab of a trimming tool, a spirochaete was successfully isolated from a blade which trimmed cattle number 2, a BDD symptomatic cow. The isolate, named SWC2 (Genbank accession: KF736097), was identified as sharing 100% 16S rRNA gene sequence identity with the *T phagedenis*- like DD spirochaete strain T320A (Genbank accession: EF061261), previously isolated from a dairy cow BDD lesion in the UK (Evans *et al.* 2008). Upon phylogenetic tree analysis, the treponeme isolate clustered with its distinct DD phylogroup, the *T. phagedenis* spirochaetes (Figure 7.4).

Table 7.1: PCR detection of DD Treponema phylogroups on hoof trimming blades after trimming DD symptomatic and asymptomatic cattle, and after subsequent disinfection of the blade.

Cattle number	**Farm**	**DD status**	**Other foot diseases**	***After trimming***				***After disinfection***			
				DD treponemes [a]			***Treponema* (genus-wide)**	**DD treponemes [a]**			***Treponema* (genus-wide)**
				1	**2**	**3**		**1**	**2**	**3**	
1	A	+		+	-	-	+	-	-	-	-
2	A	+		+	+	-	+	+	-	-	+

3	A	+		+	+	-	+	-	-	-	-
4	A	+		+	-	-	+	+	-	-	+
5	A	+		+	-	-	+	+	-	-	+
6	A	+		-	+	-	+	-	-	-	-
7	A	+		+	+	+	+	-	-	-	-
8	B	+		+	+	+	+	-	-	-	-
9	C	+		+	+	+	+	-	-	-	-
10	C	+		+	+	+	+	-	+	+	+
11	C	+		+	+	+	+	+	-	-	+
12	C	+		-	+	-	+	-	-	-	-
13	D	+		+	+	+	+	-	-	-	-
14	D	-	NHSU[b]	+	+	+	+	-	-	-	-
15	D	+		+	+	-	+	-	+	-	+
16	D	-		-	-	-	+	-	-	-	-
17	D	-		+	+	+	+	-	-	-	-
18	E	+		+	+	+	+	-	-	-	-
19	E	+		+	+	-	+	+	-	-	+
20	E	+		-	-	+	+	-	-	-	-
21	F	-		-	-	-	+	-	-	-	-
22	F	-		-	-	-	-	-	-	-	-
23	F	-		-	-	-	+	-	-	-	-
24	F	-		-	-	-	+	-	-	-	-

[a] Groups 1, 2 and 3 are *T. medium- like*, *T. phagedenis*- like and *T. pedis* spirochaetes respectively, which are routinely found in DD lesions.
[b] Non healing sole ulcer

Table 7.2: PCR detection of DD Treponema phylogroups on hoof trimming blades after trimming DD symptomatic and asymptomatic sheep, and after subsequent disinfection of the blade.

Sheep number	**Farm**	**DD status**	**Other foot diseases**	***After trimming***				***After disinfection***			
				DD treponemes[a]			***Treponema* (genus-wide)**	**DD treponemes[a]**			***Treponema* (genus-wide)**
				1	**2**	**3**		**1**	**2**	**3**	
1	A	+	footrot	-	-	+	+	-	-	-	-
2	A	+		-	-	+	+	-	-	-	-
3	A	+		+	+	+	+	+	+	+	+
4	A	+		+	+	+	+	-	-	-	-
5	A	+		-	-	-	+	-	-	-	-
6	A	+		+	+	+	+	-	-	-	-
7	A	-	footrot	-	-	+	+	-	-	-	-
8	A	-	SH[b]	-	-	-	+	-	-	-	-
9	B	+		+	+	+	+	+	+	+	+
10	B	+		+	+	+	+	+	+	-	+
11	B	+		+	+	+	+	+	+	-	+
12	B	-		-	-	-	+	-	-	-	+
13	B	-		+	-	+	+	-	-	-	+

[a] Groups 1, 2 and 3 are *T. medium* - like, *T. phagedenis*- like and *T. pedis* spirochaetes respectively, which are routinely found in DD lesions.
[b] Shelly Hoof

Table 7.3: A comparison of PCR detection rates of DD Treponema phylogroups on trimming equipment after it was used to trim DD symptomatic and asymptomatic cattle and sheep.

Animal[a]	**DD status**	***Treponema* group**[b]			***Treponema***
		1	**2**	**3**	**(genus-wide)**
Cattle	BDD+	14/17 **(82%)**	13/17 **(76%)**	8/17 **(47%)**	17/17 **(100%)**
	BDD-	2/7 **(29%)**	2/7 **(29%)**	3/7 **(43%)**	6/7 **(86%)**
Sheep	CODD+	6/9 **(67%)**	6/9 **(67%)**	8/9 **(89%)**	9/9 **(100%)**
	CODD-	1/4 **(25%)**	0/4 **(0%)**	2/4 **(50%)**	22/56 **(39%)**

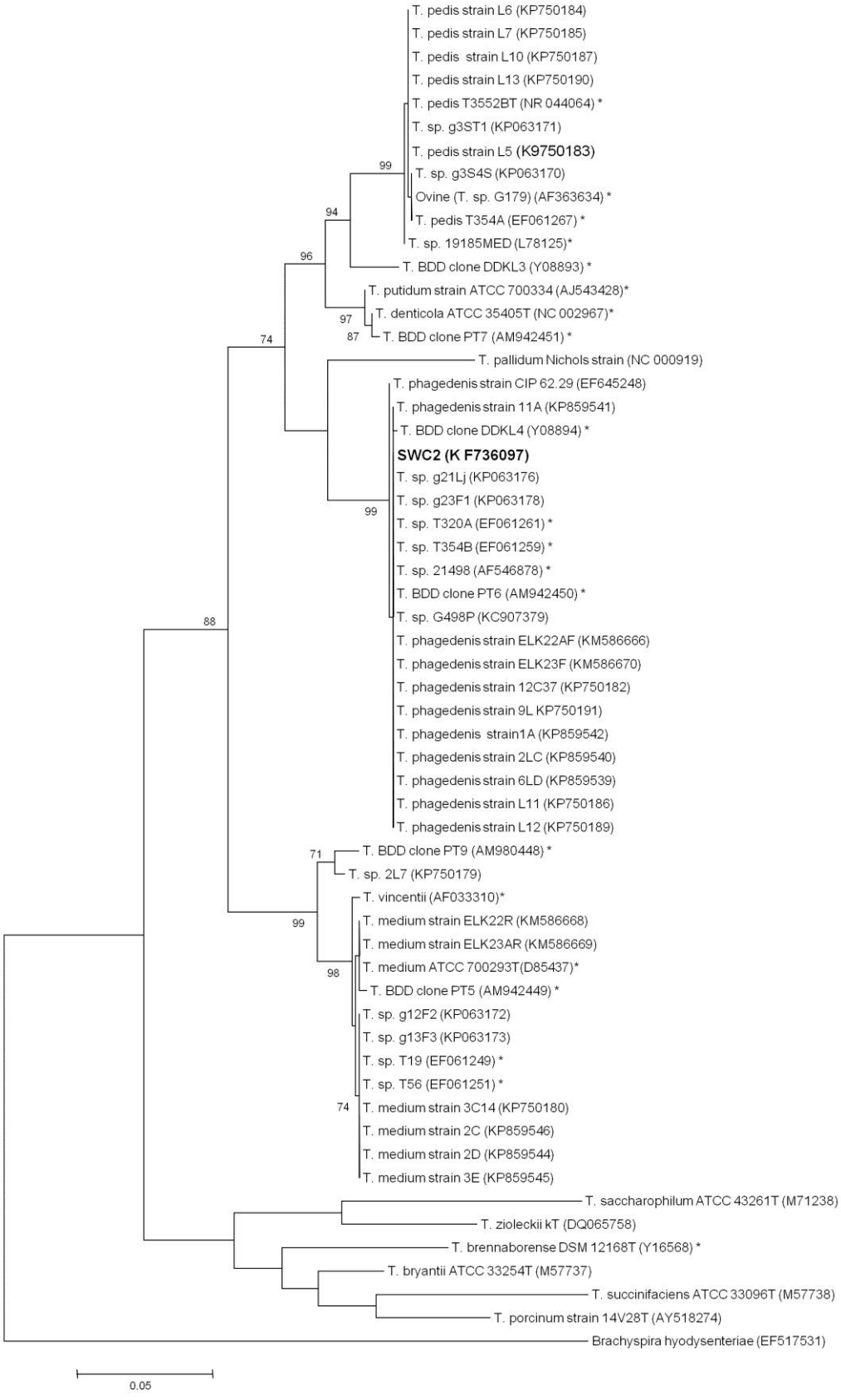
[a]Animal which had its hoof trimmed and subsequently had trimming equipment investigated for treponeme bacteria.
[b]Groups 1, 2 and 3 are *T. medium- like, T. phagedenis*- like and *T. pedis* spirochaetes, respectively.

Table 7.4 A summary of the PCR detection rates of DD Treponema phylogroups on trimming equipment after trimming, compared with the detection rates after subsequent disinfection of the equipment.

Trimming equipment[a]	***Treponema* group**[b]			***Treponema***
	1	**2**	**3**	**(genus-wide)**
After trimming	23/37 **(62%)**	21/37 **(57%)**	20/37 **(54%)**	36/37 **(97%)**
After disinfection	9/37 **(24%)**	6/37 **(16%)**	3/37 **(8%)**	13/37 **(35%)**

[a]The state of the trimming equipment tested, i.e. tested after trimming the animal hoof or after subsequent disinfection of the equipment.
[b]Groups 1, 2 and 3 are *T. medium- like, T. phagedenis*- like and *T. pedis* spirochaetes, respectively.

Figure 7.4: Phylogenetic tree showing the relationship between the treponeme strain isolated here from a piece of trimming equipment used to trim a DD symptomatic cow (shown in bold) and other DD associated and commensal treponeme 16S rRNA gene sequences. A maximum likelihood tree based on 16S rRNA gene sequence comparisons of ~1,200 aligned bases. Bootstrapped 10,000 times, and only bootstrap values above 70% are shown for clarity. = previously reported 16S rRNA gene sequences from BDD lesions.*


T. pedis strain L6 (KP750184)
T. pedis strain L7 (KP750185)
T. pedis strain L10 (KP750187)
T. pedis strain L13 (KP750190)
T. pedis T3552BT (NR 044064) *
T. sp. g3ST1 (KP063171)
T. pedis strain L5 (K9750183)
T. sp. g3S4S (KP063170)
Ovine (T. sp. G179) (AF363634) *
T. pedis T354A (EF061267) *
T. sp. 19185MED (L78125)*
T. BDD clone DDKL3 (Y08893) *
T. putidum strain ATCC 700334 (AJ543428)*
T. denticola ATCC 35405T (NC 002967)*
T. BDD clone PT7 (AM942451) *
T. pallidum Nichols strain (NC 000919)
T. phagedenis strain CIP 62.29 (EF645248)
T. phagedenis strain 11A (KP859541)
T. BDD clone DDKL4 (Y08894) *
SWC2 (K F736097)
T. sp. g21Lj (KP063176)
T. sp. g23F1 (KP063178)
T. sp. T320A (EF061261) *
T. sp. T354B (EF061259) *
T. sp. 21498 (AF546878) *
T. BDD clone PT6 (AM942450) *
T. sp. G498P (KC907379)
T. phagedenis strain ELK22AF (KM586666)
T. phagedenis strain ELK23F (KM586670)
T. phagedenis strain 12C37 (KP750182)
T. phagedenis strain 9L KP750191)
T. phagedenis strain1A (KP859542)
T. phagedenis strain 2LC (KP859540)
T. phagedenis strain 6LD (KP859539)
T. phagedenis strain L11 (KP750186)
T. phagedenis strain L12 (KP750189)
T. BDD clone PT9 (AM980448) *
T. sp. 2L7 (KP750179)
T. vincentii (AF033310)*
T. medium strain ELK22R (KM586668)
T. medium strain ELK23AR (KM586669)
T. medium ATCC 700293T(D85437)*
T. BDD clone PT5 (AM942449) *
T. sp. g12F2 (KP063172)
T. sp. g13F3 (KP063173)
T. sp. T19 (EF061249) *
T. sp. T56 (EF061251) *
T. medium strain 3C14 (KP750180)
T. medium strain 2C (KP859546)
T. medium strain 2D (KP859544)
T. medium strain 3E (KP859545)
T. saccharophilum ATCC 43261T (M71238)
T. zioleckii kT (DQ065758)
T. brennaborense DSM 12168T (Y16568) *
T. bryantii ATCC 33254T (M57737)
T. succinifaciens ATCC 33096T (M57738)
T. porcinum strain 14V28T (AY518274)
Brachyspira hyodysenteriae (EF517531)
99
94
96
97
87
74
99
88
71
99
98
74
0.05

7.4 Discussion

The high detection rate of DD *Treponema* phylogroups on trimming blades soon after trimming cattle and sheep hooves from DD cases suggests this may be a significant and worrying route for the transmission of DD between animals and, possibly, between farms. It would appear that these *Treponema* phylogroups may be able to adhere to the blades used to trim ruminants' hooves, with our study showing all but one blade harboured at least one of the three DD *Treponema* phylogroups after trimming a DD symptomatic animal. The detection rates of treponemes after trimming cattle and sheep were very high, indicating that the treponemes adhere to the knife, and it would appear they are consistently able to do this.

Blades that had been used to trim three BDD asymptomatic cattle and two CODD asymptomatic sheep were positive for DD *Treponema* phylogroups. Some of these had other lesions present on the foot trimmed, whereby treponemes may have been involved, or an early lesion/undiagnosed DD lesion may have been present on these animals. All the blades used to trim the animals from the BDD negative herd were negative for all three DD *Treponema* phylogroups.

The very high detection rates of DD treponeme phylogroups on the trimming equipment was not expected, and raises general questions about transmission of pathogens by metal devices and other farm equipment. *Treponema* species, such as *Treponema socranskii* and *Treponema denticola*, are common pathogens associated with periodontal disease in human beings (Riviere *et al.* 1995; You *et al.* 2013). It has been shown that periodontal disease associated *Treponema* can adhere to metal, where treponemes were found on metallic orthodontic brackets in the human mouth, and one study even suggested that metal brackets can also act as niches for gram negative pathogenic bacteria (Nelson-Filho *et al.* 2011). A more recent study by Andrucioli *et al.* (2012) experimentally fixed metal orthodontic brackets in human patients for 30 days, and then detected *T. socranskii* on 94.4% and *T. denticola* present on 77.8% of

the brackets. These findings of *Treponema* species on metal surfaces is consistent with our detection of DD *Treponema* species on metallic trimming instruments on farms.

However there are several factors which need to be considered when interpreting these results. A very small sample size was used in this study, and therefore to make any conclusions from the results, a larger sample size would be necessary. Additionally, environmental contamination can not be ruled out as a reason for treponeme positive results. Although this would be unlikely as these bacteria are primarily only found on the feet of affected animals, it cannot be ruled out as a possibility.

The isolate obtained from a blade after trimming a DD animal, which shared 100% sequence identity to the *T. phagedenis*- like DD spirochaete strain T320A (Genbank accession: EF061261), shows that bacteria can survive on the blade and, therefore, have the potential to infect the next animal to come into contact with the blade. This highlights how important the disinfection of trimming equipment is in terms of DD transmission. Isolation of DD treponemes has been possible from fragments of horn trimmed from DD symptomatic animals (Sullivan *et al.* unpublished data) and, therefore, it is probably unsurprising that we were able to isolate one of the phylogroups from the knife which has just been in direct contact with the hoof. It must be noted that these spirochaetal bacteria are extremely difficult to culture from animal samples, and this is the first such isolation from the farm environment and not directly from bovine tissues. This technical issue may be why we were unable to isolate a treponeme from a blade after disinfection. Equally, the disinfection of the instrument may be successfully killing the bacteria, but along with the disinfection technique, is not fully removing the DNA from the instrument, therefore, giving a PCR-positive result for the bacteria but culture not being unsuccessful. Further work would need to be undertaken to fully understand whether treponemes are consistently alive on instruments after trimming, for how long, and also whether after disinfection they may be alive and transmissible on the blade.

Our results may explain previous studies findings on the association of foot trimming and BDD, such as the results found by Wells *et al.* (1999). Here, we have shown that DD treponemes are, in fact, on the blades of trimming equipment, and so possibly explaining their previous findings. Additionally, Holzhauer *et al.* (2006) found, in their cross-sectional study to evaluate the prevalence of DD and associated risk factors, that cows that were trimmed >12 months before the study (during regular trimming) were at lower risk for BDD when compared with cows that were trimmed at shorter intervals. One possible reason for this, in light of our new data, may be less frequent trimming reduces the frequency of contact with DD *Treponema* present on the hoof trimming equipment. In terms of studies focusing on the disease prevalence in sheep, a recent investigation by Angell *et al.* (2014), found an increased risk of CODD in sheep when foot trimming was used as a treatment for the disease. Additionally, it could be that hoof trimming exposes parts of normal tissues in a way that they are more susceptible to invasion by bacteria (from whatever reservoir of infection) causing foot infections in cattle and sheep.

