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CONTENTS

1. Abstract

Digital dermatitis (DD) is a superficial infectious dermatitis of the digital skin of cattle and sheep. 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. 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. There is also limited information on the causative bacteriological agents of CODD. Furthermore, no definitive transmission routes or infection reservoirs of have thus far been delineated.

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. Upon 16S rRNA gene sequence analysis all isolates showed a high similarity to representatives of each treponeme phylogroup isolated from dairy cattle BDD lesions. Additionally, the same treponeme bacteria were detected and isolated from a new undefined foot disease in dairy goats in the UK.

The host GI tract and hoof trimming equipment were investigated as potential transmission routes and infection reservoirs of DD. 1/40 sheep gingival tissues were positive for DD-associated treponemes, and 3/40 sheep rectal tissues. 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. 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. Trimming equipment was tested after being used to trim cattle and sheep hooves. Of the blades used to trim DD symptomatic animals, 25/26 were found to be positive for at least one of the DD *Treponema* phylotypes. A *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 appears to be 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.

2. Introduction

2.1. Digital Dermatitis

2.1.1. 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.

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

2.2.1. 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).

2.2.2. 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 (£38,820), 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)).

2.2.3. 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.

2.2.4. 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.

2.2.5. 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 ofthe 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, infection, nutrition and metabollic disorders (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.	Metabolic disturbances and 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	Walking on rough ground. Corium gets penetrated (Smith and Broderson *et al.* 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 (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

2.2.6. 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. *D. 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

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

2.3.1. 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.

2.3.2. 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.

2.4. Epidemiology

2.4.1. Introduction and spread of digital dermatitis

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).

2.4.2. 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).

2.4.3. Seasonality

A seasonal effect has commonly been reported in BDD, with peak morbidity during the housing period which could probably 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.

2.4.4. 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.

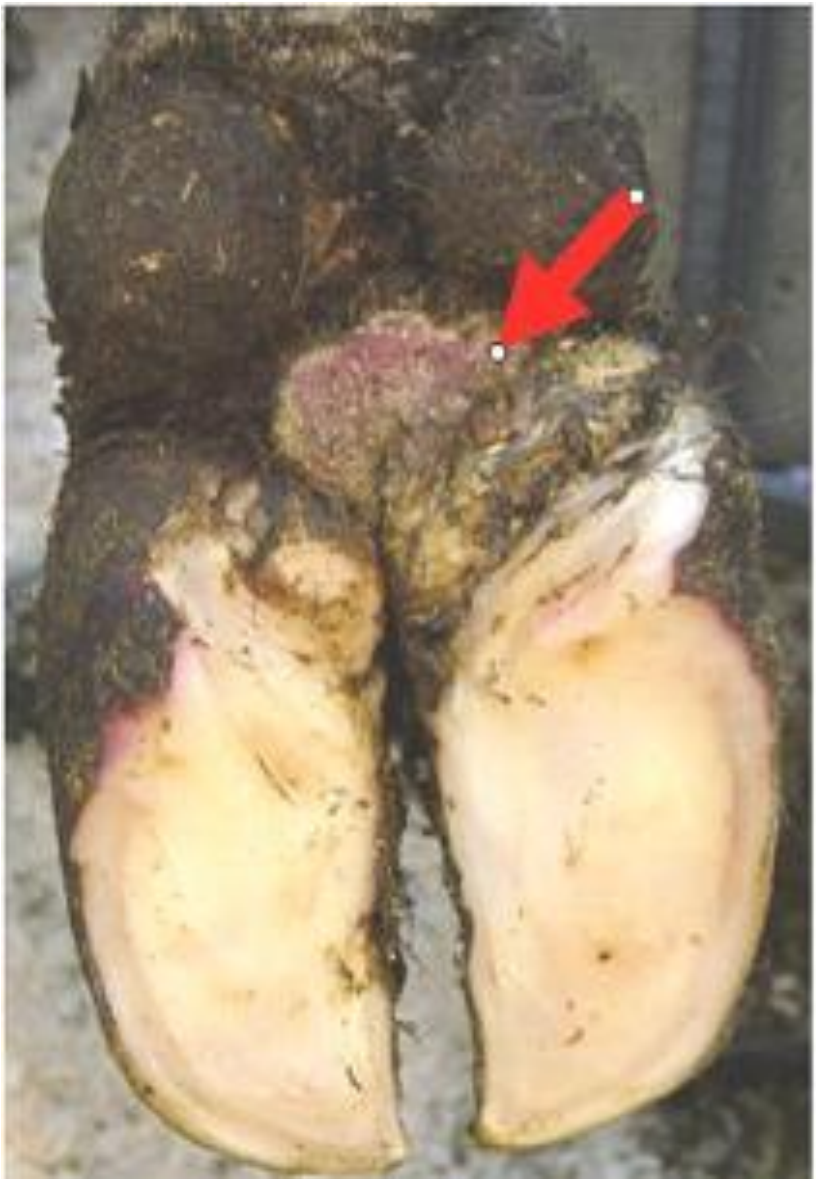
2.5. Clinical signs and management

2.5.1. Clinical manifestations of BDD in Dairy cattle

BDD 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. DD 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)



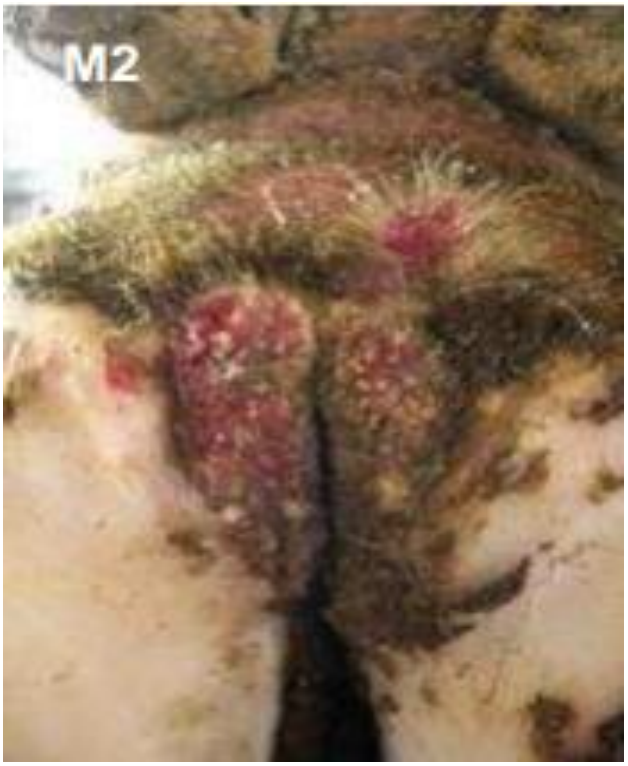
Cows with BDD are often severely lame and may walk on their toes (Blowey and Sharp 1988; Read and Walker 1998). Sometimes the animals may shake the affected foot or shift their weight from one foot to another (Bassett *et al.* 1990). These symptoms can be a good way to distinguish affected animals. However, 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 6th stage (M4.1) is sometimes used to describe a chronic lesion which is showing signs of a subclinical infection again (Döpfer *et al.* 1997).

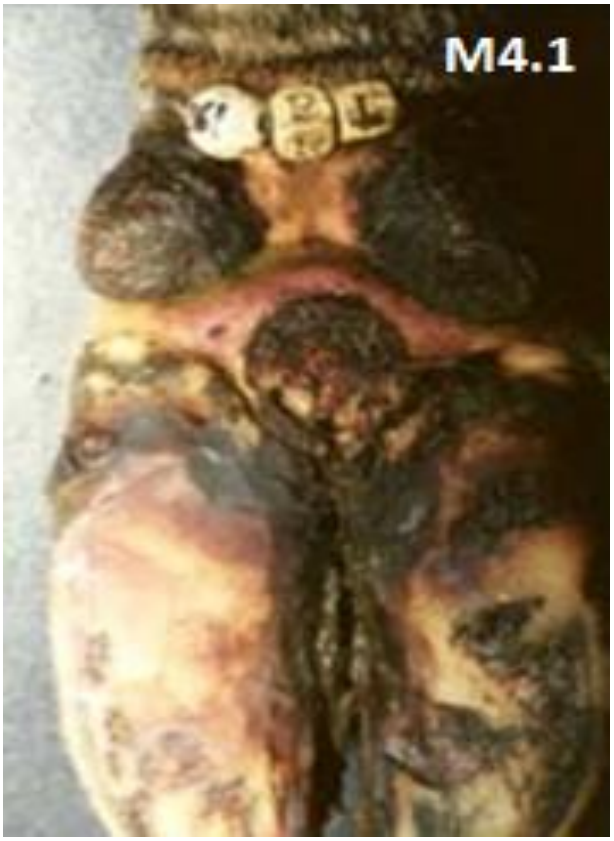
Figure 1.2: The Different presentations of BDD lesions described by the M0-M4.1 stages; detailed descriptions given in the text (M1, M2, M4, M4.1, Source: Döpfer et al. 1997).