While this study does not prove the transmission of DD from animal to animal, or farm to farm, by foot trimming tools, it does indeed show that the primary causative bacteria of DD are present on the knife, and are present on occasions even after disinfection. Hence, DD treponemes may be present on the blade when trimming the next animal's hoof and so may transmit the disease. We do not yet know how long the bacteria may survive on the knife, and whether it would be long enough to transmit across to other premises during a cattle foot trimmer's working day (or week). Our results clearly showed that the routine disinfection method used was not always adequate at removing DD treponemes from the trimming equipment used, with 11/37 (30%) of blades (sheep and cattle combined) remaining positive for one or more DD *Treponema* phylogroups after routine disinfection. The method of knife disinfection used was considered to be a simple technique that might be used by practical hoof trimmers and vets. Therefore, this study does not just highlight how important disinfecting trimming equipment is between each

foot, but it may be that a more thorough disinfection agent or routine may be needed. Additionally, if the animal requires treatment trimming in a case of DD, it would be imperative to thoroughly clean and disinfect the blade after use.

Although only a postulation, it could be possible that while trimming an animal's hoof (eg, for treatment of white line disease), the presence of DD *Treponema* on the knife could enable passage of these bacteria deep into the corium and give rise to diseases such as the newly reported non-healing horn lesions and the increasingly reported lesions on the front coronary band of cattle feet and not in the interdigital spaces at the rear of the foot. This speculation has further significance since it has been reported that DD *Treponema* have been highly associated with, and the likely causal agents, of the non-healing horn lesions such as non-healing white line disease and non-healing toe necrosis (Evans *et al.* 2011a).

Further research is also needed to identify appropriate disinfection protocols of foot trimming equipment to ensure it is effective, with a view to preventing the transmission of DD. While disinfecting a relatively small blade is quite feasible, the routine disinfection of equipment such as foot shears may be difficult due to the washing off of lubricant. Additionally, power tools may be difficult to disinfect and alternative disinfection protocols may need to be considered, although the presence of DD treponemes on such equipment has not yet been studied.

Thus far, several attempts at detecting the causative *Treponema* phylogroups in the farm environment have proved unsuccessful, although the bovine gingival and rectal tissues have been identified as potential infection reservoirs (Evans *et al.* 2012). While the contribution of the GI tract appears convincing, the results from this study suggest that foot trimming equipment may be a major route of transmission throughout cattle and sheep herds, and suggests it may be spread via touch and, therefore, have a similar route of transmission to other closely related diseases such as yaws. Yaws is a tropical infection of the

skin, bones and joints caused by the spirochaete *Treponema pallidum* subspecies *pertenue*, which is considered to be transmitted by skin-to-skin contact with an infective lesion (Mitjà *et al.* 2012, 2013). Therefore, ruminant DD transmission may, in fact, be more similar to the non-venereal human treponematoses than previously considered, with touch as a major route of transmission.

This new data has, for the first time, identified treponemes outside animal tissues on farm equipment, and suggests a mode of transmission of DD in ruminants. The cleaning and disinfection of hoof trimming equipment between animals and between farms is highlighted to be extremely important, and may even limit the spread of DD. Further work is necessary to confirm that hoof trimming blades are an important route of transmission of DD between animals. Additionally, finding out how long the bacteria are able to survive on the knife would be invaluable information and useful to veterinarians and hoof trimmers to limit the spread of DD from farm to farm. In conclusion, this data suggests that thorough disinfection of hoof trimming equipment between feet, between animals and between farms should be strongly recommended to attempt to reduce the transmission of DD.

Chapter 8

Serological studies of beef cattle to determine their exposure to digital dermatitis- associated *Treponema* spp.

8.1 Introduction

Large numbers of spirochaetes can be observed within BDD lesions providing evidence towards their active contribution to the pathogenesis of BDD (Choi *et al.* 1997; Collighan and Woodward 1997; Döpfer *et al.* 1997; Demirkan *et al.* 1998; Moter *et al.* 1998). This hypothesis is further substantiated by studies showing that serum samples from dairy cattle with BDD contain elevated levels of antibody to *Treponema* antigens (Demirkan *et al.* 1999; Murray *et al.* 2002; Trott *et al.* 2003).

Whilst circulating antibodies against treponemes in cattle with BDD can be detected and reach high levels, they do not appear to be protective (Walker *et al.* 1997; Demirkan *et al.* 1999; Vink 2006) as they can stay at high levels and the disease frequently recurs. This lack of protection could be for several reasons. For example, the work of Scholey *et al.* (2013) showed that in cases of BDD a significant upregulation of anti-inflammatory cytokines occurred that potentially could suppress local immune responses. This may explain why

systemic immunity to treponemes appears to have a small/no effect against the occurrence or reoccurrence of BDD. Additionally, *Treponema phagedenis*-like spirochaetes have been found to have an immunosuppressive effect on bovine macrophages and negative effects on the innate immune response, as well as wound repair, which may explain the persistent nature of the lesions (Zuerner *et al.* 2007).

More information is needed to fully understand the pathogenesis and the role the immune system plays in the susceptibility to, formation of and persistence of BDD lesions. Dhawi *et al.* (2005) developed an enzyme-linked immunosorbent assay (ELISA) assay to detect anti-treponeme antibodies in the sera of cattle and sheep against treponeme isolates and used Western blotting to detect antigen reactivity in sera. Cattle with BDD had increased seropositivity rates to treponemes isolated from both cattle and sheep. In some cattle herds, significant correlations were shown between antibodies to bovine DD treponemes and CODD treponemes and in other herds, there was no cross reaction, suggesting the presence of more than one treponeme in BDD. Western blotting against both treponeme antigens showed that they frequently manifested different antigen epitopes, but some minor bands were common to both microorganisms. That study (Dhawi *et al.* 2005) supports the hypothesis that there are a number of treponemes in UK farms, which could be involved in the pathogenesis of BDD in dairy cattle.

The perceived lack of effectiveness of injected antibiotics, combined with their requirement for milk withdrawal after treatment has meant that topical antibiotic treatment and footbathing is far more commonly used for the treatment of BDD. Unfortunately however, there remains no known single effective treatment for DD that can eliminate this severe disease (Laven and Logue 2006). Taken all together, it appears as though an effective vaccine against BDD may be one of the only ways to limit/eliminate BDD in cattle. This gives more reason for investigation into the immunological response of ruminants to treponemes to enable future vaccine design.

Digital dermatitis in beef cattle has only recently been reported (Chapter 4; Sullivan *et al.* 2013, 2015a) and there is no data on the nature of immune responses to treponemes in beef cattle. Beef cattle BDD lesions, according to anecdotal reports from vets/farmers, may have increased in prevalence in recent years. Prevalence estimates of BDD in dairy cattle herds range from between 20- 30% (Brown *et al.* 2000; Cramer *et al.* 2008; Barker *et al.* 2009) but can be as high as 62% (460/742) (Nielsen *et al.* 2012). However, previous data (Brown *et al.* 2000; Chapter 4; Sullivan *et al.* 2013), appears to suggest a lower within-herd BDD prevalence rate in beef cattle (0.5-21%), than is commonly found in dairy cattle. This may be due to beef cattle having shorter lives than dairy cows which may increasingly contract lesions as they get older. However, there may be other reasons. Whether the small amount of prevalence data so far is an underestimate of the true prevalence of BDD in these animals, or whether beef animals are truly contracting the disease less than dairy cattle is currently unknown. If the latter is true, the question remains whether this is due to a lack of exposure to the *Treponema* bacteria due to differing farm management systems for beef cattle, or that beef cattle are able to mount a protective immune response which dairy cattle have been shown unable to generate. If the immune response in these beef animals indicates that the animals have been exposed but responded in an effective manner then this could lead to exceedingly useful data for future vaccine design.

This study aimed to analyse beef cattle sera samples to identify their immunological responses using ELISA assays and Western blotting against treponeme antigens from a variety of *Treponema* isolates. By comparing BDD positive and negative herd responses, and using comparisons to previous dairy cattle immunological data, it was hoped to identify whether beef cattle have a more effective immune response or whether there is a lower exposure of beef cattle to DD treponemes.

8.2 Materials and Methods

8.2.1 Collection of blood samples and farm information

Beef cattle were blood sampled from four different farms between March and November 2014. These farms were located in Gloucestershire (Gloucestershire farm 1, 2, 3) and Monmouthshire (farm 4). From these farms a total of 100 blood samples were obtained.

Farm 1 was a beef-rearing unit, with around 120 beef cattle, and sampled cattle were 2.5-3 years of age, all Belgium Blue Friesian crosses or Aberdeen Angus Friesian crosses. Beef cattle were housed in groups of approximately 30 animals, in straw yards with a feed fence at the front. Some did go out to graze, but the animals sampled were all housed. Farm 2 was a finishing unit, finishing around 3000 animals each year. Animals sampled from this farm were 1-2 years of age housed mainly indoors and of one of the following breeds; Belgium Blue cross Friesian, Aberdeen Angus cross Friesian or Limousin cross Friesian. Both Farm 1 and Farm 2 had BDD present on their farms; however, exact prevalence estimates at the time of sampling were not available.

Farm 3 consisted of around 90 – 100 milking cows, and around 180 beef stock. Beef animals sampled animals were 1-1.5 years of age and were Friesian beef bulls. Dairy cows on the farm were housed in cubicles; however, the beef animals that were sampled were in straw yards. The cows went out to graze in summer but the bull beef (from which samples were obtained) stay housed all year due to the bovine tuberculosis risk if they go out to graze. Farm 4 consisted of 80 cows in milk, but had 100 heifers, as the herd was hoping to expand to 120 milking cows. Beef calves were retained, giving a total stock of 250. Both farm 3 and farm 4 were BDD negative farms, with no BDD present in any animals on site. Both farms had been BDD free for over 30 years. From farm 1, farm 2, farm 3 and farm 4, n= 27, n= 38, n= 10 and n= 25 blood samples were collected from beef animals, respectively.

Cows blood sampled were chosen randomly on the day as the attending vet (RB) was performing other veterinary routine treatments on these animals. This was convenient as these animals were already being put through a race and therefore blood sampling could easily be performed. On farms 1 and 2 some animals were reported with BDD at the time of sampling, farm 1; *n*= 1, farm 2; *n*= 5; however, in most cases it was not possible to comprehensively ascertain the BDD status due to the difficulty of routinely lifting all of the feet for inspection.

8.2.2 Treponeme antigen preparations

Antigens were prepared according to Method 2.8.2. Treponemes which were used in the antigen preparation for subsequent immunological studies are listed in Table 8.1. Due to all three *Treponema* phylogroups being consistently isolated from BDD lesions in dairy and beef cattle (Stamm *et al.* 2002; Evans *et al.* 2008; 2009a; 2009b; Chapter 4; Sullivan *et al.* 2015a) , it was decided to separately prepare antigens from all three phylogroups isolated from beef cattle BDD lesions for investigation with beef sera. Additionally, for validity and also to identify antigenic variation within treponeme phylogroups, two additional antigen preparations of each phylogroup were prepared from DD treponemes isolated from dairy cattle BDD lesions and sheep CODD lesions. This consisted of *T. medium*- like, *T. phagedenis*- like and *T. pedis* isolates from sheep CODD lesions, dairy cow BDD lesions and beef cow BDD lesions, giving a total of nine isolates.

8.2.3 Quantification of protein concentrations in antigen preparations

Protein concentrations of antigen preparations were determined to allow the correct and consistent concentrations of proteins to be used in ELISA assays and Western blotting. Protein concentrations were determined according to Method 2.8.3.

Table 8.1: Treponema isolates used to prepare antigens for subsequent immunological studies. Their corresponding 16S rRNA gene Genbank accession number is listed in the right hand column.

***Treponema* isolate**	**Lesion *Treponema* isolate was obtained**	**Location isolated**	**Genbank accession number**
T. medium- like strain T19	Dairy cow BDD lesion	Merseyside, UK	EF061249
T. medium- like strain 2C	Beef cow BDD lesion	Gloucestershire, UK	KP859546
T. medium- like strain g1OV11	Sheep CODD lesion	Gloucestershire, UK	KP063154
T. phagedenis- like strain T320A	Dairy cow BDD lesion	Merseyside, UK	EF061261
T. phagedenis- like strain 6LD	Beef cow BDD lesion	North Wales, UK	KP859539
T. phagedenis- like strain g2F9	Sheep CODD lesion	Conwy, UK	KP063160
T. pedis strain T3552B	Dairy cow BDD lesion	Merseyside, UK	EF061268
T. pedis strain L13	Beef cow BDD lesion	Gloucestershire, UK	KP750190
T. pedis strain g3S4S	Sheep CODD lesion	Shropshire, UK	KP063171

8.2.4 Trichloroacetic acid (TCA) protein precipitation

This was used to concentrate protein solutions in order to obtain better visualisation of proteins from antigen preparations on one-dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE) gels. TCA protein precipitation was performed as per Method 2.8.4.

8.2.5 Enzyme-linked immunosorbent assays (ELISAs)

ELISA assays were carried out according to Method 2.8.5. ELISA data was analysed using Microsoft Excel 2010 (v14.0)**.** Linear regression was used for correlation coefficient determinations. ELISA antibody titre results were expressed on a numerical scale of 0-9, based on the known positive sera result on each ELISA plate. Following ELISA results, a subset of beef animal sera was analysed using Western Blotting.

8.2.6 One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE)

1D SDS-PAGE was used to view the protein profiles of treponeme antigen preparations and as the initial step for Western Blotting. For this a 12% resolving SDS-polyacrylamide gels and 5% SDS-polyacrylamide stacking gels were used. Gels were prepared and run according to Method 8.2.6.

8.2.7 Western Blotting

For Western blotting, proteins were first separated using 1D SDS-PAGE as described Method 8.2.6. After resolving spirochaete proteins in the SDS-PAGE gel, the electrophoretic transfer of proteins was carried out and subsequent Western blotting steps according to Method 2.8.7.

8.3 Results

8.3.1 ELISA assays

The cut off for seropositivity was defined as mean+ 3*Standard Deviation (SD) for control animal sera in each ELISA assay ie against each specific antigen. SD is a measure that is used to quantify the amount of variation or dispersion of a set of data values. Stand deviation was worked out using the following formula:

$$\sigma = \sqrt{\frac{\sum (x - \bar{x})^2}{n}}$$

σ = standard deviation

$\sum$ = sum of

x = each value in the data set

$\bar{x}$ = mean of all values in the data set

n = number of value in the data set

Each serum sample was defined as seropositive or seronegative for IgG_1 and IgG_2 to each treponeme strain (Table 8.2). Analysis of quantitative data was also performed.

In terms of antibodies to IgG_1, 64/100 (64%) of sera were seropositive to at least one of the treponeme strains, farm 1; 23/27 (85%), farm 2; 36/38 (95%), farm 3; 1/10 (10%), farm 4; 4/25(16%). Combined, 59/65 (89%) of sera samples from BDD positive farms (farms 1 and 2) were seropositive to at least one treponeme strain, and only 5/35 (14%) of sera from BDD negative farms (farms 3 and 4) were seropositive for to at least one treponeme strain. As can be seen from Table 8.2, many were IgG_1 and IgG_2 seropositive to all three treponeme phylogroups, including all nine treponeme strains.

The rate of detection of antibodies to IgG_2 appeared to be similar to that of IgG_1 with 49/100 (49%) of sera being seropositive to at least one of the treponeme strains, farm 1; 23/27 (85%), farm 2; 25/38 (66%), farm 3; 0/10 (0%), farm 4; 1/25 (4%). Combined, 48/65 (74%) of sera samples from BDD positive farms (farms 1 and 2) were seropositive to at least one treponeme strain, and only 1/35 (3%) of sera from BDD negative farms (farms 3 and 4) were seropositive to at least one treponeme strain.

The IgG_1 and IgG_2 seropositivity rates to all nine treponeme strains were calculated for all cows, broken down by farm (Table 8.3). For BDD positive farms, the farm seropositivity rate to any one treponeme strain ranged from 66%- 82% and 37%- 66% for IgG_1 and IgG_2 response, respectively. For BDD negative farms, the farm seropositivity rate to any one treponeme strain ranged from 0%- 16% and 0%- 4% for IgG_1 and IgG_2 response, respectively.

Table 8.2: Seropositivity to spirochaetes amongst beef cattle from DD positive and DD negative farms.

Sera	IgG_1									IgG_2								
	Treponeme strain[a]									Treponeme strain[a]								
	D 1	B 1	S 1	D 2	B 2	S 2	D 3	B 3	S 3	D 1	B 1	S 1	D 2	B 2	S 2	D 3	B 3	S 3
1	3.1	2.1	2.1	1.8	2.1	2.5	3.2	4.9	2.7	1.1	1.2	1.6	1.2	1.4	1.0	1.3	1.6	1.2
2	2.0	1.0	1.4	1.4	1.3	1.8	2.3	1.9	2.1	1.4	1.3	1.5	1.2	1.1	0.9	1.3	1.0	1.1
3	2.1	1.5	2.5	2.6	2.6	6.2	2.3	4.3	2.7	1.9	2.9	3.3	2.4	2.5	4.1	1.7	8.8	1.7
4	4.5	4.5	1.5	8.2	8.3	8.8	4.7	4.1	5.7	2.3	1.3	1.8	4.1	3.9	3.5	2.7	1.0	3.7
5	2.3	1.3	1.3	1.4	1.3	5.8	1.5	3.0	1.8	1.4	1.4	1.8	1.4	1.1	2.4	1.2	2.0	1.1
6	2.2	1.2	1.2	1.1	1.2	1.6	1.6	1.1	1.7	1.3	1.3	1.8	1.5	1.1	1.1	1.4	0.9	1.0
7	5.5	5.5	6.6	8.0	8.4	8.9	4.4	3.0	6.7	4.6	6.6	6.7	8.2	8.6	8.6	3.9	4.7	5.5
8	4.4	4.4	3.4	1.7	1.9	2.1	2.5	2.3	3.0	1.9	1.9	2.1	1.7	1.5	1.5	1.4	1.3	1.5
9	3.0	1.0	2.0	1.4	1.4	2.5	2.2	1.6	2.1	1.3	1.3	1.6	1.2	1.3	1.4	1.3	1.0	1.1
10	2.6	1.6	1.6	1.0	1.0	1.5	1.4	1.6	1.7	1.6	1.6	1.8	1.1	1.1	0.9	1.3	1.2	1.1
12	2.3	1.1	1.1	1.0	1.1	1.3	1.5	1.2	1.6	1.4	1.4	1.6	1.2	1.1	0.8	1.2	1.0	1.0
13	3.9	3.9	3.7	8.0	8.6	8.6	4.6	6.1	6.1	5.7	5.6	6.0	9.0	8.8	8.4	5.3	3.5	5.2
14	3.4	2.4	1.6	6.9	7.2	6.9	4.2	4.1	4.0	5.3	5.2	3.9	9.0	8.9	9.0	6.0	4.0	4.7
15	2.3	1.3	1.7	1.1	1.2	1.7	1.5	1.3	1.7	2.3	2.3	2.5	1.5	1.9	0.9	1.3	1.0	1.3
16	2.2	1.2	1.6	1.1	1.1	1.4	1.4	1.2	1.5	2.2	2.2	2.4	1.1	1.4	0.9	1.2	1.0	1.0
17	4.7	4.7	4.6	8.3	8.7	6.9	4.8	5.3	7.6	2.4	2.4	2.6	5.9	5.3	2.1	2.9	1.2	2.4
18	3.2	3.2	2.1	1.2	1.3	2.0	2.1	1.8	2.3	1.5	1.5	1.7	1.2	1.5	1.6	1.5	1.1	1.1
19	4.6	3.6	3.6	5.9	6.5	5.8	4.2	4.1	4.1	2.4	2.4	2.6	2.4	2.5	2.1	1.9	1.4	1.7
21	4.9	3.9	3.9	7.7	8.8	3.4	4.5	5.0	7.1	3.6	2.6	2.8	3.9	3.9	1.5	2.8	1.9	2.6
22	4.5	3.5	1.8	4.6	4.2	7.6	3.7	6.2	5.5	3.5	3.5	2.8	3.2	3.3	2.8	2.8	4.3	3.9
23	2.7	2.7	2.7	1.2	1.1	1.6	1.7	1.5	1.9	2.2	2.2	2.4	1.3	1.7	1.1	1.3	1.0	1.1
25	2.5	2.5	2.4	1.2	1.2	2.5	1.5	1.4	1.8	2.3	2.3	2.6	1.2	1.2	0.9	1.2	1.0	1.0
26	2.3	2.3	2.2	1.2	1.0	1.4	1.4	1.3	1.7	2.3	2.3	2.6	1.3	1.4	0.9	1.4	1.1	1.0
27	2.5	2.5	2.6	1.3	1.2	1.5	1.3	1.3	1.7	2.2	2.2	2.4	1.1	1.0	0.8	1.2	1.0	0.9
28	3.5	2.5	2.5	4.0	4.4	6.3	3.8	4.2	4.6	2.6	1.6	1.8	1.3	1.1	1.1	1.4	1.0	1.7
29	2.6	1.6	2.6	1.8	2.3	5.9	2.2	3.9	2.5	2.5	2.5	3.7	3.9	4.5	5.3	2.9	8.6	2.8
30	2.3	1.5	1.5	1.0	1.1	1.5	1.7	1.4	1.8	1.9	1.9	2.2	1.3	1.1	1.2	2.0	1.0	1.2
A1	3.2	2.2	2.2	1.5	1.5	2.3	2.2	1.7	3.0	2.4	1.4	1.6	1.3	0.9	1.2	1.5	0.9	1.2
A2	3.2	3.2	3.3	4.3	5.2	8.8	3.1	2.0	8.8	2.2	3.2	3.8	2.2	2.4	5.4	2.0	1.1	4.9
A3	3.4	3.4	1.9	3.1	3.2	3.4	3.1	2.3	4.7	2.1	2.1	2.3	1.6	4.7	1.1	1.6	0.9	1.2
A4	4.7	4.7	4.2	6.1	6.8	8.2	4.6	2.9	8.1	3.6	3.5	3.9	5.6	5.8	5.8	3.5	3.6	9.0
A5	3.4	3.4	3.3	5.8	7.2	8.3	3.1	2.5	4.9	3.2	3.4	2.2	5.1	4.9	3.3	3.1	3.8	6.6
A6	2.9	2.3	1.7	1.6	1.6	2.5	2.3	2.3	4.2	1.9	1.9	2.1	1.1	1.0	0.9	1.3	1.0	1.2
A7	4.0	4.0	3.0	6.4	7.7	8.7	4.0	3.4	8.3	5.1	4.9	5.4	5.4	5.4	5.6	4.0	8.4	8.3
A8	2.3	2.3	1.9	1.2	1.3	1.4	1.9	1.3	2.0	2.4	2.4	2.6	1.3	1.5	2.4	2.1	1.1	1.1
A9	5.5	6.5	6.4	3.4	4.1	6.3	3.1	1.8	5.8	3.3	4.3	4.5	1.9	1.9	1.7	1.9	1.0	2.3
A10	2.5	2.5	2.5	1.2	1.5	2.3	2.2	1.7	1.8	3.2	2.2	2.4	1.3	1.4	1.4	1.8	1.2	1.4
A11	4.3	4.3	2.3	2.5	2.8	3.6	1.9	1.5	3.0	2.4	2.4	2.6	1.6	1.6	1.1	1.6	0.9	1.0

A12	2.5	4.5	2.5	3.1	3.3	2.7	2.0	3.8	3.0	2.3	2.3	2.9	1.6	1.6	1.1	1.4	1.2	1.1
A13	2.7	3.7	2.7	2.7	2.8	3.6	1.8	1.5	2.6	1.4	1.4	1.1	1.7	1.5	1.1	1.5	0.9	1.0
A14	1.4	1.3	1.3	1.2	1.6	1.6	1.0	1.3	1.4	0.8	0.9	1.9	0.9	1.4	0.9	1.5	0.9	1.1
A15	2.1	2.0	2.0	1.1	1.4	1.4	1.0	1.4	1.5	0.8	0.9	1.9	0.9	1.4	1.0	1.4	1.0	1.2
A16	2.7	2.6	2.6	2.5	2.9	3.3	2.2	1.2	2.2	0.8	0.9	1.9	1.5	2.0	2.0	1.6	0.9	1.5
A17	4.2	4.2	4.2	5.0	6.1	8.8	4.2	3.7	5.8	3.3	3.5	3.4	3.6	4.1	6.4	3.1	2.9	6.7
A18	3.7	3.7	3.7	4.3	2.9	5.4	4.0	3.6	4.8	2.2	2.3	2.8	3.1	2.6	6.5	3.0	2.0	2.5
A19	2.5	2.3	2.3	1.1	1.2	2.8	1.9	1.3	1.7	0.8	0.9	1.9	0.9	1.1	1.0	1.3	0.9	1.2
A20	2.6	2.5	2.5	1.1	1.4	2.2	2.2	1.2	1.7	0.8	0.9	1.9	1.0	1.3	0.9	1.3	1.0	1.2
A21	2.3	2.2	2.2	1.1	1.4	1.1	1.9	1.5	1.9	0.8	0.9	1.8	0.9	1.5	0.9	1.4	1.1	1.3
A22	1.8	1.7	1.7	1.1	1.4	2.3	1.7	1.3	1.5	0.7	0.9	1.9	0.7	1.2	0.9	1.2	0.9	1.1
A23	1.1	3.0	3.0	1.3	1.4	1.6	1.5	2.0	2.1	1.0	2.3	3.3	0.8	1.3	1.1	1.2	1.1	1.4
A24	2.3	1.1	1.6	1.0	1.2	1.2	1.3	1.1	1.3	0.8	0.9	1.9	0.8	1.1	0.8	1.2	0.9	1.0
A25	3.0	4.9	4.9	6.9	7.5	9.0	3.5	4.2	6.0	1.0	2.1	2.8	2.5	3.0	3.9	1.7	1.0	5.7
A26	3.6	2.5	2.5	3.2	3.5	2.2	2.4	3.2	3.8	0.8	1.0	2.7	3.0	3.8	1.3	3.0	1.0	2.9
A27	2.8	2.7	2.7	3.2	3.6	4.7	2.0	3.3	4.7	0.8	0.9	1.9	1.6	2.3	1.9	1.4	1.0	6.1
A28	3.2	2.8	2.8	3.0	3.4	3.8	2.2	2.4	3.0	1.4	1.5	5.5	1.8	2.2	2.3	1.4	1.1	1.7
A29	2.7	1.6	1.6	1.2	1.5	1.3	1.7	1.2	1.5	0.8	0.9	1.9	0.9	1.4	0.9	1.2	0.8	1.1
A30	2.1	2.0	2.0	1.3	1.9	3.8	2.3	1.4	1.9	1.2	1.3	2.3	1.0	1.7	1.7	1.4	1.0	1.3
A31	3.0	2.9	2.9	1.5	2.3	2.8	2.1	1.3	2.6	1.0	1.2	1.2	0.9	2.4	1.4	1.4	0.9	1.9
A32	2.1	2.0	2.0	1.4	1.6	1.8	1.8	1.6	2.0	0.7	1.9	1.9	0.9	1.2	0.9	1.2	1.0	1.1
A33	1.7	1.6	1.6	1.1	1.7	1.2	1.6	1.3	1.5	0.7	1.8	1.8	0.9	1.2	0.9	1.2	1.0	1.0
A34	3.8	3.6	3.6	2.2	3.0	2.3	2.1	2.1	2.2	1.3	2.4	3.4	1.0	1.3	0.9	1.3	1.0	1.2
A35	1.9	2.8	2.8	2.6	2.6	2.9	2.0	1.3	2.5	1.2	3.3	4.3	1.1	1.3	1.2	1.3	1.0	1.6
A36	1.9	1.8	1.8	1.5	2.0	3.4	1.9	1.3	1.7	0.8	0.9	1.9	0.9	1.4	1.4	1.2	1.0	1.2
A54	7.2	7.1	7.1	9.0	8.4	9.0	7.4	5.3	8.6	5.0	6.2	6.1	7.3	8.1	8.8	5.4	3.1	7.2
A65	1.5	1.4	1.5	1.0	1.3	1.4	1.0	1.2	1.4	0.8	1.9	2.2	1.0	1.3	0.9	0.9	1.0	1.1
J1	0.7	0.6	1.0	0.4	0.6	0.5	0.1	0.6	0.7	0.8	0.9	1.5	1.1	0.8	0.8	0.8	0.9	0.6
J2	1.3	1.2	1.1	0.5	0.7	1.0	0.3	0.9	0.9	1.0	1.2	1.6	0.8	1.0	0.9	0.9	1.0	0.6
J3	0.6	0.5	1.1	0.6	0.8	0.6	0.4	0.5	0.7	0.8	1.0	0.6	0.9	0.9	0.8	0.8	1.0	0.5
J4	1.1	0.9	0.8	0.7	0.8	1.2	0.5	0.7	1.0	1.0	1.2	1.2	0.9	0.9	1.0	1.0	0.9	0.7
J5	0.6	1.5	1.3	0.7	0.8	0.6	0.3	0.6	0.7	0.8	0.9	0.9	1.0	0.9	0.8	0.9	0.9	0.6
J6	1.2	0.7	0.6	0.9	1.3	0.6	0.5	0.8	0.9	1.1	1.2	1.4	1.0	1.0	0.8	0.8	0.9	0.6
J7	0.7	0.6	0.7	0.6	1.0	0.7	0.4	0.6	0.7	0.7	0.8	0.6	0.9	1.0	0.9	0.9	0.9	0.5
J8	1.0	0.9	0.8	0.5	0.9	1.0	0.9	1.0	1.2	0.9	1.0	1.2	0.9	0.8	0.9	0.9	1.0	0.6
J9	1.4	1.3	1.1	0.6	0.6	0.5	0.0	0.8	0.8	0.9	1.1	1.3	0.9	0.7	0.8	0.9	0.9	0.6
J10	0.8	0.7	1.2	0.5	0.6	0.8	0.6	1.0	0.9	0.8	0.9	1.2	0.9	0.9	0.9	0.8	1.0	0.5
1	1.2	1.1	1.0	0.7	0.8	1.0	0.5	0.7	0.9	0.8	0.9	1.2	0.9	1.7	0.9	1.0	1.0	0.5
2	1.1	1.4	1.2	0.7	0.8	1.1	0.4	0.7	0.8	0.8	1.0	1.2	0.9	0.8	1.0	0.9	0.9	0.5
3	0.9	0.8	0.7	1.5	0.7	1.3	1.0	0.8	1.6	0.8	0.9	1.1	1.3	1.9	1.0	1.1	0.9	0.8
4	1.1	1.4	1.3	0.8	1.0	0.7	0.3	0.6	0.8	0.7	0.8	0.7	0.9	0.9	0.7	0.9	0.8	0.5
5	0.8	1.5	1.4	0.7	0.9	0.3	0.3	0.6	0.7	0.7	0.8	1.1	0.9	0.9	0.8	0.8	0.8	0.5
6	0.5	0.6	0.4	0.8	1.0	1.0	0.1	0.7	1.1	0.8	1.1	1.1	1.2	1.4	0.9	0.9	1.0	1.0
7	0.5	0.6	0.5	0.9	1.1	0.7	0.1	0.7	1.1	0.8	0.9	0.8	1.0	1.1	0.8	0.9	0.9	0.8
8	0.4	0.6	0.6	1.0	0.8	0.6	0.1	0.7	1.2	0.8	1.1	1.0	0.8	1.1	0.9	0.9	1.0	0.7

9	0.4	0.6	0.5	0.6	1.0	0.6	0.4	0.5	1.0	0.7	0.8	0.8	1.0	1.0	0.7	0.8	0.9	0.6
10	0.4	0.7	0.5	0.7	1.0	0.7	0.2	0.6	1.3	0.7	0.9	0.8	1.1	1.0	0.7	0.8	0.8	0.7
11	0.4	0.5	0.4	0.7	1.1	0.8	0.5	0.7	1.3	0.8	0.9	0.8	1.1	1.0	0.8	0.8	0.9	0.7
12	0.4	0.5	0.6	0.6	1.0	0.7	0.8	0.5	1.0	0.8	1.1	1.0	1.0	1.0	0.9	0.8	0.9	0.8
13	0.4	0.5	0.6	0.5	1.1	0.7	0.1	0.6	0.9	0.8	0.9	1.0	1.1	1.1	1.0	0.9	0.9	0.8
14	0.5	0.6	0.5	0.7	0.9	0.8	0.3	0.6	0.9	0.9	1.0	0.9	1.0	1.3	1.3	0.9	0.9	0.8
15	0.5	0.6	0.6	1.1	0.9	0.8	0.0	0.7	0.9	0.8	0.9	0.8	1.0	1.3	0.9	0.9	0.9	0.7
16	0.4	0.5	0.6	0.8	0.7	0.7	0.3	0.6	1.0	0.8	1.2	1.1	1.1	1.5	1.0	1.1	0.9	1.0
17	0.6	0.7	1.3	1.2	1.5	1.3	0.3	0.8	1.4	0.7	0.8	0.7	1.1	1.1	0.9	0.9	0.8	0.6
18	0.7	0.8	1.4	1.1	1.7	1.9	0.6	0.8	1.5	0.9	1.0	1.0	1.1	1.1	0.9	0.9	0.8	0.8
19	0.4	0.5	0.6	0.5	1.3	0.8	0.5	0.5	1.0	0.8	0.9	0.8	1.1	1.0	0.8	0.8	0.8	0.7
20	0.4	0.5	0.5	0.7	1.2	1.1	0.5	0.6	1.1	0.8	0.9	0.8	1.0	1.0	0.9	0.8	0.9	0.6
21	0.3	0.5	0.5	0.6	1.3	0.8	0.1	0.6	1.2	0.8	1.0	0.8	1.1	1.0	0.9	0.8	0.9	0.7
22	0.5	0.6	0.5	0.9	1.1	1.5	0.6	0.7	1.0	0.8	0.9	0.8	1.1	1.1	0.8	0.9	0.9	0.7
23	0.6	0.7	1.1	1.1	1.1	1.3	0.2	0.8	1.2	0.7	0.8	0.7	1.1	1.2	0.7	0.8	0.8	0.6
24	0.5	0.8	0.7	1.0	1.1	1.2	0.1	0.8	1.4	0.8	1.2	1.3	1.1	1.5	1.0	0.9	0.8	0.7
25	0.4	0.5	0.5	0.6	1.0	1.0	0.5	0.6	1.1	0.7	0.8	0.7	1.1	1.2	1.1	0.9	0.8	0.7

[a] Treponeme strains have been abbreviated according to the following abbreviations: D1, B1, S1, D2, B2, S2, D3, B3, S3 refer to dairy cattle (D), beef cattle (B) and sheep (S) DD lesion isolates, 1 refers to *T. medium*- like, 2; *T. phagedenis*- like and 3; *T. pedis* spirochaete isolates. Full isolate names in Table 8.1.
Green highlighting indicates seropositivity, blank indicates seronegativity. Number shows level of seropositivity based on scale 0-9.
Bold horizontal borders signal the start of sera samples from a different farm. Double line vertical border separates IgG_1 and IgG_2 seropsitivity responses to treponemes.
Sera highlighted in yellow are sera obtained from BDD positive cattle (BDD present on at least 1 or more feet).