M1


M2


M3


M4



2.5.2. 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.

2.5.3. 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).

2.5.4. 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.

2.6. 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* spp. 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.

2.7. Introduction to the Spirochaetes

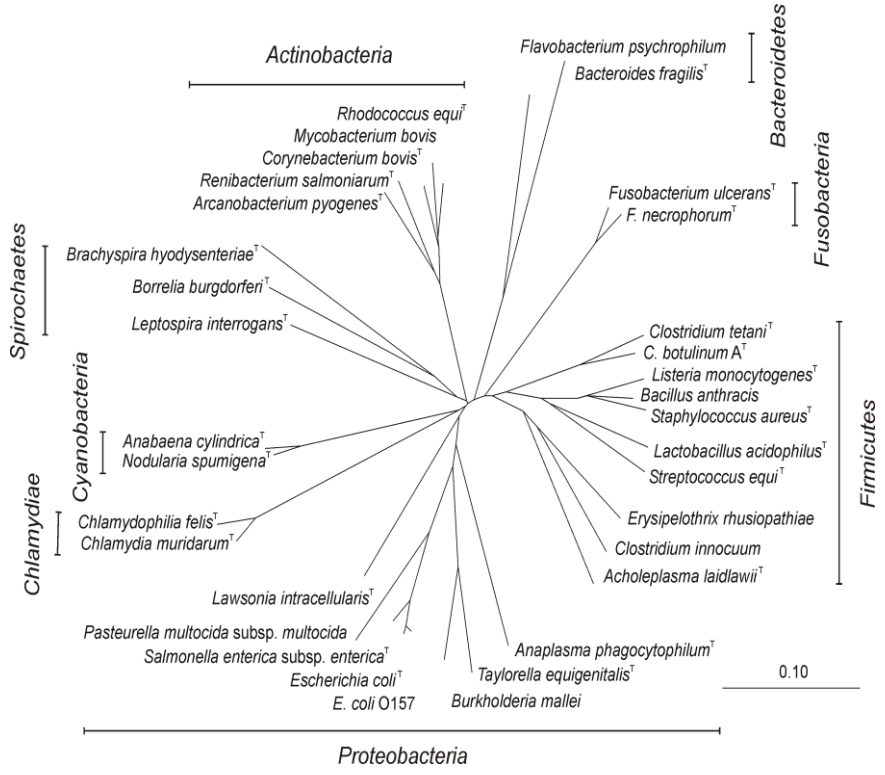
2.7.1. Spirochaete phylogeny

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.*

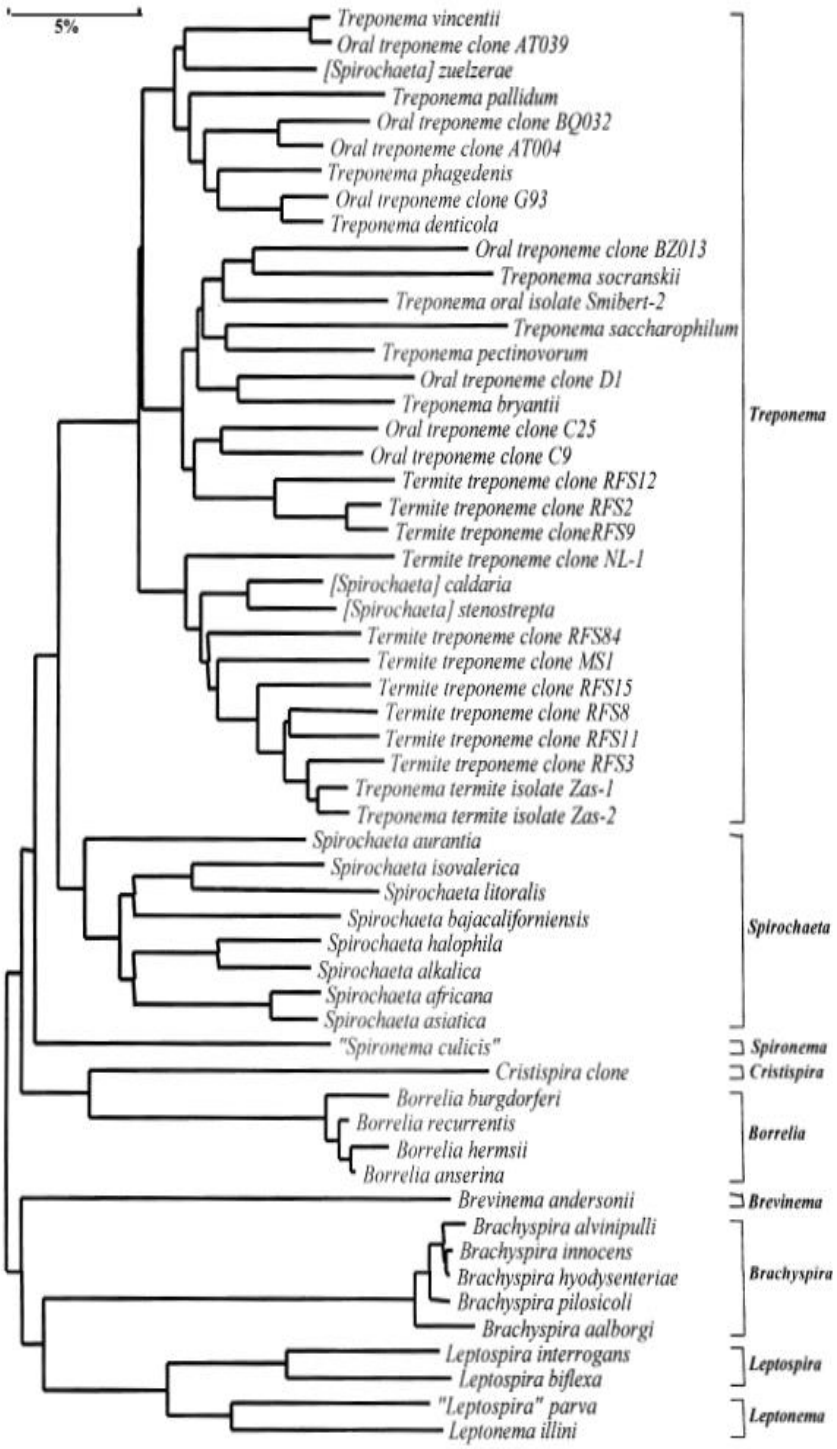
1995). *Cristispira* contains spirochaetes which are large in size and live in aquatic environments (Leschine *et al.* 2001). *Spironema* includes spirochaetes from the mosquito (Paster and Dewhirst 2000).

Figure 1.4: A radial tree illustrating bacterial phylogeny based on 16S rDNA. Only some phyla are represented and each phylum is based 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).

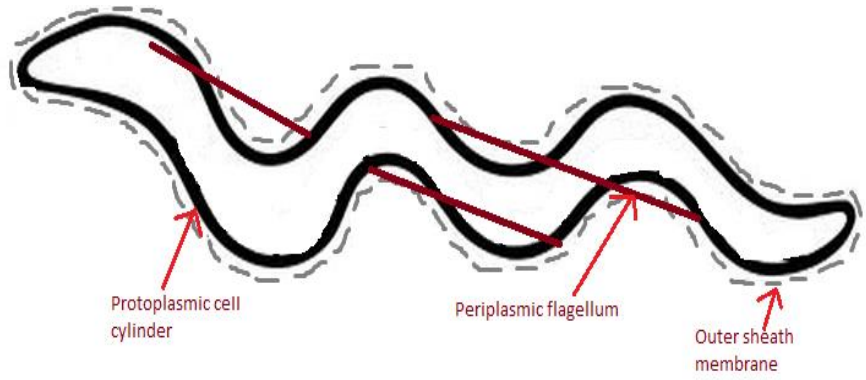


2.7.2. Spirochaete structure and biology

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).

2.7.3. 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).

2.7.4. 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 termite utilize atmospheric nitrogen as a nutrient source (Lilburn *et al.* 2001).

2.7.5. Antibiotic resistance of spirochaetes

A useful trait of the spirochaetes is their resistance to the antibiotic Rifampin (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.

2.7.6. 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).

2.7.7. 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).

2.8. The Genus *Treponema*

2.8.1. 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.

2.8.2. 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).

2.8.3. 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.

2.9. Treponemes and Digital Dermatitis

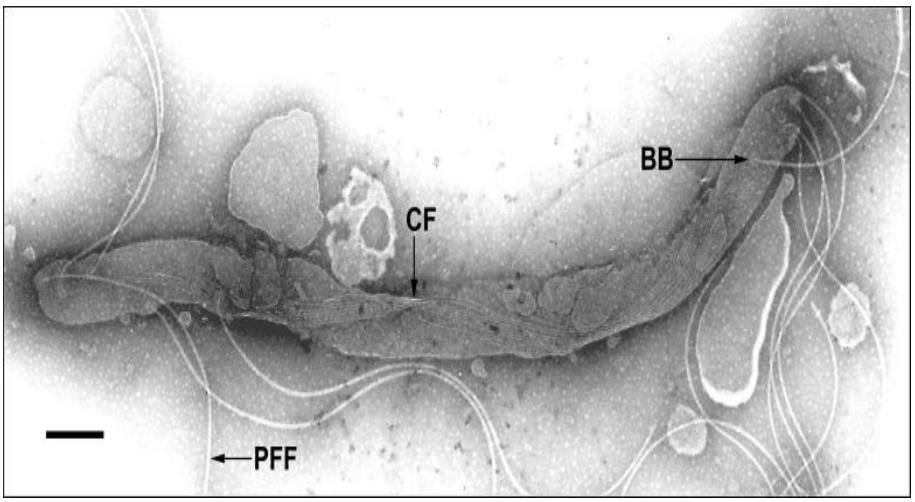
2.9.1. Aetiology of BDD

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.

2.9.2. 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.

2.9.3. 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.

2.9.4. 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.

2.10. 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.

3. Materials and methods

3.1. 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) 11.5g 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 ^{o}C 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.

3.2. Collection and transportation of clinical samples

3.2.1. 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.

3.2.2. 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).

3.2.3. 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).

3.2.4. 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.

3.2.5. 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).

3.3. Bacterial culture

3.3.1. 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.

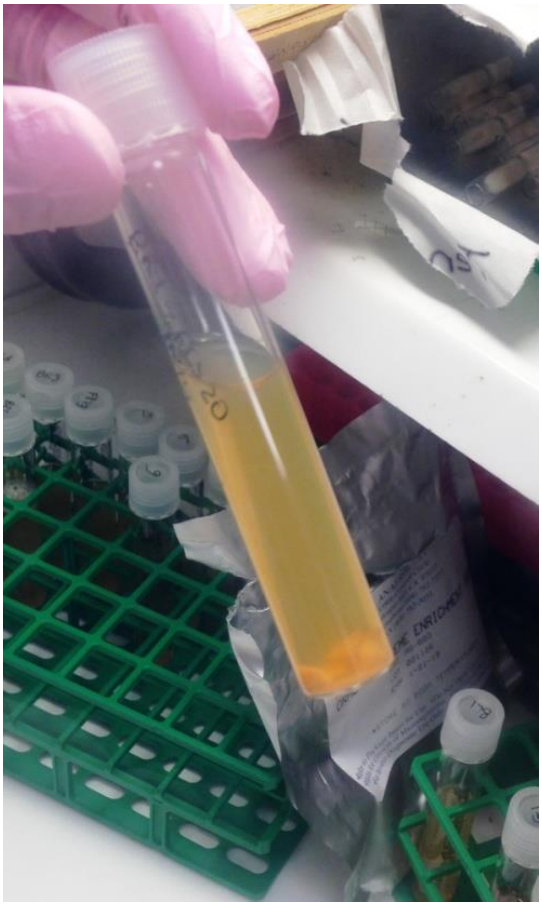
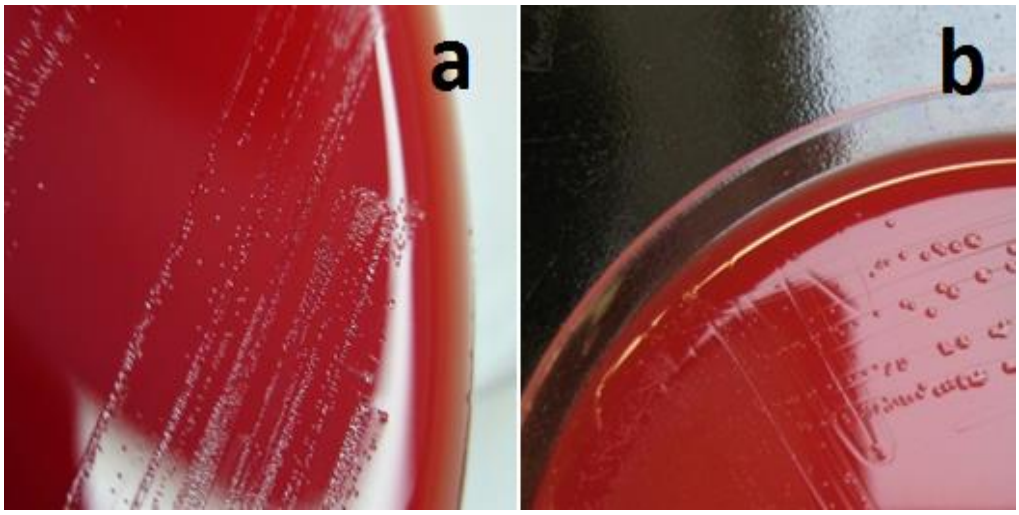


Figure 2.2: a) and b) show translucent, circular, convex colonies typical of treponeme colonies on blood agar plates.



3.3.2. Storage of treponeme cultures

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

3.4. DNA extraction

3.4.1. 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.

3.4.2. 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.

3.4.3. 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.

3.5. Polymerase Chain Reaction (PCR) assays

3.5.1. 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).

3.5.2. 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.

3.5.3. 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.

3.5.4. 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.

3.5.5. *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*.

3.5.6. 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*.

3.5.7. 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).

3.6. Gene sequencing and assembly

3.6.1. 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.

3.6.2. 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.

3.7. 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).

3.8. Statistical analyses

3.8.1. 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.

3.8.2. 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.

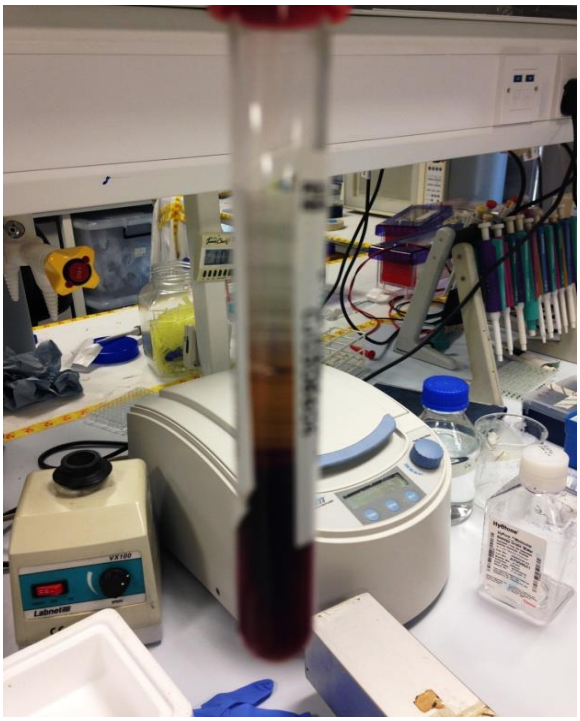
This was carried out using GraphPad InStat Software, Version 3.10 (GraphPad Software, USA). In all analyses, an associated probability (P-value) of < 0.05 was considered significant.