Table 8.3: Seropositivity rates of farms to treponeme strains

Treponeme strain[a]	**IgG_1 /IgG_2 response[b]**	**Farm 1**	**Farm 2**	**Farm 3**	**Farm 4**
D1	**1**	19/27 (70%)	29/38 (76%)	1/10 (10%)	4/25 (16%)
	2	12/27 (44%)	17/38 (45%)	0/10 (00%)	0/25 (0%)
B1	**1**	19/27 (70%)	29/38 (76%)	1/10 (10%)	4/25 (16%)
	2	12/27 (44%)	17/38 (45%)	0/10 (0%)	0/25 (0%)
S1	**1**	19/27 (70%)	26/38 (68%)	1/10 (10%)	4/25 (16%)
	2	11/27 (41%)	17/38 (45%)	0/10 (0%)	0/25 (0%)
D2	**1**	17/27 (63%)	28/38 (74%)	0/10 (0%)	2/25 (8%)
	2	13/27 (48%)	17/38 (45%)	0/10 (0%)	0/25 (0%)

B2	**1**	17/27 (63%)	31/38 (82%)	0/10 (0%)	2/25 (8%)
	2	16/27 (60%)	17/38 (45%)	0/10 (0%)	1/25 (4%)
S2	**1**	18/27 (67%)	31/38 (82%)	1/10 (10%)	3/25 (12%)
	2	13/27 (48%)	19/38 (50%)	0/10 (0%)	0/25 (0%)
D3	**1**	18/27 (67%)	25/38 (66%)	1/10 (0%)	4/25 (16%)
	2	15/27 (56%)	15/38 (39%)	0/10 (0%)	0/25 (0%)
B3	**1**	17/27 (63%)	27/38 (71%)	1/10 (10%)	4/25 (16%)
	2	18/27 (67%)	14/38 (37%)	0/10 (0%)	0/25 (0%)
S3	**1**	18/27 (67%)	28/38 (74%)	1/10 (10%)	4/25 (16%)
	2	18/27 (67%)	16/38 (42%)	0/10 (0%)	0/25 (0%)

[a] Treponeme strains have been abbreviated according to the following abbreviations: D1, B1, S1, D2, B2, S2, D3, B3, S3 refer to dairy cattle (D), beef cattle (B) and sheep (S) DD lesion isolates, 1 refers to *T. medium*- like, 2; *T. phagedenis*- like and 3; *T. pedis* spirochaete isolates. Full isolate names in Table 8.1.

[b] IgG_1 /IgG_2 response has been abbreviated to 1 and 2, respectively.

All known DD positive animals were seropositive for IgG_1 against at least one treponeme strain, excluding one animal (A65) which showed neither a significant IgG_1 nor IgG_2 response to any treponeme strain. Half (3/6) of the known DD positive animals, although showing an IgG_1 response to at least one treponeme strain, did not show an IgG_2 response to any treponeme isolate.

Using linear regression analysis to produce correlation coefficients (*r* values) from the data, made it possible to understand whether there was a correlation between IgG_1 response and IgG_2 response to each treponeme strain, allowing comparisons between responses to each treponeme strain within a phylogroup and between phylogroups. R values range from -1.0 to +1.0. The closer r is to +1 or -1, the more closely the two variables are related (+ integer; positively correlated, - integer; negatively correlated). In all analyses, an associated probability (*P*- value) of < 0.05 was considered significant.

When sera IgG_1 and IgG_2 antibody responses to each treponeme strain were compared, all responses showed a strong positive correlation, (all r values were > 0.7, and *P* values < 0.001), indicating a positive relationship between IgG_1 and IgG_2 responses to all purified treponeme antigens. Table 8.4 lists r and *P*

values for IgG_1 responses and IgG_2 responses to each treponeme strain. As examples, Figures 8.1 and 8.2 show the linear regression correlation for IgG_1 and IgG_2 antibodies against *T. phagedenis*- like strain T320A and *T. pedis* strain g3S4S, respectively.

Table 8.4: Correlation coefficients (r values) and associated probability (P values) for IgG_1 versus IgG_2 antibody response to each treponeme strain.

Treponeme strain [a]	**r value**	***P* value**
D1	0.754	*< 0.001*
B1	0.732	*< 0.001*
S1	0.772	*< 0.001*
D2	0.857	*< 0.001*
B2	0.842	*< 0.001*
S2	0.810	*< 0.001*
D3	0.833	*< 0.001*
B3	0.532	*< 0.001*
S3	0.858	*< 0.001*

[a] Treponeme strains have been abbreviated according to the following abbreviations: D1, B1, S1, D2, B2, S2, D3, B3, S3 refer to dairy cattle (D), beef cattle (B) and sheep (S) DD lesion isolates, 1 refers to *T. medium*- like, 2; *T. phagedenis*- like and 3; *T. pedis* spirochaete isolates. Full isolate names in Table 8.1.

When the different isolates of each phylogroup were compared, there was a strong positive correlation for IgG_1 and IgG_2 antibody response to each of the isolates from each phylogroup, i.e levels of IgG_1 and IgG_2 Anti- *T. medium*-like strain T19, show a positive correlation with levels of IgG_1 and IgG_2 Anti-*T. medium*- like strain 2C (IgG_1, IgG_2; r = 0/905, $P < 0.001$; r = 0.912 $P < 0.001$, respectively). This was true for all strains of the same phylogroup (see Table 8.5). Figure 8.3 shows the correlation between sera levels of Anti- *T. phagedenis*- like strain T320A versus Anti- *T. phagedenis*- like strain 6LD levels (IgG_1) and Figure 8.4 shows the correlation between sera levels of Anti-

T. phagedenis- like strain 6LD versus Anti- *T. phagedenis*- like strain g2F9 levels (IgG_2).

Additionally, the IgG_1 and IgG_2 response to isolates from different phylogroups also showed a strong positive correlation, i.e there was a positive correlation for antibody response levels against *T. medium*- like strains and *T. phagedenis*- like (both IgG_1 and IgG_2 responses). This was true for all combinations. Table 8.6 provides corresponding r and *P* values. As examples, Figure 8.5 shows the correlation between sera levels of Anti- *T. pedis* strain T3552B versus Anti- *T. medium*- like strain T19 antibody levels (IgG_1) and Figure 8.6 shows the correlation between sera levels of anti- *T. pedis* strain g3S4S versus Anti- *T. phagedenis*- like strain g10V11 antibody levels (IgG_1).

ELISA antibody titre results were expressed on a numerical scale of 0-9, and the frequency of each antibody titre level in response to each treponeme isolate for each farm is shown in Table 8.7 (IgG_1) and Table 8.8 (IgG_2). For the DD negative farm sera, on Farm 3 only one sera had an antibody response of 3 on the scale, all other BDD negative farm sera were below 3, and Farm 3 has no titre level over 1. BDD positive farms had much higher antibody titre levels, with all sera levels 1 or above. There appeared generally lower titre levels for IgG_2 antibody response; however, as also seen in the linear regression graphs, all IgG_1 and IgG_2 titres for all treponeme antigens were positively correlated (see Table 8.4 for r values).

Figure 8.1: The linear regression analysis of IgG_1 and IgG_2 antibodies against T. phagedenis- like strain T320A.

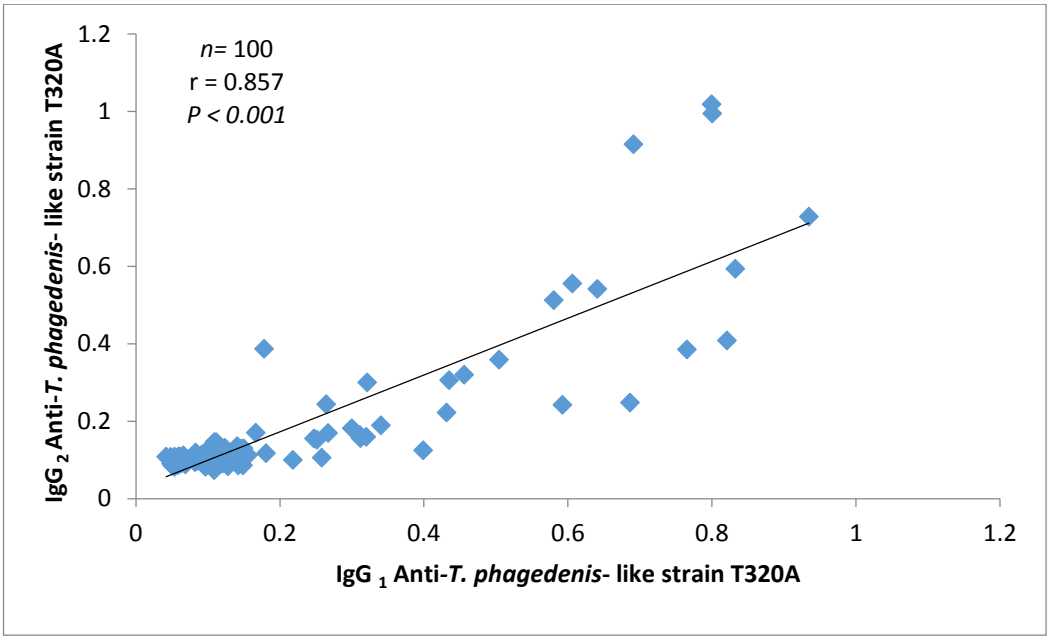


Figure 8.2: The linear regression analysis of IgG_1 and IgG_2 antibodies against T. pedis strain g3S4S.

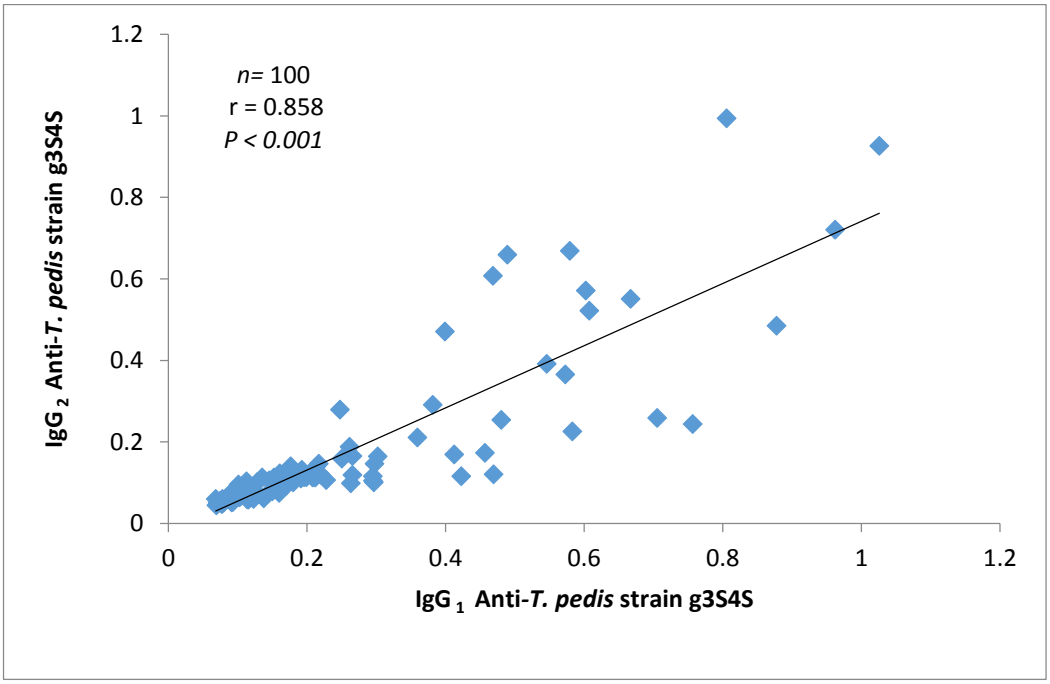


Figure 8.3: The linear regression analysis of IgG1 antibodies against T. phagedenis- like strain 6LD versus T. phagedenis- like strain T320A.

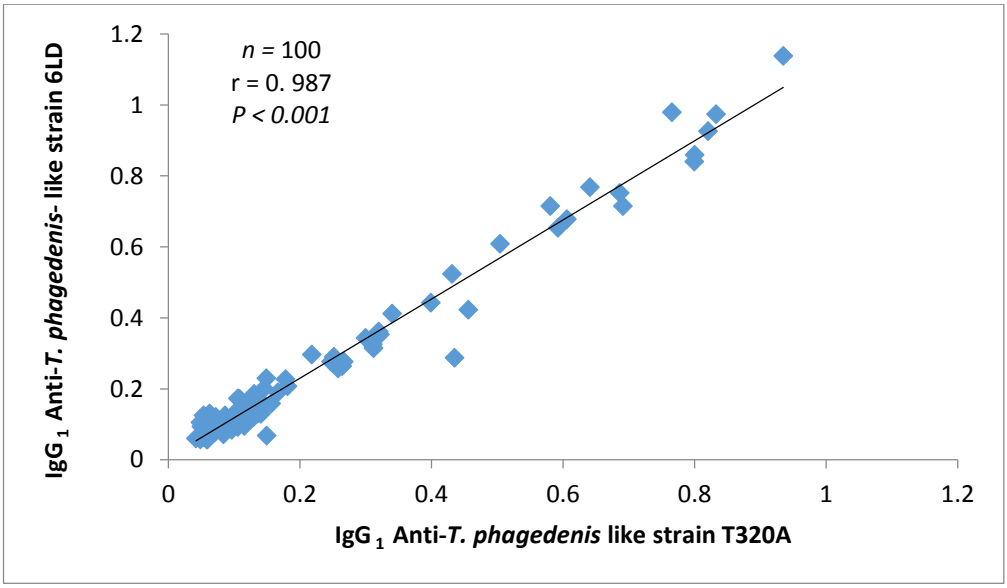


Figure 8.4: The linear regression analysis of IgG2 antibodies against T. phagedenis- like strain g2F9 versus T. phagedenis- like strain 6LD.

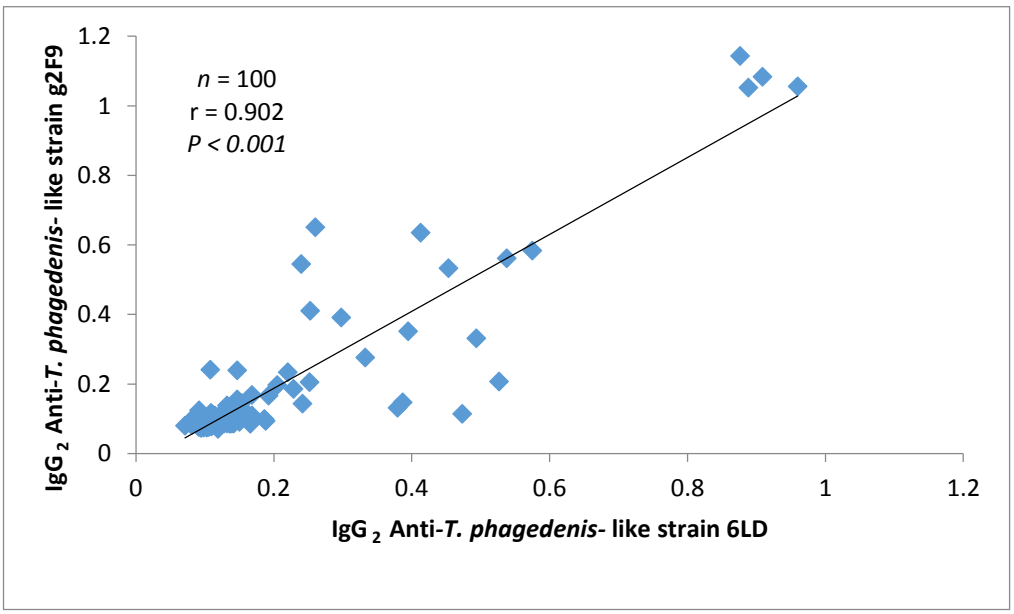


Figure 8.5: The linear regression analysis of IgG$_1$ antibodies against T. pedis strain T320A versus T. medium- like strain T19.