3.9. Serological methods

3.9.1. 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.



3.9.2. 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.

3.9.3. 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.

3.9.4. 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.

3.9.5. 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

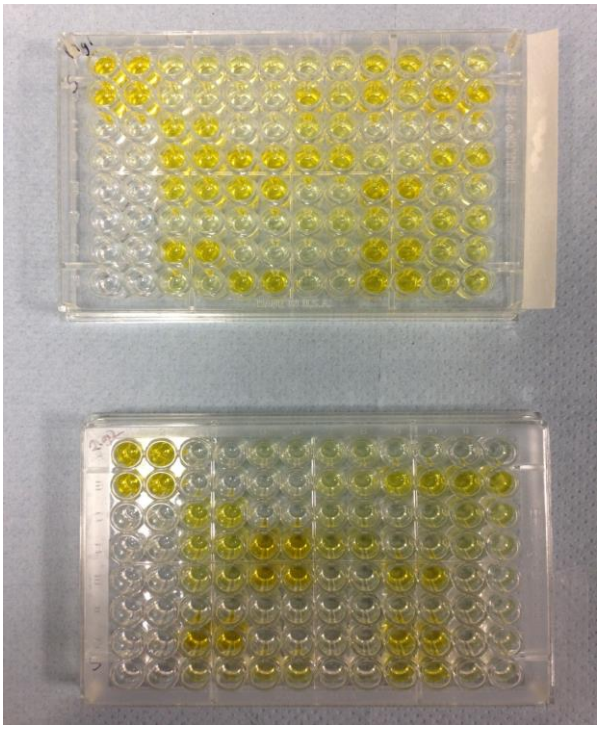
then incubated for 1 h at 37 °C and overnight at 4 °C. Unbound antigen was removed by washing three times with PBST. Sera samples were diluted to 1/100 in PBST (0.05% (v/v)) (determined optimum dilution) and 100 µl pipetted into ELISA plate wells in duplicate. All plates included positive and negative 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.



3.9.6. 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 ℃ 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

3.9.7. 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.

4. Results

4.1. CODD bacteriology and DD in goats Results

4.1.1. CODD investigation

4.1.1.1 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 3.1 and 3.2, 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 3.1 and 3.2, 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 3.1: 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 3.2.

Table 3.2: PCR detection of treponemes, D. nodosus and F. necrophorum in healthy foot tissue biopsies.

Sample	Biopsy date	Details (Farm, location[a], sheep number[b])	Result					
			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 3.1.

4.1.1.2 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 3.3.

Table 3.3: Associations between bacteria present in CODD lesions from PCR data analysis (Chi-squared analysis (P values).

		***Treponema* group[a]:**			***D. nodosus***	***F. necrophorum***
***Treponema* group[a]:**		**1**	**2**	**3**		
	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 (*P*= 0.9727). 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.

4.1.1.3 Culture of spirochaetes and phylogenetic analysis of spirochaete isolates

Spirochaetes were successfully isolated from a high proportion of CODD lesions (Table 3.1). 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

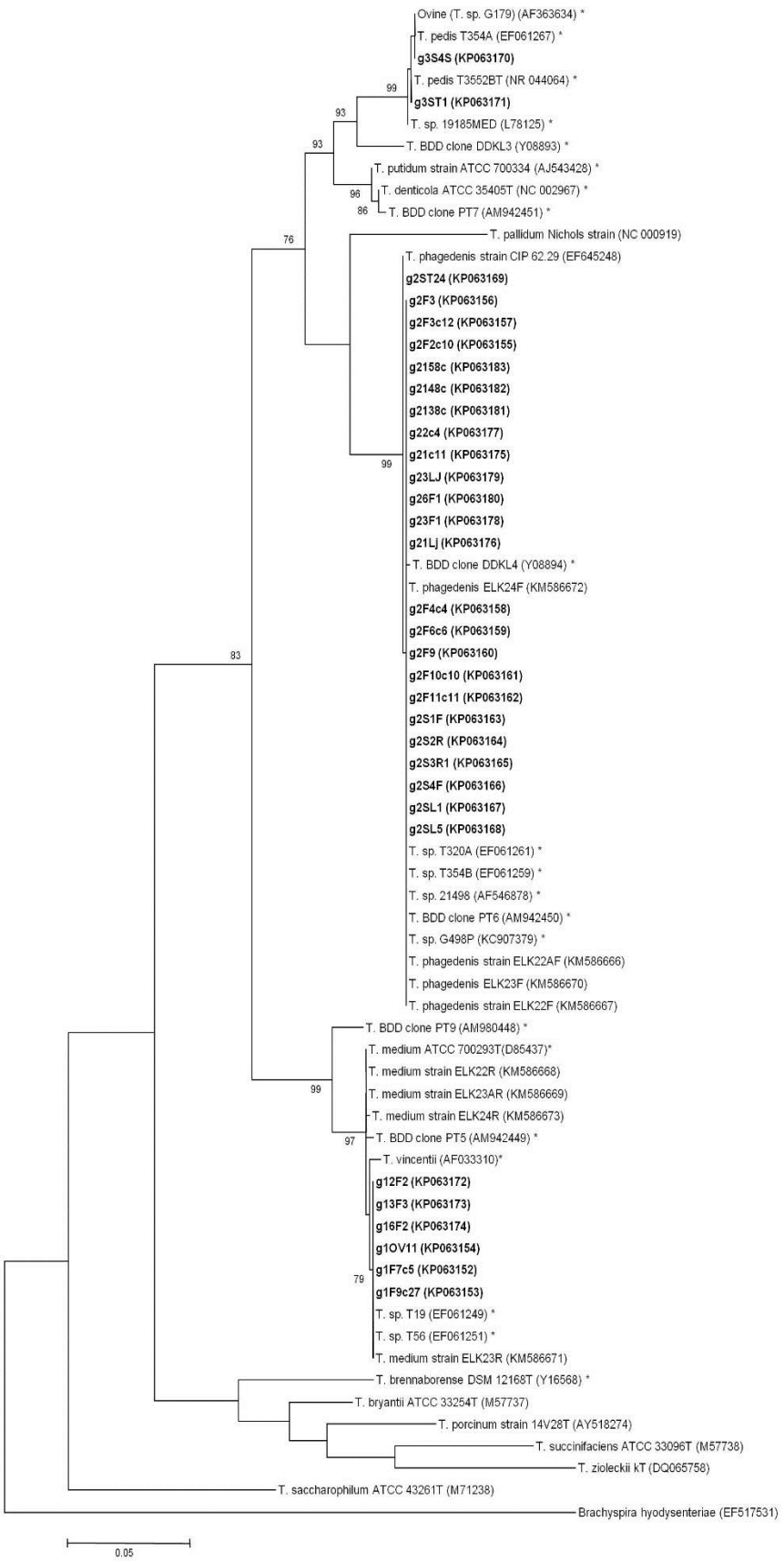
(EF645248) which both differ from the dairy cow DD isolate, *T. phagedenis*- like DD spirochaete strain T320A, by a single nucleotide substitution.

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

4.1.2. Goat lesions with an unknown aetiology

4.1.2.1 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.

4.1.2.2 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 3.4.

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.

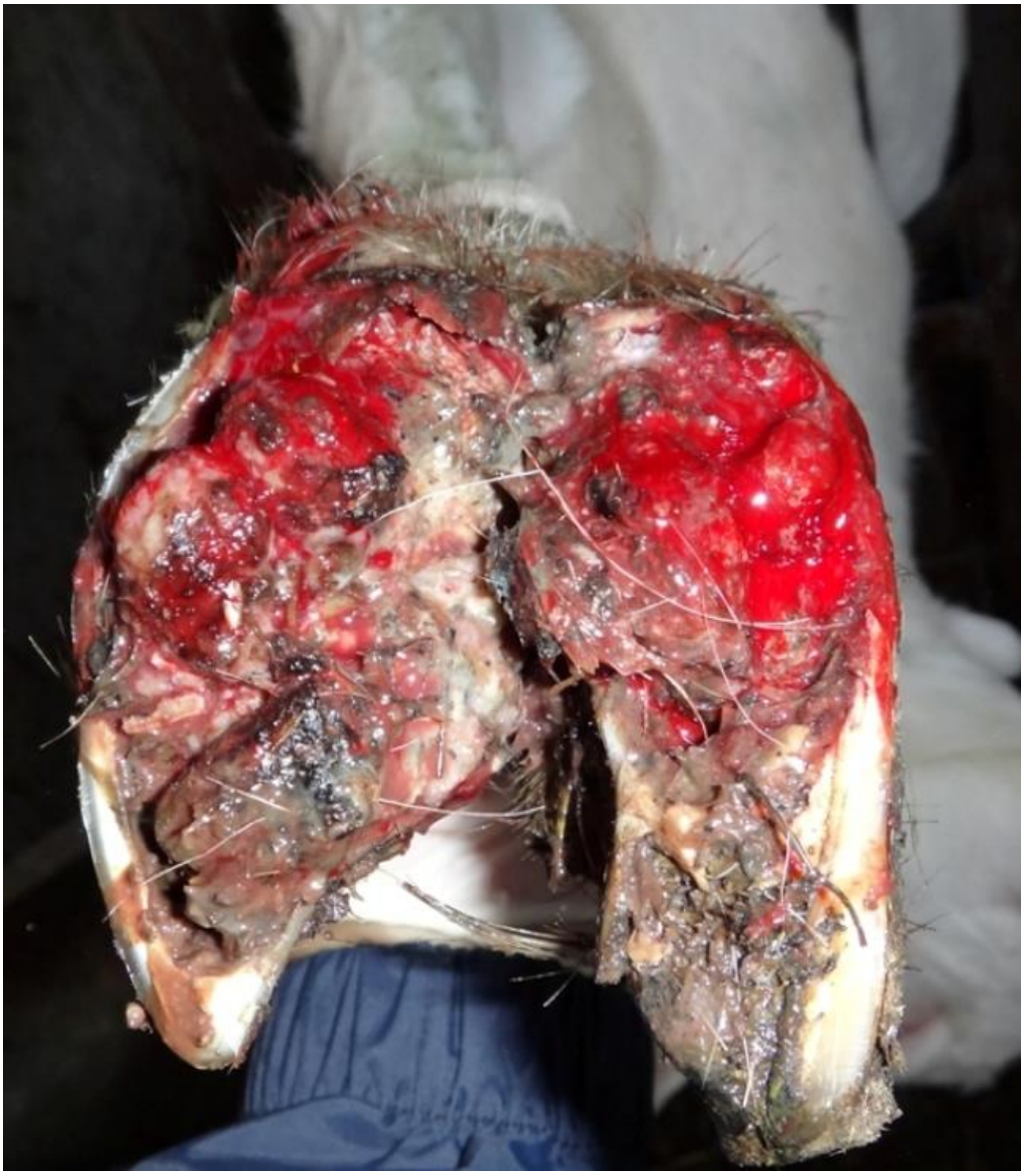


Table 3.4: PCR detection and isolation of treponemes in goat foot lesion biopsies (samples 1–10) and healthy goat foot tissues (samples 11–20).

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.

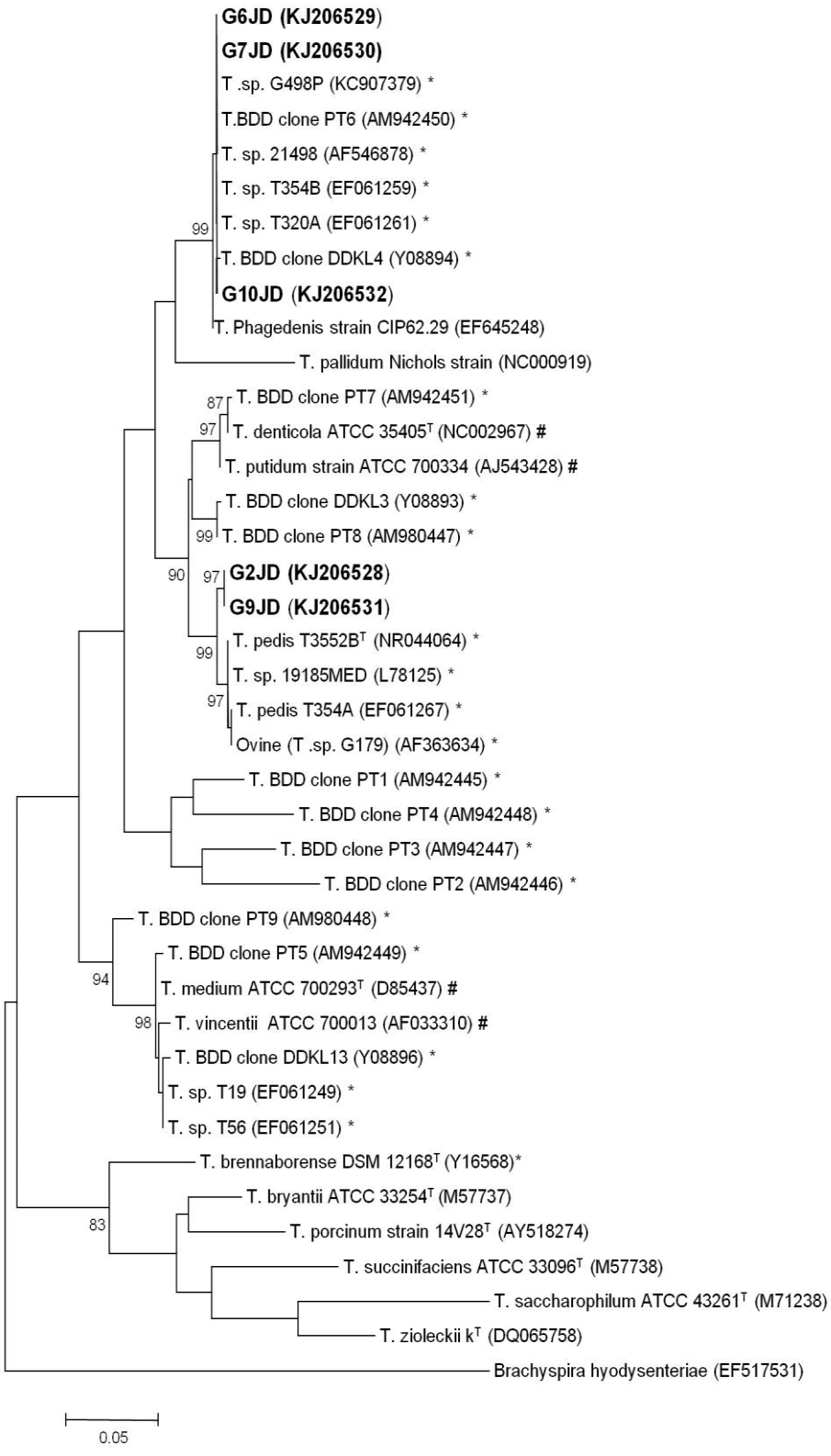
4.1.2.3 Culture of spirochaetes and phylogenetic analysis of spirochaete isolates

Spirochaetes were successfully isolated from 5/10 (50%) of cultured lesion samples (Table 3.4). 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

4.2. BDD in beef cattle Results

4.2.1. Clinical description of bovine digital dermatitis 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.

4.2.2. 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.

4.2.3. 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 ($P = 0.9004$). 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.

4.2.4. 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.