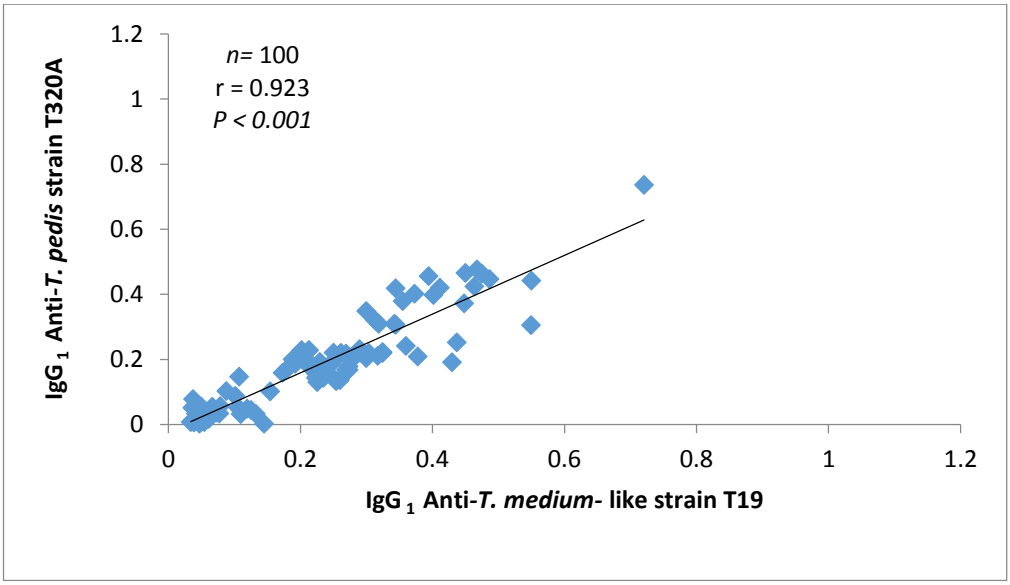


Figure 8.6: The linear regression analysis of IgG$_1$ antibodies against T. pedis strain g3S4S versus T. phagedenis- like strain g2F9.

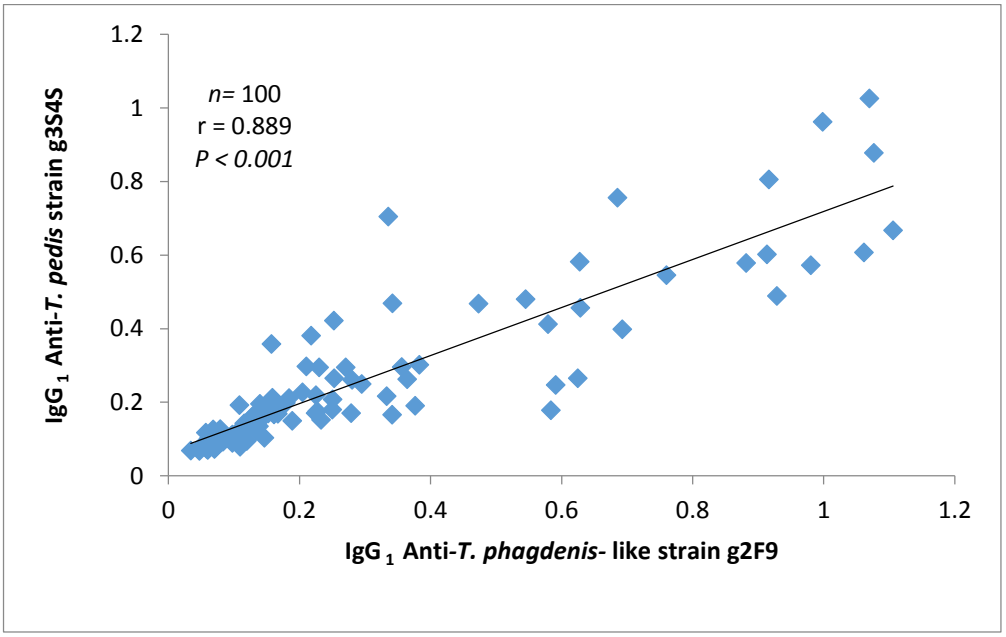


Table 8.5: (a)- (c); Correlation coefficients (r values) and associated probability (P values) for sera IgG$_1$ antibody response to each treponeme strain versus sera IgG$_1$ antibody response to each other treponeme isolate from the same phylogroup. (d)- (f); Correlation coefficients (r values) and associated probability (P values) for sera IgG$_2$ antibody response to each treponeme strain versus sera IgG$_2$ antibody response to each other treponeme isolate from the same phylogroup. Treponeme strains have been abbreviated according to the following abbreviations: D1, B1, S1, D2, B2, S2, D3, B3, S3 refer to dairy cattle (D), beef cattle (B) and sheep (S) DD lesion isolates. Full isolate names in Table 8.1.

(a) IgG$_1$ antibody responses to *T. medium*- like strains

	D1	B1	S1
D1	-	0.905 (P< 0.001)	0.903 (P< 0.001)
B1	-	-	0.940 (P< 0.001)
S1	-	-	-

(b) IgG$_1$ antibody responses to *T. phagedenis*- like strains

	D2	B2	S2
D2	-	0.987 (P< 0.001)	0.887 (P< 0.001)
B2	-	-	0.869 (P< 0.001)
S2	-	-	-

(c) IgG$_1$ antibody responses to *T. pedis* strains

	D3	B3	S3
D3	-	0.860 (P< 0.001)	0.883 (P< 0.001)
B3	-	-	0.767(P< 0.001)
S3	-	-	-

(d) IgG$_2$ antibody responses to T. medium- like strains

	D1	B1	S1
D1	-	0.912 (P< 0.001)	0.778 (P< 0.001)
B1	-	-	0.883 (P< 0.001)
S1	-	-	-

(e) IgG$_2$ antibody responses to *T. phagedenis*- like strains

	D2	B2	S2
D2	-	0.965 (P< 0.001)	0.910(P< 0.001)
B2	-	-	0.902 (P< 0.001)
S2	-	-	-

(f) IgG$_2$ antibody responses to *T. pedis* strains

	D3	B3	S3
D3	-	0.591 (P< 0.001)	0.580 (P< 0.001)
B3	-	-	0.578 (P< 0.001)
S3	-	-	-

Table 8.6: (a)- (c); Correlation coefficients (r values) and associated probability (P values) for sera IgG$_1$ antibody response to each treponeme phylogroup strain isolated from the same host species (e.g. beef BDD lesion) versus sera IgG$_1$ antibody response to each of the other treponeme phylogroup strains isolated from the same host species. (d)- (f); Correlation coefficients (r values) and associated probability (P values) for sera IgG$_2$ antibody response to each treponeme phylogroup strain isolated from the same host species versus sera IgG$_1$ antibody response to each of the other treponeme phylogroup strains isolated from the same host species. Treponeme strains have been abbreviated according to the following abbreviations: D1, B1, S1, D2, B2, S2, D3, B3, S3 refer to dairy cattle (D), beef cattle (B) and sheep (S) DD lesion isolates. Full isolate names in Table 8.1.

(a) IgG$_1$ antibody responses to dairy cattle BDD treponeme isolates from each phylogroup.

	D1	D2	D3
D1	-	0.788 (P< 0.001)	0.923 (P< 0.001)
D2	-	-	0.891 (P< 0.001)
D3	-	-	-

(b) IgG$_1$ antibody responses to beef cattle BDD treponeme isolates from each phylogroup.

	B1	B2	B3
B1	-	0.790 (P< 0.001)	0.701 (P< 0.001)
B2	-	-	0.800 (P< 0.001)
B3	-	-	-

(c) IgG$_1$ antibody responses to sheep CODD treponeme isolates from each phylogroup.

	S1	S2	S3
S1	-	0.766 (P< 0.001)	0.792 (P< 0.001)
S2	-	-	0.889 (P< 0.001)
S3	-	-	-

(d) IgG$_2$ antibody responses to dairy BDD treponeme isolates from each phylogroup.

	D1	D2	D3
D1	-	0.835 (P< 0.001)	0.881 (P< 0.001)
D2	-	-	0. 926 (P< 0.001)
D3	-	-	-

(e) IgG$_2$ antibody responses to beef BDD treponeme isolates from each phylogroup.

	B1	B2	B3
B1	-	0.828 (P< 0.001)	0.591 (P< 0.001)
B2	-	-	0.577 (P< 0.001)

B3	-	-	-

(f) IgG_2 antibody responses to sheep CODD treponeme isolates from each phylogroup.

	S1	S2	S3
S1	-	0.752 (P< 0.001)	0.672 (P< 0.001)
S2	-	-	0.757 (P< 0.001)
S3	-	-	-

Table 8.7: ELISA IgG_1 antibody titre results expressed on a numerical scale of 0-9, and the frequency of each antibody titre level in response to each treponeme isolate for each farm is shown. Abbreviations: F1; farm 1, F2; farm 2, F3; farm 3, F4; farm 4. (a)- (c); (a) IgG_1 antibody titre results in response to T. medium- like treponeme isolates, (b) IgG_1 antibody titre results in response to T. phagedenis- like treponeme isolates, (c) IgG_1 antibody titre results in response to T. pedis treponeme isolates.

(a)

	T. medium- like strain T19				*T. medium*- like strain 2C				*T. medium*- like strain g1OV11			
ELISA titre scale	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
0	0	0	0	16	0	0	0	3	0	0	0	4
1	1	2	10	9	6	3	10	22	4	1	10	21
2	9	15	0	0	8	15	0	0	10	19	0	0
3	8	12	0	0	6	9	0	0	8	11	0	0
4	5	6	0	0	5	6	0	0	3	4	0	0
5	4	2	0	0	2	3	0	0	1	1	0	0
6	0	0	0	0	0	1	0	0	0	1	0	0
7	0	1	0	0	0	1	0	0	1	1	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0

(b)

	T. phagedenis- like strain T320A				*T. phagedenis*- like strain 6LD				*T. phagedenis*- like strain g2F9			
ELISA titre scale	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
0	0	0	2	1	0	0	0	0	0	0	2	1
1	11	11	8	24	11	7	10	25	4	7	8	23
2	4	11	0	0	4	12	0	0	9	9	0	1
3	2	8	0	0	2	9	0	0	3	9	0	0
4	2	1	0	0	3	3	0	0	0	3	0	0

5	1	1	0	0	0	1	0	0	0	2	0	0
6	1	4	0	0	0	1	0	0	5	1	0	0
7	1	1	0	0	2	2	0	0	2	0	0	0
8	5	0	0	0	1	2	0	0	1	0	0	0
9	0	1	0	0	4	1	0	0	3	7	0	0

(c)

	T. pedis strain T3552B				*T. pedis* strain L13				*T. pedis* strain g3S4S			
ELISA titre scale	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
0	0	0	7	17	0	0	0	0	0	0	0	0
1	5	4	3	8	7	4	10	25	0	6	10	23
2	11	24	0	0	6	24	0	2	14	12	0	2
3	2	4	0	0	2	4	0	0	4	7	0	0
4	6	3	0	0	6	3	0	0	2	2	0	0
5	3	1	0	0	4	1	0	0	2	4	0	0
6	0	0	0	0	2	1	0	0	2	3	0	0
7	0	1	0	0	0	1	0	0	2	0	0	0
8	0	0	0	0	0	0	0	0	1	1	0	0
9	0	0	0	0	0	0	0	0	0	3	0	0

Table 8.8: ELISA IgG_2 antibody titre results expressed on a numerical scale of 0-9, and the frequency of each antibody titre level in response to each treponeme isolate for each farm is shown. Abbreviations: F1; farm 1, F2; farm 2, F3; farm 3, F4; farm 4. (a)- (c); (a) IgG_2 antibody titre results in response to T. medium- like treponeme isolates, (b) IgG_2 antibody titre results in response to T. phagedenis- like treponeme isolates, (c) IgG_2 antibody titre results in response to T. pedis treponeme isolates.

(a)

	T. medium- like strain T19				*T. medium*- like strain 2C				*T. medium*- like strain g1OV11			
ELISA titre scale	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
0	0	0	3	15	0	0	3	10	0	0	3	9
1	8	19	7	10	7	16	7	15	4	6	7	16
2	12	9	0	0	13	13	0	0	12	17	0	0
3	2	7	0	0	3	6	0	0	7	8	0	0
4	2	1	0	0	1	1	0	0	2	3	0	0
5	2	2	0	0	1	1	0	0	0	3	0	0
6	1	0	0	0	1	1	0	0	1	1	0	0

7	0	0	0	0	1	0	0	0	1	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0

(b)

	T. phagedenis- like strain T320A				*T. phagedenis*- like strain 6LD				*T. phagedenis*- like strain g2F9			
ELISA titre scale	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
0	0	0	3	5	0	0	1	3	0	0	3	3
1	13	20	7	20	12	19	9	21	12	23	7	22
2	6	10	0	0	4	10	0	1	8	5	0	0
3	1	3	0	0	4	2	0	0	1	2	0	0
4	3	1	0	0	2	2	0	0	2	2	0	0
5	0	2	0	0	2	3	0	0	1	1	0	0
6	1	1	0	0	0	1	0	0	0	3	0	0
7	0	1	0	0	0	0	0	0	0	1	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	3	0	0	0	3	1	0	0	3	1	0	0

(c)

	T. pedis strain T3552B				*T. pedis* strain L13				*T. pedis* strain g3S4S			
ELISA titre scale	F1	F2	F3	F4	F1	F2	F3	F4	F1	F2	F3	F4
0	0	0	7	16	0	0	6	4	0	0	6	4
1	12	20	3	9	10	20	4	21	9	21	4	20
2	6	10	0	0	9	10	0	0	8	7	0	1
3	4	6	0	0	2	6	0	0	3	2	0	0
4	1	1	0	0	4	1	0	0	2	0	0	0
5	3	1	0	0	1	1	0	0	4	1	0	0
6	1	0	0	0	0	0	0	0	1	2	0	0
7	0	0	0	0	0	0	0	0	0	3	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	2	0	0	0	0	2	0	0

8.3.2 SDS-PAGE and Western Blotting

SDS-PAGE profiles of whole-cell lysates of each treponeme isolate can be seen in Figure 8.7. Protein staining showed that the banding patterns of whole-cell lysates were quite similar amongst the three strains from each phylogroup, and with some differences between phylogroups.

Both IgG_1 and IgG_2 antibodies were detected by Western blotting. Both positive and negative sera to treponemes (identified by ELISA) were used in the Western blotting analysis. Twelve ELISA positive and twelve ELISA negative sera were selected for analysis for antibodies to each treponeme strain. The molecular weights on the NCS were estimated from the protein standard weight markers.

ELISA- positive sera from cows with BDD, and ELISA- positive sera from cows on BDD positive farms (but DD status unknown) presented similar banding patterns, for both IgG_1 and IgG_2 staining. Additionally, there were very limited differences in terms of IgG_1 and IgG_2 band staining for both positive and negative sera.

Upon Western blotting analysis, staining patterns for IgG_1 and IgG_2 antibodies against strains within each phylogroup were almost identical. Limited differences were seen, which is what would be expected from the ELISA assay results.

Overall, the major positively stained bands for all western blots, for all nine treponeme strains, were at 30-32 kDa and 12-14 kDa for both IgG_1 and IgG_2. The 30-32 kDa was present in the banding pattern from both ELISA positive and negative sera; however, the 12-14 kDa band was mainly seen in ELISA positive sera. Additionally, a common band only for the *T. phagedenis*- like strains, was at ~55kDa, present in the banding pattern from both ELISA positive and negative sera. An additional common band for sera tested against the *T. medium*- like and *T. pedis* treponeme strains was at ~53 kDa.