4.2.5. 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.*

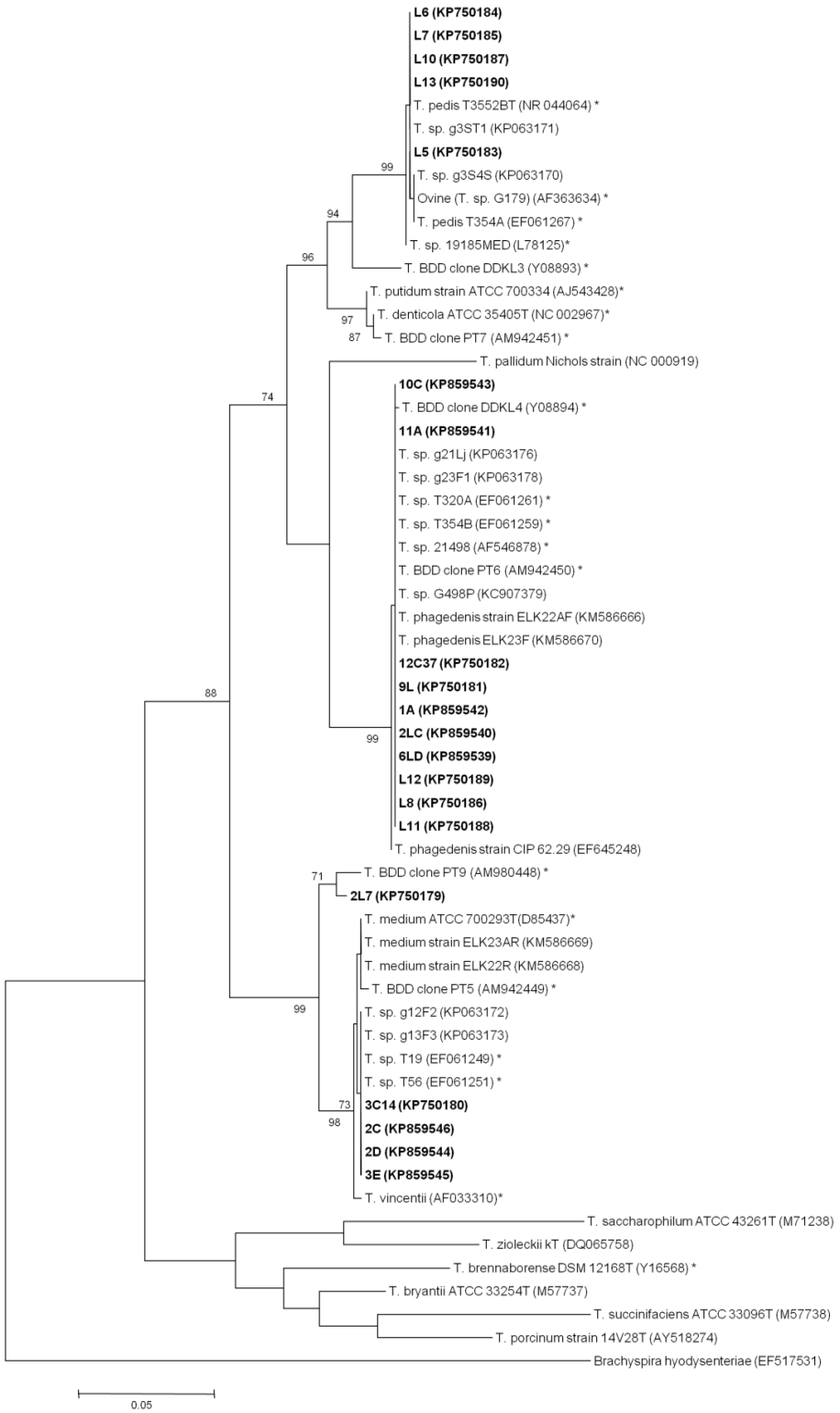

L6 (KP750184)
L7 (KP750185)
L10 (KP750187)
L13 (KP750190)
T. pedis T3552BT (NR 044064) *
T. sp. g3ST1 (KP063171)
L5 (KP750183)
T. sp. g3S4S (KP063170)
Ovine (T. sp. G179) (AF363634) *
T. pedis T354A (EF061267) *
T. sp. 19185MED (L78125)*
T. BDD clone DDKL3 (Y08893) *
T. putidum strain ATCC 700334 (AJ543428)*
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.

4.3. Investigating the GI tract for treponemes metaenomically- Results

4.3.1. 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.

4.3.2. 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

4.3.3. 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 5. 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.

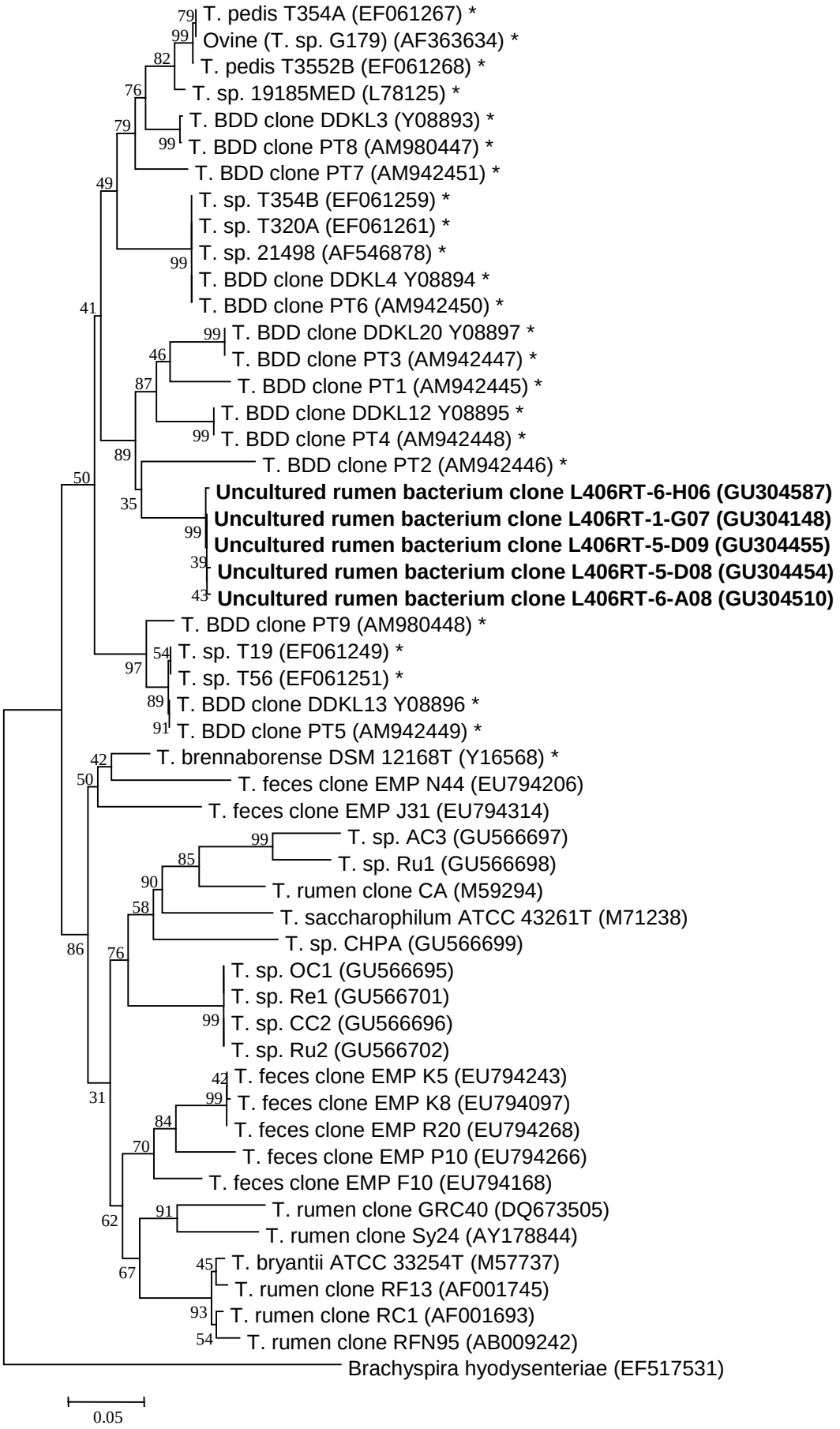
	T.BDD clone PT1[a]	**T.BDD clone PT2[a]**	**T.BDD clone PT3[a]**	**T.BDD clone PT4[a]**	**DDKL-12**	**DDKL- 20 (420)**
L406RT-1-G07 (1)	0.908(1325)	0.892(1325)	0.903(1325)	0.900(1325)	0.877(580)	0.873
L406RT-6-H06 (1)	0.907(1325)	0.891(1325)	0.906(1325)	0.902(1325)	0.879(580)	0.873
L406RT-5-D08 (1)	0.909(1325)	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) *
T. pedis T3552B (EF061268) *
T. sp. 19185MED (L78125) *
T. BDD clone DDKL3 (Y08893) *
T. BDD clone PT8 (AM980447) *
T. BDD clone PT7 (AM942451) *
T. sp. T354B (EF061259) *
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)
T. BDD clone PT9 (AM980448) *
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)
T. feces clone EMP K5 (EU794243)
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 5.3: 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.*

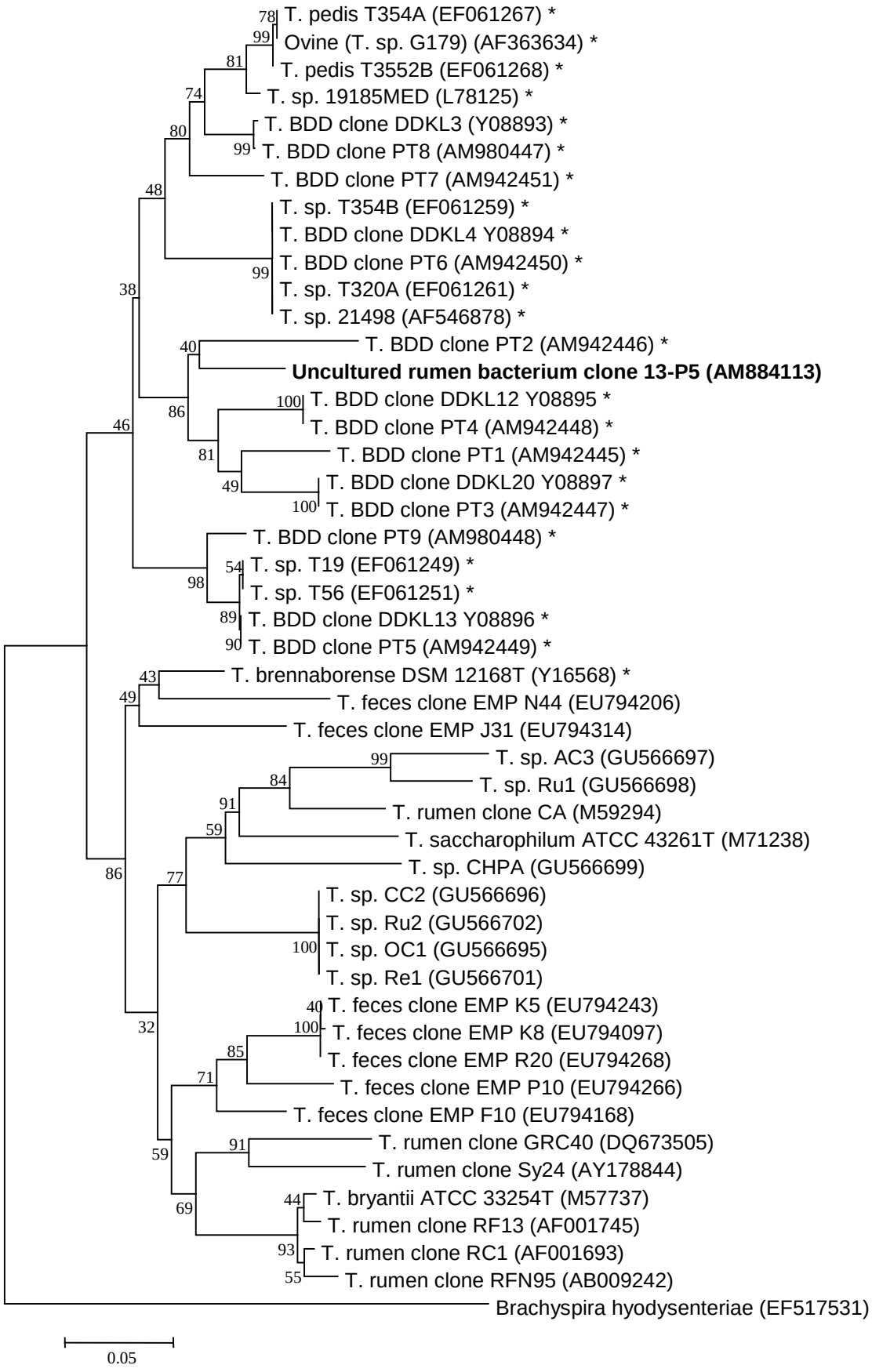
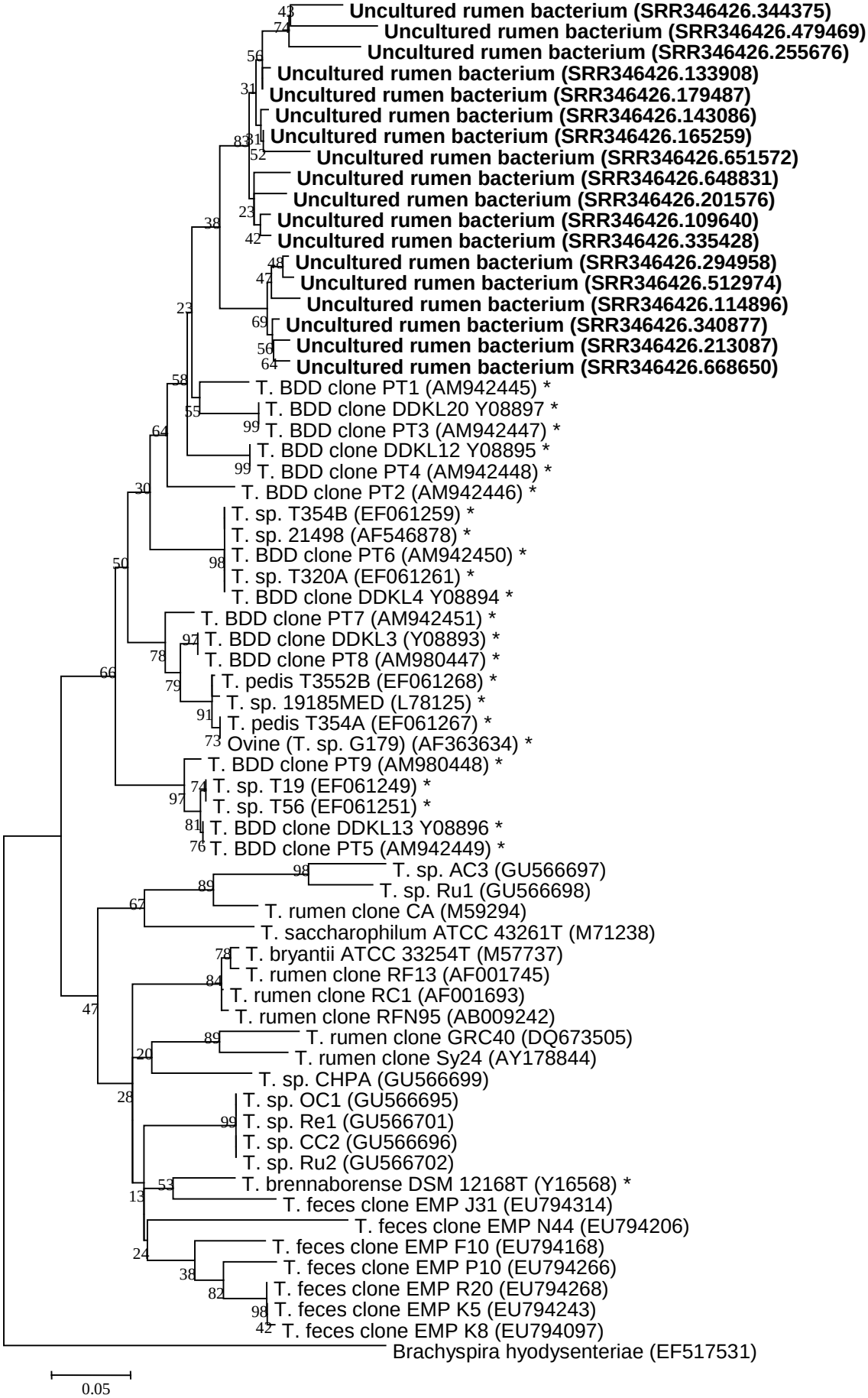