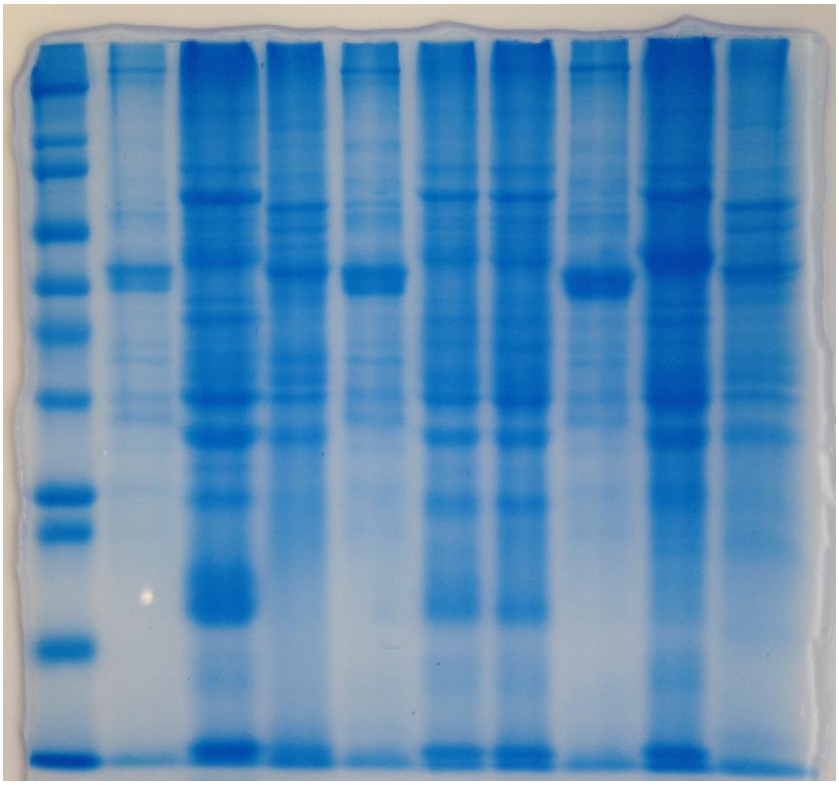
Against *T. medium*- like treponemes, the most common IgG_1 and IgG_2 band staining was detected at ~53kDa (11/12 ELISA positive sera for IgG_1 and IgG_2, 8/12 of negative sera for both IgG_1 and IgG_2), 30-32 kDa (5/12, 4/12 ELISA positive sera for IgG_1 and IgG_2, respectively, and 4/12 of negative sera for both IgG_1 and IgG_2), and 12-14 kDa (11/12 ELISA positive sera for IgG_1 and IgG_2, 0/12 of negative sera for both IgG_1 and IgG_2). Figure 8.7 shows a Western blot NCS with band staining for IgG_1 in response to *T. medium*- like strain 2C. As

can be seen from Figure 8.8, most of the seropositive sera on this western blot bound a band at ~53kDa and 12-14 kDa; however, the seronegative sera tested did not show a band at 12-14 kDa and only a few sera bound a band present at ~53kDa.

The most common IgG_1 and IgG_2 band staining against *T. phagdenis*- like treponemes, was detected at ~55kDa (11/12 ELISA positive sera for IgG_1 and IgG_2 , 7/12 of negative sera for both IgG_1 and IgG_2), 30-32 kDa (4/12, 5/12 ELISA positive sera for IgG_1 and IgG_2, respectively, and 4/12 of negative sera for both IgG_1 and IgG_2), and 12-14 kDa (11/12 ELISA positive sera for IgG_1 and IgG_2 , 0/12 of negative sera for both IgG_1 and IgG_2 apart from 1/12 negative sera had a band for IgG_1 against *T. phagedenis*- like strain g1OV11). Additionally, a band at 32-34 kDa occurred for IgG_1 and IgG_2 in 5/12 of the ELISA positive sera, and 5/12 of the ELISA negative sera. Figure 8.9 shows the reaction of seropositive sera samples IgG_2 antibodies against *T. phagedenis*- like strain T320A, with bands at ~55kDa, 12-14 kDa, 30-32 kDa and 32-34 kDa.

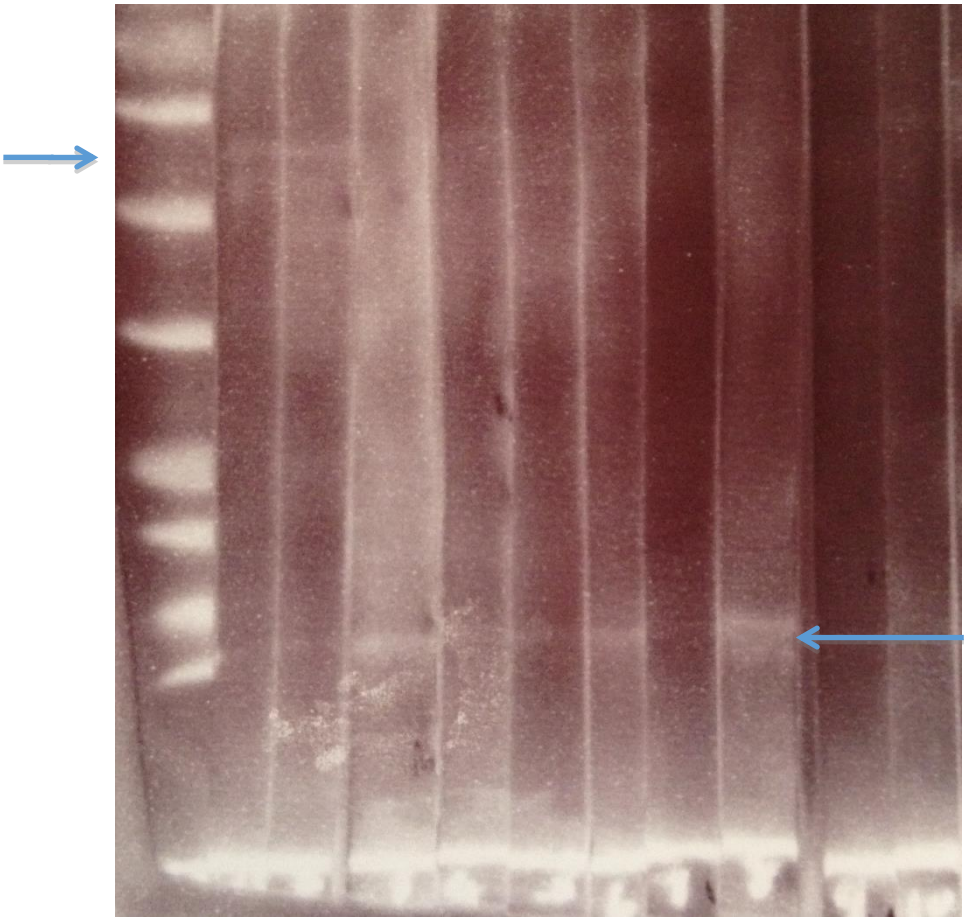
Against *T. pedis* treponemes, the most common IgG_1 and IgG_2 band staining was detected at ~53kDa (10/12 ELISA positive sera for IgG_1 and IgG_2, 8/12 of negative sera for both IgG_1 and IgG_2), 30-32 kDa (5/12, 4/12 ELISA positive sera for IgG_1 and IgG_2, respectively, and 5/12 of negative sera for both IgG_1 and IgG_2), and 12-14 kDa (11/12 ELISA positive sera for IgG_1 and IgG_2 , 1/12 of negative sera for both IgG_1 and IgG_2).

Figure 8.7: SDS-PAGE protein profiles of whole-cell lysates of each treponeme isolate. Lane numbers are listed below. Abbreviations: Prot; protein marker. Lanes 1-9; T. medium- like strain T19, T. medium like strain 2C, T. medium- like strain g1OV11, T. phagedenis- like strain T320A, T. phagedenis- like strain 6LD, T. phagedenis- like strain g2F9, T. pedis strain T3552B, T. pedis strain L13, T. pedis strain g3S4S, respectively.



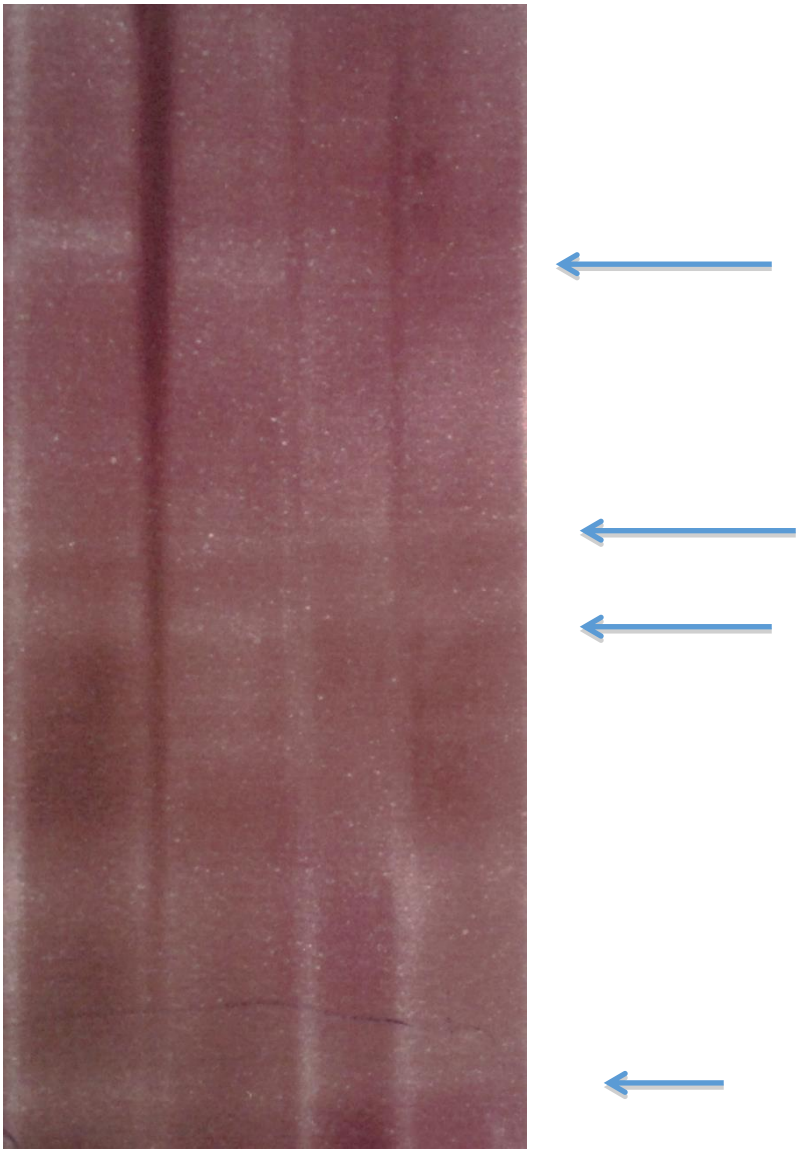
Prot 1 2 3 4 5 6 7 8 9

Figure 8.8: A western blot NCS with IgG_1 monoclonal antibodies to T. medium- like strain 2C. Colour has been inverted to show protein bands more clearly. Lane numbers are listed along the bottom of the NCS picture. Abbreviations: Prot; protein markers. Lanes 1 and 9 are seronegative cow sera; 2-8 and 10 are seropositive sera. Top left hand arrow marks the ~53 kDa band, and the bottom right hand arrow marks the 12-14 kDa band.



Prot 1 2 3 4 5 6 7 8 9 10

Figure 8.9: IgG_2 antibody reaction of seropositive sera samples against T. phagedenis- like strain T320A. Colour has been inverted to show protein bands more clearly. Lane numbers are listed along the bottom of the NCS picture. Bands of interest at ~55kDa, 12-14 kDa, 30-32 kDa and 32-34 kDa can be seen to be present, from top right to bottom right sequentially.



1 2 3 4

8.4 Discussion

The lack of reported cases of BDD in beef cattle could imply that these animals are contracting the disease less than that of dairy cattle. Additionally, from the data published so far (Brown *et al.* 2000; Chapter 4; Sullivan *et al.* 2013), it would appear that beef cattle are experiencing consistently lower herd prevalence levels. The question this study aimed to uncover is whether, this could be due to a lack of exposure to *Treponema* bacteria, or do BDD negative animals from BDD negative farms show the same exposure rate to treponemes but do not subsequently contract BDD. Due to the isolation of these treponemes being extremely difficult, it is only recently that treponeme isolate and phylogeny data has started to increase, and therefore serological investigations against all three treponeme phylogroups, and strains isolated from several animals, could only now be performed.

Previous studies have shown anti-spirochaete serological responses in BDD cattle, which were not often detected in unaffected cows. Antibodies against *T. phagedenis*- like and *T. medium*- like spirochaetes have been detected (Walker *et al.* 1997; Dermirkan *et al.* 1999; Trott *et al.* 2003; Dhawi *et al.* 2005; Elliot *et al.* 2009). However, recurrent infection is common in treated cows, for example 33% of Holstein dairy cows in southern Californian study (Read and Walker 1998) and 41% of dairy cows in a Japanese study (Ohtake *et al.* 1999). This supports the idea that the immune response to treponemes is not protective. It has been hypothesised that this may be due to antigenic variations among treponeme phylogroups, allowing them to go undetected by the animals immune system. However, this study, having analysed the immune response to three isolates of each treponeme phylogroup, detected very little in the way of differences in sera responses in ELISA or Western blotting analysis between strains from each phylogroup. This could be due to beef animals being exposed to many different strains of treponemes and therefore mount a response to each, or due to the similarity of strains within a phylogroup causing a cross reaction in assays. The lack of diversity at the 16S rRNA gene level would indicate that it is the latter.

It is quite clear from the ELISA assay results that BDD negative farms appear to be avoiding exposure to treponemes, apart from in a few cases. Farm 3 only had 1/10 beef animals seropositive to any of the treponeme isolates, and farm 4 had 4/25 seropositive to treponeme isolates. In contrast, the lowest farm seropositivity rate for any one isolate for sera samples from farms 1 and 2 (BDD positive farms) was 37% (Table 8.3). These results are consistent with previous serological investigations of antibody responses in dairy cattle to treponeme isolates where sera from cows with and without lesions on BDD positive farms showed significant levels of antibodies against treponemal isolates, whereas antibody levels were very low in sera of cows on BDD free farms (Demirkan *et al.* 1999; Dhawi *et al.* 2005; Moe *et al.* 2010). Additionally, there appeared to be no difference between ELISA seronegative samples staining on western blotting between seronegative animals from BDD positive and BDD negative farms, indicating again that it is the lack of exposure to treponemes that may be keeping certain farms BDD free.

Although BDD positive farms showed a high herd seropositivity rate for all treponeme isolates, the animals known to definitely have the disease at the time of sampling had generally higher ELISA antibody titres to treponemes. Animal A54 for example, a BDD positive beef animal had the highest antibody titres of all sera samples. So, although animals presumed to be healthy (and not suffering from BDD) which are on farms with endemic BDD often show seropositivity there does appear to be a difference in titre levels between these and some of the animals which actually had BDD at the time of sampling. However, this does again indicate that the immune response response has failed to be protective because clinical disease has developed. Interestingly, animal 10, a BDD positive cow was only seropositive against the *T. medium*-like treponemes (according to ELISA results), therefore it appears to have only been exposed to just one phylogroup of DD- associated treponemes. This could indicate that it may only take exposure to one phylogroup of treponemes to result in disease. Additionally, one animal which had BDD at the time of sampling showed no significant IgG_1 or IgG_2 response to any of the treponeme

groups. This could indicate that several more treponeme species play a role in lesions, and this animal was infected by a novel treponeme species, or that it was not able to mount a significant immune response for unknown reasons.

The present study supports the findings of previous dairy cattle studies in that spirochaete antibodies could be detected in sera samples from clinical cases of BDD in beef cattle and also in cattle without clinical signs in BDD positive herds. Both normal and BDD beef cattle showed evidence of infection with treponemes, even those which had no lesions at the time of sample collection (Walker *et al.* 1997; Demirkan *et al.* 1999; Dhawi *et al.* 2005). Dhawi *et al.* (2005), observed seropositive responses in 24% of DD negative cattle against DD- treponeme antigens, despite the farm having no history of BDD. Like our data, this could indicate that the animals on this farm may have had undetected early lesions or been in preclinical stages of the disease. However, more likely, that they had been exposed to treponemes but require other environmental/microbiological factors to develop clinical DD.

Dhawi *et al.* (2005), showed that dairy cows with BDD and sheep with CODD had antibodies to treponemes isolated from both species. In an extension of this work, here it can be seen that beef cattle also have antibodies to treponemes, and again in agreement to the previous study, antibodies to treponeme strains isolated from different species; sheep CODD lesions, dairy cow BDD lesions and beef cow BDD lesions. Further to this, when sera was exposed to all three phylogroup antigens, most sera showed an antibody response to all three phylogroups, and sometimes to all nine isolates. On its own, this would suggest that either all the treponeme strains from the same phylogroup used in this study are very antigenically similar or that the majority of cattle have been exposed to all of the strains (or to closely related strains). Additionally, when sera showed a significant antibody response against a strain from a certain treponeme phylogroup, in most cases the same sera also showed the same response to a different treponeme strain from the same phylogroup (isolated from a different host species). This was true for both ELISA and Western blotting results.

The Western blotting data confirmed the results obtained by ELISA, and showed that beef, dairy cattle and sheep treponeme isolate antigens are often similar to each other in terms of surface protein expression in that they shared several polypeptide bands. However, there were bands that appeared phylogroup specific, including the 55kDa protein for the *T. phagedenis*- like spirochaetes. Prominent bands found upon western blot analysis included, 30-32 kDa and 32-34 kDa and 55kDa for the *T. phagedenis* isolates. Similarly, in a previous study by Demirkan *et al.* (1999) BDD positive dairy cattle sera were analysed by western blotting against antigens of treponemes (including *T. phagedenis*- like spirochaete antigens) and demonstrated reactivity with 34 kDa, 41kDa, and 55-kDa antigens. However, a 60kDa *T. phagedenis*- like antigen was the one most reactive in cattle from BDD positive farms in a study by Moe *et al.* (2010), and Trott *et al.* (2003) detected an 80kDa band. These differences in the recognition of bacterial antigens may be caused by differences in treponeme isolates from different geographical locations, as isolates in this study were all obtained from animal lesions in the UK and little differences were noted between them.

In a pronounced difference to this study, Demirkan *et al.* (1999), found no significant IgG_1 antibodies against the DD treponemes tested, which included *T. medium*- like and *T. phagedenis*- like spirochaetes. However, more recent studies (Elliot *et al.* 2007, 2009) detected IgG_1 and IgG_2 responses, with a slight skew towards IgG_1. This is true for the results found in this study where both IgG_1 and IgG_2 responses were detected, but IgG_1 detection rates were higher and titre levels also appear to be higher. However, there was also variation in responses, with some sera showing a strong IgG_1 response to treponemes and no significant IgG_2, and others an IgG_2 response and no significant IgG_1 response. Additionally, it appears from this study that they positively correlated. Although there have been differences in IgG_1 and IgG_2 responses found in different studies, it does appear that these are the more consistently detected anitbodies in response to treponemes. Whereas, IgM for

example, has failed to be detected in a number of studies against treponeme antigens (Demirkan *et al.* 1999; Elliot *et al.* 2009).

Neither of the BDD negative farms used in this study had bought in cattle for at least the last 30 years. Both farms produce their own cattle replacements (rather than buying in when stock is lost) in order to reduce their need for other cattle from other herds coming onto their farms. From the data in this study, and the fact that once BDD is on a farm it is usually virtually impossible to eradicate, it may be possible that by not buying in cattle and therefore reducing chances of farm-to–farm transmission of external bacteria such as pathogenic treponemes, the exposure of cattle to treponemes is avoided, and subsequently BDD development too. Previous studies have shown that BDD can enter BDD free herds when apparently healthy cattle are bought-in from BDD positive farms (Brizzi 1993; Read and Walker 1994).

The strong immunological response to treponemes seen in beef cattle provides more evidence implicating these bacterial species in BDD pathogenesis, possibly as the initiating aetiological agent of BDD in this and other host species. However, it does appear this response is also not protective in beef cattle, as has previously been demonstrated in dairy cattle. Therefore, the perceived lower prevalence of BDD in beef cattle does not appear to be due to a protective immune response in these animals, but more likely and suggested from this current studies data, due to a lack of exposure to BDD treponemes. As previously suggested, this may be due to differing farm management systems for beef cattle, their relatively short life span or, quite possibly, because beef farmers do not look for the clinical signs and lesions.

This study strengthens the evidence towards a primarily IgG_1 and IgG_2 response to treponemes, and that DD negative farms appear to be DD negative due to a lack of exposure to the pathogenic treponemes. In the future it would be useful to know the BDD status of the entire herd that was blood sampled on a BDD positive farm, as it was not possible to do so in this present study, and

therefore hard to interpret antibody titre levels in some cases. Additionally, serological studies using treponemes actually isolated from the same animal's blood sampled would also be an improvement on this study to investigate specific responses to treponeme phylogroups.

The serological data is very much aligned with the presence of BDD lesions, but not completely associated. Hence, none of the serological data presented here would support the use of serology for diagnostic or prognostic assays for early or subclinical detection of BDD in beef (or dairy) cattle.

Chapter 9

Discussion

9.1 Digital dermatitis in beef cattle and sheep

Lameness in cattle and sheep has serious animal welfare and economic implications (Marshall *et al.* 1991; Enting *et al.* 1997; Hernandez *et al.* 2001; Warnick *et al.* 2001), especially when the cause of lameness is poorly understood. Even with over 40 years of research on BDD in dairy cattle, the causative agents, infection reservoirs and transmission of DD have remained largely a mystery until relatively recently. With BDD now a worldwide problem and controlling BDD proving difficult, more knowledge in these areas is paramount. Moreover, the spread of CODD in sheep within the UK (Naylor *et al.* 1998) and into the Republic of Ireland (Sayers *et al.* 2009), means that both BDD and CODD need to be more thoroughly understood to have any chance of control or eradication. Indeed, the fear must be that such lesions associated with treponemal infections will now spread into sheep in other countries and possibly into other additional host species.

There has been a large amount of research conducted on lameness in dairy cattle, for several important reasons. The breeding of these animals to produce large quantities of milk has made them the focus of many welfare concerns. Additionally, the controversial price wars between supermarkets have led to more pressure on farmers to produce enough milk for their farms to be financially viable. Dairy animals are also seen daily due to milking practices, and are often walked in and out of a milking parlour where lameness can easily be observed and then treated. For these reasons, and probably many more,

lameness in dairy cattle has been widely reported and been the focus of media and research attention. There have been countless studies focusing on the causes of lameness, economic consequences of lameness and the subsequent welfare issues. Of these there has been various studies focusing primarily on BDD.

There is little doubt that beef cattle have been neglected in terms of veterinary research on BDD. Upon the start of this study it was surprising to find no definitive case report of the disease in beef animals. This was particularly interesting given that upon visiting UK farms, countless vets/farmers say they have seen/treated BDD in beef cattle. It may be expected that beef cattle, being of similar and often crosses with dairy cattle breeds, would suffer many of the same diseases. Bovine digital dermatitis has been found to be highly associated with the hygiene of farms and slurry levels (Rodríguez-Lainz *et al.* 1999; Nowrouzian and Radgohar 2011). Beef animals are fed different diets to dairy cattle, often housed differently (or outdoors), and so are exposed to very different environments. Therefore, inference that BDD is present, or exactly the same disease in beef cattle as in dairy cattle, could be considered over-presumptuous. However, as suspected from these anecdotal reports, our findings did show that the beef animals investigated did appear to be suffering from what clinically appeared to be BDD, and upon PCR and culture analysis provided the same bacterial findings as in dairy cattle BDD.

This new data is important to the beef cattle industry, as now that it is known that beef cattle are suffering from the same type of lesions as dairy cattle it will help with diagnosis, treatment, and widespread awareness. It could be considered that the most important of these, is the public awareness gained from the published reports of the disease in beef cattle produced from this work (Sullivan *et al.* 2013; 2015a). Beef cattle BDD has obviously been an overlooked disease and it is unknown whether these animals also have been suffering with the disease for over 40 years as with dairy cattle, therefore this project will hopefully help raise awareness for the disease in the veterinary and farming community. BDD has been shown to increase the risk of culling

(Bruijnis *et al.* 2012), and with beef animals worth a large amount per head, it is unlikely to be favourable for beef animals to be culled due to an infectious lameness. Additionally, due to the weight loss, disease susceptibility and reduced fertility associated with lameness (Greenhough *et al.* 1981; Van Arendonk 1985; Lucey *et al.* 1986; Collick *et al.* 1989; Lee *et al.* 1989; Groehn and Kaneene 1992; Hernandez *et al.* 2001; Garbarino *et al.* 2004) and DD in dairy cows (Argáez-Rodriquez *et al.* 1997; Hernandez *et al.* 2001; Losenger *et al.* 2006; Relun *et al.* 2013), it is likely to be high on the agenda of beef cattle farmers to avoid beef cattle infection with BDD. From informal discussions with farm animal vets in practice, they have reported that when they have come across what they assumed to be BDD in beef cattle, it has often appeared more severe or at the later lesion stages commonly observed in dairy cattle. This in their opinion was due to farmers/vets not observing early lesions in beef animals and therefore not treating them. It is interesting to speculate whether lack of close contact with these animals leads to a lack of early diagnosis and therefore successful treatment. It is hoped that through the resulting publications on BDD in beef cattle more farmers and vets will be looking for the disease and therefore early treatment and a better outcome for the animals involved is more likely.

What was lacking in the present study was a full epidemiological investigation into the prevalence of BDD in beef cattle herds, which would have been of great value. This would enable a greater understanding of the weight of the burden of BDD on these animals and the beef cattle industry, as well as clues to further infection reservoirs and risk factors of the disease. Unfortunately, this is easier said than done, as handling of beef cattle can be extremely difficult and, as found in this project, lifting the feet of a significant amount of beef cattle (which are rarely handled) requires a large amount of time and safety precautions. Although farmer estimates were used where possible, these are not always accurate and therefore further epidemiological investigations on BDD in beef cattle herds in the UK would be very beneficial.

Treponemes have now been isolated from the tissue of many BDD lesions of dairy cow feet (Walker *et al.* 1995; Trott *et al.* 2003; Evans *et al.* 2008, 2009a, 2009b; Pringle *et al.* 2009). This, together with the isolation of a large number of treponemes from beef cattle BDD lesions and CODD lesions of sheep feet, makes it was possible to genetically compare these bacteria. On the basis of the 16S rRNA gene, these same pathogenic bacteria are found in all three livestock animals. Although it is thought that DD originally spread from dairy cattle to sheep, it has not been scientifically proven. However, findings that almost genetically identical treponemes are found in both cow BDD and sheep CODD lesions provides a clear link between the two diseases. Work by Angell *et al.* (2014) provided epidemiological data demonstrating that there was an association between the presence of CODD in sheep where cattle with BDD were also present on farms. This provides yet more evidence supporting this theory. This infection link between the diseases poses large transmission concerns. Beef and sheep are often farmed together in the UK and, even when managed in separate buildings/pasture areas, still come into contact when animals are being moved or rotated around different pastures. Although we do not yet know how long treponemes may survive in the environment, and exactly where, this does raise concerns for cross-species transmission. Again, now that this data is published, detailing the isolation of large amounts of genetically similar treponeme bacteria found in both beef, dairy and sheep DD lesions, the farming community can be more aware and make precautionary management changes accordingly.

At the 16S rRNA gene level, limited differences can be seen between treponemes isolated from sheep, dairy and beef DD lesions, and as published for dairy cattle BDD in the UK and USA (Stamm *et al.* 2002; Evans *et al.* 2008, 2009a), these treponemes fall into three distinct phylogroups, *T. medium*- like, *T. phagedenis*- like and *T. pedis.* The isolation and subsequent 16S rRNA gene sequencing of over 100 treponemes from DD lesions from dairy and beef cattle and sheep, which this study contributes substantially to, is a large addition to treponeme research as a whole, especially given that the successful growth and

isolation of just one treponeme can be extremely difficult. Such data is key to distinguish between treponeme species and phylogenetically relate individual bacteria so as to further understand transmission routes. The 16S rRNA gene is regarded as a highly conserved gene (Woese 1987). This current project focused entirely on the 16S rRNA gene, as many studies into spirochaetes and specifically treponemes have in the past. Such studies of a single locus does not allow for comprehensive delineation of microbial population biology and associated transmission and infection cycles. Future work needs to focus on multi locus sequence typing (MLST) (Maiden *et al.* 1998) of these isolates or given recent advances in sequencing technology, the entire genomes of DD treponemes could be investigated (Bratcher *et al.* 2014) as little genomic data is currently available.

The value of culture, isolation and subsequent genotyping, when studying bacteria is invaluable. This is especially true when previously only a limited number of the bacteria have been grown and isolated. However, the bias of the culture techniques cannot be overlooked. Treponemes are fastidious bacteria and notoriously difficult to culture (Paster and Dewhirst 2000). It is surprising that, we are commonly able to isolate three phylogroups of treponemes from DD lesions. Whilst this can be regarded a successful outcome, and could be due to these three being the most prominent bacterial species in lesions; it should also be considered that this culture technique may only specifically target and enable growth of these species, possibly at the expense of other treponemal species in the samples. Future metagenomic studies are needed to thoroughly understand the bacteriology of the lesions, and particularly throughout the different stages of lesion development, as has now been done for dairy cattle BDD lesions (Krull *et al.* 2014; Zinicola *et al.* 2015). The results from these studies found treponeme frequency/presence changes throughout the stages of BDD infection. These data supported BDD as a polymicrobial disease, with active BDD lesions in USA dairy cattle having a distinct microbiome dominated by treponemes including *T. denticola, T. putidum, T. medium, T. phagedenis, T. maltophilum* and *T. paraluiscuniculi*.

Such data would be extremely interesting to compare with the same information produced for sheep and beef cattle DD lesions, and would again provide greater aetiological information.

9.2 Infection reservoirs of DD and considerations for control of the disease

Initial studies trying to identify DD treponemes in the GI tract failed (Evans *et al.* 2011b). Furthermore the characterisation and comparative studies of GI treponemes with DD treponemes described them as very different (Evans *et al.* 2011b). However, the evidence promoting the GI tract as an infection reservoir of DD treponemes has increased since 2012 when Evans *et al.* first demonstrated that the bovine GI tract could harbour DD treponemes. As an extension from this work, this project confirmed these results by detecting the same DD treponeme phylogroups in the same GI tract tissues (Chapter 6; Sullivan *et al.* 2015d). Interestingly though, various other studies have now recently detected one or more of the commonly associated cultivatable DD treponeme phylogroups in rumen fluid and faecal/slurry samples. This presents several questions;

- Are all of these areas potential infection reservoirs?
- Are some of these areas more important for DD treponeme load/transmission than others?
- Are there other tissues, both GI and non-GI, that may also carry these DD treponemes?
- What role do these DD treponemes play whilst in these tissues/fluids?
- Are the DD treponeme bacteria actually alive in all of these areas, or are they simply being shed/are in the environment but are not transmissible?

Currently, it is unknown whether all of these GI tract tissues and fluids are actually contributing to the survival and spread of the DD treponeme bacteria. However, what this present study has provided is the knowledge that at least

one DD treponeme phylogroup can be alive in GI tract tissue, in this case rectal tissue. This provides the first evidence of live DD treponemes in any healthy host tissue. The isolation of this treponeme was importantly from a CODD positive sheep, a host species of which the GI tract has not previously been investigated as a reservoir for DD treponemes.

This study focused on two GI tract tissues and on faecal samples, primarily due to the previous findings of Evans *et al.* (2012) in dairy cattle, as when this study commenced this was the only evidence implicating the GI tract as an infection reservoir for DD treponemes at this time. If the GI tract is a significant infection reservoir of DD treponemes, and allows the spread of these into the environment, it seems unlikely that in the entire GI tract they are only harboured in two GI tissues. Therefore, there may be more GI tract tissues that are harbouring DD treponeme bacteria which we failed to investigate. However, Evans *et al.* (2012), did investigate a large number of tissues and fluids from dairy cows with BDD, and failed to detect any DD treponemes outside of the rectal, gingival and rumen tissue. To confirm this, a more comprehensive host tissue investigation would be useful, focusing on as many tissues as possible and including a full GI tract tissue survey of a large number of cows and sheep, including both DD symptomatic and DD asymptomatic animals.

The role treponemes play in these GI tissues is presumed to be non-pathogenic, as macroscopically no internal lesions/infections have currently been reported. Histological experiments would provide more of an insight into what exactly is the DD treponemes role whilst present in these GI tract tissues.

The epidemiological data produced by Wells *et al.* (1999), detailing a link between BDD prevalence and the frequency and hygienic practices of hoof trimming, were too interesting to ignore. It was surprising to find that no microbiological research had followed to uncover whether this was a potential transmission route, especially when no current transmission routes of DD were known. The small study carried out here to investigate trimming equipment for

the presence of treponemes, produced results which were not totally surprising (Chapter 7; Sullivan *et al.* 2014). This equipment is a material which comes into direct contact with the body part infected with the bacteria, and therefore contamination of the equipment with treponemes seemed likely. However, even so this was a breakthrough finding which brought the use of trimming equipment and the lack of routine disinfection of this equipment, into the spotlight. This was for good reason, as farmers often employ external hoof trimmers to come onto their farms to trim their cattle, and sometimes sheep, feet. Additionally, along with routine trimming, these animals may have their hooves trimmed as a treatment for foot disorders, therefore possibly contaminating the knife with infectious bacteria. This study hopefully highlighted to farmers, vets and hoof trimmers the importance of disinfection and hygienic practices, on farm between each animal and between farms.

What this study failed to provide was a timeline of treponeme survival on trimming equipment. Although treponemes were isolated from knife blades, showing that they are capable of surviving at least a small amount of time on the knife, this was only for the time between trimming to swabbing the knife. Investigations into the length of survival of treponemes on trimming equipment would be extremely useful as it would enable farmers/hoof trimmers/vets to know if there is a possibility of transmission via this route not just between animals trimmed on any given farm but also between farms visited. Additionally, an investigation of the effectiveness of different disinfectants at killing treponeme bacteria on trimming equipment would also be of potential use, as this study did show that even after disinfection treponemes could still be detected on equipment in some cases.

The importance of the Wells *et al.* (1999) study cannot be overlooked. It may bridge the gap between the microbiological data provided here and the subsequent epidemiological consequences for DD transmission. The use of a cattle/sheep hoof trimmer can be common practice in the UK and such a hoof trimmer may attend multiple farms on the same day. This poses concern as the epidemiological data found that dairy cattle farms which used a primary hoof

trimmer who also trimmed on other operations were 2.8 times more likely to have >5% herd incidence of BDD, compared to herds which used a hoof trimmer who did not trim on other farming operations (Wells *et al.* 1999). Additionally, it would be interesting to ascertain how many hoof trimmers in the UK wash their equipment between animals or farms. Wells *et al.* (1999) discovered that farms using a hoof trimmer who did not wash their equipment with water between cows were 1.9 times more likely to have >5% herd incidence of BDD than herds that used a hoof trimmer who washed equipment between cows. This data would infer that a large reduction in herd BDD incidence may be possible by improving the sanitation of equipment used to trim hooves on farms.

When interpreting the epidemiological data provided by Wells *et al.* (1999) it is important to recognize that bringing a hoof trimmer onto the operation that also trimmed cows on other operations might have been an effect rather than a cause. However, nevertheless taken together with the data from this project it highlights that these associations found by Wells *et al.* (1999) show a potential transmissibility among cows via hoof-trimming equipment with increased DD incidence as a possible result. This emphasizes the extremely contagious nature of DD and the importance of attempting to break the chain of transmission through disinfection by hoof trimmers, farmers and veterinarians.

This study, although preliminary, may also tell us something about treponeme biology. It has been thought for a long time that treponemes are highly anaerobic bacteria (Radolf and Lukehart 2006). The isolation of live bacteria from a knife blade may indicate they are a much more versatile bacteria than first thought. Indeed, this adaptation to an aerobic or partially aerobic environment, probably evolutionary, may well go a long way to explaining how the disease has spread so rapidly between animals in a herd, between farms and even between species of host.

It has to be highlighted though that although this study provided preliminary data showing treponemes being present on trimming equipment after trimming

DD symptomatic sheep and cattle, it does not prove transmission. This was not the aim of the study, it was to firstly investigate whether treponemes could be present on the knife after trimming DD symptomatic animals, and if they could be alive on the knife after trimming. A study whereby animals are infected with DD after having their hooves trimmed with a knife that has previously trimmed a DD symptomatic animal, would need to be carried out in order to confirm this as a DD transmission route.

If treponemes can survive on this metal for a significant amount of time then it has to be considered that they could survive on other metal equipment on farms. An example might be metal scrapers used to scrape faeces from the floors of cattle housing. It may not take exposure to the foot lesions as in the case of hoof trimming equipment, but if treponemes are live in faecal matter then equipment such as metal scrapers could be a potential way treponemes can be transmitted along areas of animal housing. On the other hand, if treponemes are not just present in faeces but are in fact alive and transmissible (no evidence for this thus far), then the likelihood of eliminating DD treponeme spread via faeces on farms would be virtually impossible.

Thus, two areas of treponemal detection, outside of the lesions themselves, provide plausible routes for treponemal transmission. In this study both GI tract tissues, and trimming equipment were found to contain live DD treponeme phylogroup bacteria and therefore are stronger evidence of treponemal involvement in DD lesion transmission than PCR detection alone (Chapter 6, 7; Sullivan *et al.* 2014, 2015d). It could be considered that contact with feet in the form of trimming equipment may not be a key DD transmission route, due to the infrequent nature of hoof trimming, compared to the potential transmission of treponemes via faeces. With larger studies, investigating more animals and more GI tissues it will be possible to soon know definitively how large a role the GI plays in the transmission of DD treponemes. Additionally, the growth of treponemes from faecal matter of cattle/sheep would produce stronger evidence for the shedding of live treponemes from GI tract tissues/fluids to faeces and then subsequent transmission.

The hypothesis arising from the GI tract tissue study is that DD treponemes may be carried in the GI tracts of a small number of individual cattle/sheep (DD symptomatic or asymptomatic) and then spread into the farm environment via faeces. This would indicate that cattle/sheep on a farm which had these individuals present would expose most, if not all, the animals to DD treponemes. Additionally, if these animals were not present, and therefore the introduction of these treponemes onto the farm never occurred, then animals would not be exposed to the treponeme bacteria. This means that increased hygiene on farm might prevent the transmission of DD, Furthermore in the future with development of better diagnostics and then subsequent removal of shedders might help allow control of the diseases.

9.3 Immunological response and potential for vaccine development

Most beef cattle from BDD positive farms showed an immunological response to DD treponemes, indicating prior exposure to these treponemes. Conversely, all beef cattle from BDD negative farms, bar a small number, showed no significant immunological responses to DD treponeme antigens.

Interestingly farmers which had managed to avoid BDD infection were convinced this was due to their lack of buying in cattle from other farms. This may link in with what was previously found in Chapter 6 whereby animals were found to have DD- associated treponemes in their GI tissues. If animals may carry treponemes in their GI tracts, it would make sense as to why bringing in clinically healthy animals (with no sign of BDD) can result in BDD outbreaks in previously healthy herds (Brizzi 1993; Read and Walker 1994). Additionally, if BDD asymptomatic animals are also carriers of DD treponemes, and therefore it is not infection with BDD that causes carrier status, then this raises the question; why do cattle not carry the bacteria? The possibility that asymptomatic cattle do carry DD treponemes and are able to

shed these on farm, would make it almost impossible to know when buying in cattle whether you were in fact bringing BDD infection onto farm. Therefore completely avoiding bringing cattle onto your farm would appear to be the most sensible way of avoiding BDD infection in your herd. Interestingly, the attending clinician identified one closed BDD negative farm as unhygienic, with animals stood in large amounts of slurry. Such conditions are normally associated with increased levels of BDD (Rodríguez-Lainz *et al.* 1999; Nowrouzian and Radgohar 2011), and recent research indicates slurry as a reservoir for treponemes (Klitgaard *et al.* 2014; Zinicola *et al.* 2015). This information although anecdotal, does again suggest that certain animals carry the bacteria, and therefore avoiding bringing cattle onto your farm, may be successfully avoiding exposure, as the BDD negative herds have successfully done in this study.

Although the immunological data produced provided vital information on the exposure versus non-exposure of cattle on BDD positive and BDD negative farms, the studies could have been improved in several ways. The unknown disease status of all animals on BDD positive farms meant that some data was hard to interpret. Ideally, all beef animal's blood sampled would have all feet lifted and BDD status determined; however, again as said above, these animals are difficult to handle and time consuming.

The strong immunological response to treponemes seen in beef cattle provides more evidence towards treponemes in DD pathogenesis. The lesions clinical appearance (Chapter 4; Sullivan *et al.* 2013, 2015a), bacteriology (Chapter 4; Sullivan *et al.* 2013, 2015a) and host immune response (Chapter 8) all mirror what has previously been found in dairy cattle in the UK (Demirkan *et al.* 1999; Murray *et al.* 2002; Trott *et al.* 2003; Klitgaard *et al.* 2008; Nordhoff *et al.* 2008; Evans *et al.* 2009b). Additionally here it can be seen that beef cattle also have antibodies to treponemes, and antibodies to treponeme strains isolated from different species; sheep CODD lesions, dairy cow BDD lesions and beef cow BDD lesions. Whether or not this is due to antigenic similarity or exposure

to all strains from each treponeme phylogroup, this raises concern for cross species transmission. Especially given that the serological data from this study showed beef cattle's immune response to be likely unprotective.

This study provided key information which would be necessary if a successful vaccine was to be developed. The ELISA assay data found many cattle are exposed to all three treponeme phylogroups, and Chapter 4 illustrated that BDD lesions can often contain one or all three phylogroups simultaneously. Cow number 10 for example (Chapter 8) was, at the time of blood sampling, DD symptomatic and yet from ELISA results it appears the cow was only exposed to *T. medium*- like spirochaetes and not to the other two DD-associated treponeme phylogroups, *T. phagedenis*- like and *T. pedis*. Therefore it may only take the presence of one DD treponeme phylogroup to cause clinical disease. A vaccine would therefore need to take this into consideration. The Western blotting data confirmed that beef, dairy cattle and sheep treponeme isolate antigens are often similar to each other in terms of surface protein expression sharing several polypeptide bands. The most prominent bands found between all three treponeme phylogroups were proteins of sizes 30-32 kDa and 12- 14 kDa. These shared antigens would be of interest in vaccine development trials as possible vaccine components.

Vaccines have shown some success for other foot diseases in ruminants, such as vaccines used to treat and prevent footrot. The main causative agent *D. nodosus* has many different serogroups currently identified (Claxton *et al.* 1983; Chetwin *et al.* 1991; Dhungyel *et al.* 2014), and many of these serogroups can be present in the same flock (Claxton 1989). In countries where footrot is endemic it has been found that isolates of *D. nodosus* can show considerable antigenic diversity (Claxton *et al.* 1983; Kingsley *et al.* 1986; Chetwin *et al.* 1991; Ghimire 1996), and therefore, similarly as discussed with a potential DD vaccine, a commercial footrot vaccine needed to have antigens of several/all serogroups. Some success has been found with a multivalent recombinant fimbrial vaccine (Footvax, Schering-Plough Animal Health Limited) which contains 10 serogroups and was released commercially in 1986

and has shown positive results as a treatment and preventative for footrot (Liardet *et al.* 1989; Duncan *et al.* 2012).

There have been a number of efforts to develop a vaccine for BDD, however there are none currently available. Two studies found promising results using inactivated bacterin vaccines. In the USA, Keil *et al.* (2002) found that adult cows and heifers showed a significant reduction in BDD prevalence when vaccinated with an inactivated *Treponema* bacterin compared to unvaccinated cows. Novartis produced a whole cell lysate vaccine for the USA market in the early 2000s. Using two Californian dairy herds, Berry *et al.* (2003) found this *Treponema* bacterin, known as TrepShield (Novartis Animal Health), to show interesting results. The vaccine led to a significantly lower occurrence of BDD in heifers when they were immunised before calving and in cows immunised during the dry period. This vaccine has since been withdrawn.

Other trials have shown less encouraging results from the use of vaccines. For example, Fidler *et al*. (2012) investigated the use of a vaccine containing *Serpens* sp. bacterin. The trials found that vaccinated dairy cows elicited an immune response to the bacterin but this did not translate into a reduction in prevalence of BDD or the severity of the BDD infections when compared to an unvaccinated control group. Interestingly this coincides with no other groups worldwide having demonstrated *Serpens* as important in the aetiology of BDD.

Staton *et al.* (2014) have considered the implications of multiple treponeme phylogroups in vaccine design and are currently attempting to identify vaccine candidate proteins for a recombinant vaccine. They are using a novel bioinformatics-centred approach, termed reverse vaccinology, which has enabled analysis of treponemal genomes. This enables them to identify proteins deemed most suitable for inclusion in a vaccine, particularly focusing on ones homologous across the three DD- associated treponeme phylogroups. Identified molecules can then be selected and synthesized and then subjected to relevant immunological investigations.

9.4 New host species suffering with DD

The worldwide importance of DD is growing, with new reports of what appears to be DD in previously unaffected host species. Goats have never been recorded to suffer from DD, and it is doubtful this is due to a lack of reporting as most, if not all, sheep foot diseases have been reported in goats. The dairy goats investigated in this study (Chapter 3; Sullivan *et al.* 2015c) were in a large amount of pain and the issue was becoming a welfare concern due to the high prevalence in the herd and severity of the lesions. Now multiple other UK dairy goat farms have been reported to be suffering from what appears to be DD (Groenevelt *et al.* 2015), so confirming the suspicion that this was not an isolated case. The detection of all three treponeme phylogroups in multiple goat lesions across farms from different geographical locations in the UK suggests this disease is spreading quickly and needs to be of high concern to dairy goat famers.

Last year, in the USA, the first report of a DD- like manifestation occurring in a wildlife species was published (Clegg *et al.* 2015). Again, as in goats, the disease in Elk showed the high similarity to CODD in terms of clinical appearance and upon PCR and culture analysis, the same DD treponeme phylogroups were detected and isolated. Not only are domesticated livestock species contracting treponeme infected foot lesions, but wild species who have the potential to spread disease over large distances are now suffering from treponeme associated foot lesions.

Although the same treponemal bacteria is found in all of these lesions, there is little evidence that these diseases have been caused by cross-species transmission. However, what is interesting is that both of these previously unaffected species have one thing in common. The goat farm investigated was previously a large dairy farm and the Elk which were investigated came from a study area which included areas grazed by domestic cattle and sheep.

Spirochaetes of the genus *Treponema* were isolated from the ear lesions and gingiva of pigs with ear necrosis during outbreaks in two organic pig herds in

Sweden (Pringle *et al.* 2009). Two years later in 2010, *T. pedis* was isolated from a sow shoulder ulcer in another herd (Pringle and Fellström 2010). More recently research at Liverpool has shown the presence of the DD- associated treponemes in ear, flank and tail lesions in this species (Unpublished data). This is further evidence of possible cross species transmission.

Hopefully with these reports being published, and more information on the possible infection reservoirs of DD, the disease can be limited from spreading to further host species.

9.5 Conclusions

Digital dermatitis has proven almost impossible to eradicate from farms, and with what we now know about DD treponemes in the GI tract and possibly faeces, and given that on farm faecal contamination can be very high and therefore hygiene levels precarious, it seems this disease will be difficult to eliminate.

However, here we have identified several possible routes of DD transmission and therefore potential to reduce the burden of the disease by certain measures. Effective disinfection of foot trimming equipment used to trim sheep and cattle could reduce transmission both on and between farms. Although the contribution of this method of transmission is unknown, from the previous epidemiological data (Wells *et al*. 1999), it appears that appropriate cleaning of tools may be key to reducing the herd incidence of DD.

In order to reduce the potential spread of DD- associated treponemes via faecal contamination, better general hygiene on farm should help to decrease the spread within herd. Further to the epidemiological studies already outlining the link between farm hygiene and DD incidence (Rodríguez-Lainz *et al.* 1999; Nowrouzian and Radgohar 2011), our data suggests the GI tract as an infection

reservoir for both symptomatic and asymptomatic animals and therefore good farm sanitation should now be seen as imperative.

The serological studies performed have suggested that BDD negative beef farms are effectively avoiding/limiting exposure to DD treponemes, and as found previously, it appears this is permitted by the lack of buying in animals (Brizzi 1993; Read and Walker 1994). Due to this, and the possibility that both symptomatic and asymptomatic animals may be carriers of DD treponemes in their GI tracts, quarantining of bought in animals and treating them with systemic antibiotics before including in herd would be useful. However it is understood that there may be limitations on this on grounds of good antibiotic stewardship and therefore the use of a probiotic to treat clinically healthy bought in animals to reduce the shedding of bacteria from GI tract may be a future product of use. Similar probiotic products have been used to reduce the shedding of *Salmonella* in chickens and pigs (Pickler *et al.* 2013; Robbins *et al.* 2013). Additionally, the development of an on farm diagnostic test to identify shedders of treponemes in faeces and then treating these animals appropriately with these or antibiotics would be an extremely useful way of limiting the potential of treponeme contaminated faeces facilitating the spread of DD.

Armed with more knowledge on the transmission routes and infection reservoirs of DD- associated treponemes it should be possible to limit the spread of treponemes on farm. A vaccine however, would be the most ideal way to control/eradicate this disease as with microbial antibiotic resistance now a high public concern (WHO 2001; Defra 2013b; Department of Health 2013) and discussions on banning the on farm use of footbaths such as formalin in the UK (Winter 2009), it appears to be the only option (along with better on farm hygiene, and foot trimming equipment disinfection) soon available to consider. Not only does DD cause significant losses per case, but animals often get concurrent infections, resulting in huge losses for farmers. Considering these large costs a reliable vaccine would be a surely be a welcome product to the livestock farming industry. The once limited information we had on DD in

beef cattle and sheep is now much larger and armed with more knowledge on the treponemes present in BDD and CODD lesions and two potential transmission routes almost completely delineated, effective actions to prevent the spread of this disease to yet more host species can be limited.

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Supporting Papers

Please find all supporting papers in the CD attached to the back of this thesis.

Papers included on the CD are listed below in chronological order.

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