78
T. pedis T354A (EF061267) *
99
Ovine (T. sp. G179) (AF363634) *
81
T. pedis T3552B (EF061268) *
74
T. sp. 19185MED (L78125) *
80
T. BDD clone DDKL3 (Y08893) *
99
T. BDD clone PT8 (AM980447) *
48
T. BDD clone PT7 (AM942451) *
T. sp. T354B (EF061259) *
T. BDD clone DDKL4 Y08894 *
99
T. BDD clone PT6 (AM942450) *
38
T. sp. T320A (EF061261) *
T. sp. 21498 (AF546878) *
40
T. BDD clone PT2 (AM942446) *
Uncultured rumen bacterium clone 13-P5 (AM884113)
100
T. BDD clone DDKL12 Y08895 *
86
T. BDD clone PT4 (AM942448) *
46
81
T. BDD clone PT1 (AM942445) *
49
T. BDD clone DDKL20 Y08897 *
100
T. BDD clone PT3 (AM942447) *
T. BDD clone PT9 (AM980448) *
54
T. sp. T19 (EF061249) *
98
T. sp. T56 (EF061251) *
89
T. BDD clone DDKL13 Y08896 *
90
T. BDD clone PT5 (AM942449) *
43
T. brennaborense DSM 12168T (Y16568) *
49
T. feces clone EMP N44 (EU794206)
T. feces clone EMP J31 (EU794314)
99
T. sp. AC3 (GU566697)
84
T. sp. Ru1 (GU566698)
91
T. rumen clone CA (M59294)
59
T. saccharophilum ATCC 43261T (M71238)
T. sp. CHPA (GU566699)
86
77
T. sp. CC2 (GU566696)
T. sp. Ru2 (GU566702)
100
T. sp. OC1 (GU566695)
T. sp. Re1 (GU566701)
40
T. feces clone EMP K5 (EU794243)
32
100
T. feces clone EMP K8 (EU794097)
85
T. feces clone EMP R20 (EU794268)
71
T. feces clone EMP P10 (EU794266)
T. feces clone EMP F10 (EU794168)
91
T. rumen clone GRC40 (DQ673505)
59
T. rumen clone Sy24 (AY178844)
44
T. bryantii ATCC 33254T (M57737)
69
T. rumen clone RF13 (AF001745)
93
T. rumen clone RC1 (AF001693)
55
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.*


Uncultured rumen bacterium (SRR346426.344375)
Uncultured rumen bacterium (SRR346426.479469)
Uncultured rumen bacterium (SRR346426.255676)
Uncultured rumen bacterium (SRR346426.133908)
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)
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Uncultured rumen bacterium (SRR346426.335428)
Uncultured rumen bacterium (SRR346426.294958)
Uncultured rumen bacterium (SRR346426.512974)
Uncultured rumen bacterium (SRR346426.114896)
Uncultured rumen bacterium (SRR346426.340877)
Uncultured rumen bacterium (SRR346426.213087)
Uncultured rumen bacterium (SRR346426.668650)
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 5. 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

4.3.4. 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).

4.4. GI tract tissue survey Results

4.4.1. *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	Location[a]	DD status[b]	Result	
				Rectal tissue	Gingival tissue

	(mo/yr)			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.		

4.4.2. *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)			**DD status[b]**		**Result**

	Collection date (mo/yr)	Location sample obtained[a]		Treponeme isolated[c]	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.

4.4.3. 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).

4.4.4. 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 ($P = 0.58$) or beef cattle ($P > 0.99$). However, interestingly there was a statistically significance association between DD status and the isolation of *T. sp.* CHPA identified ($P = 0.041$).

4.4.5. 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.*

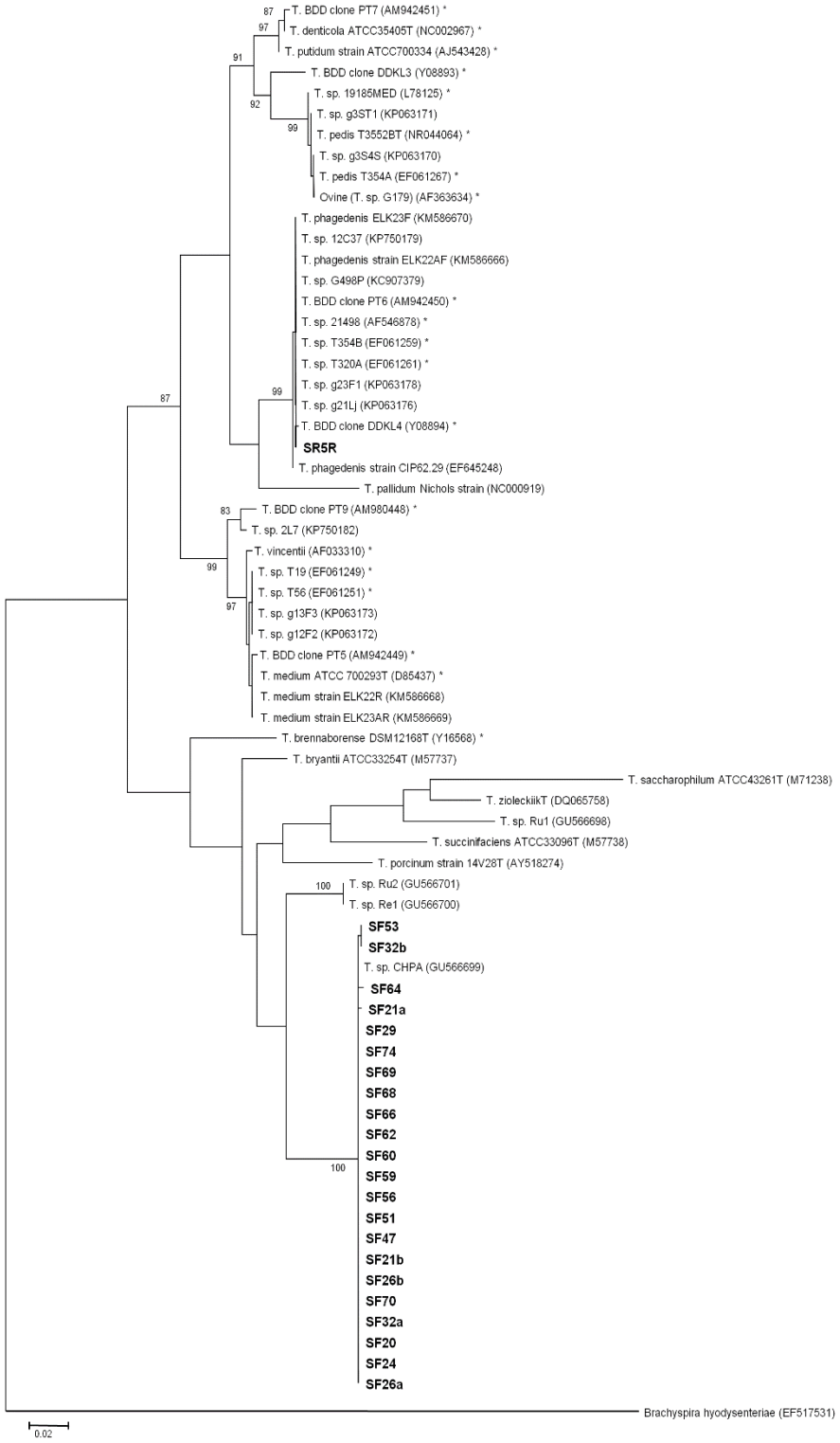


Table 6.6: A comparison of PCR detection rates of Treponema DD phylogroups in the GI tissues of dairy cattle, beef cattle and sheep.

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.

4.5. Presence of treponemes on trimming equipment Results

4.5.1. 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.

4.5.2. 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* (genus-wide)
		1	2	3	
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.

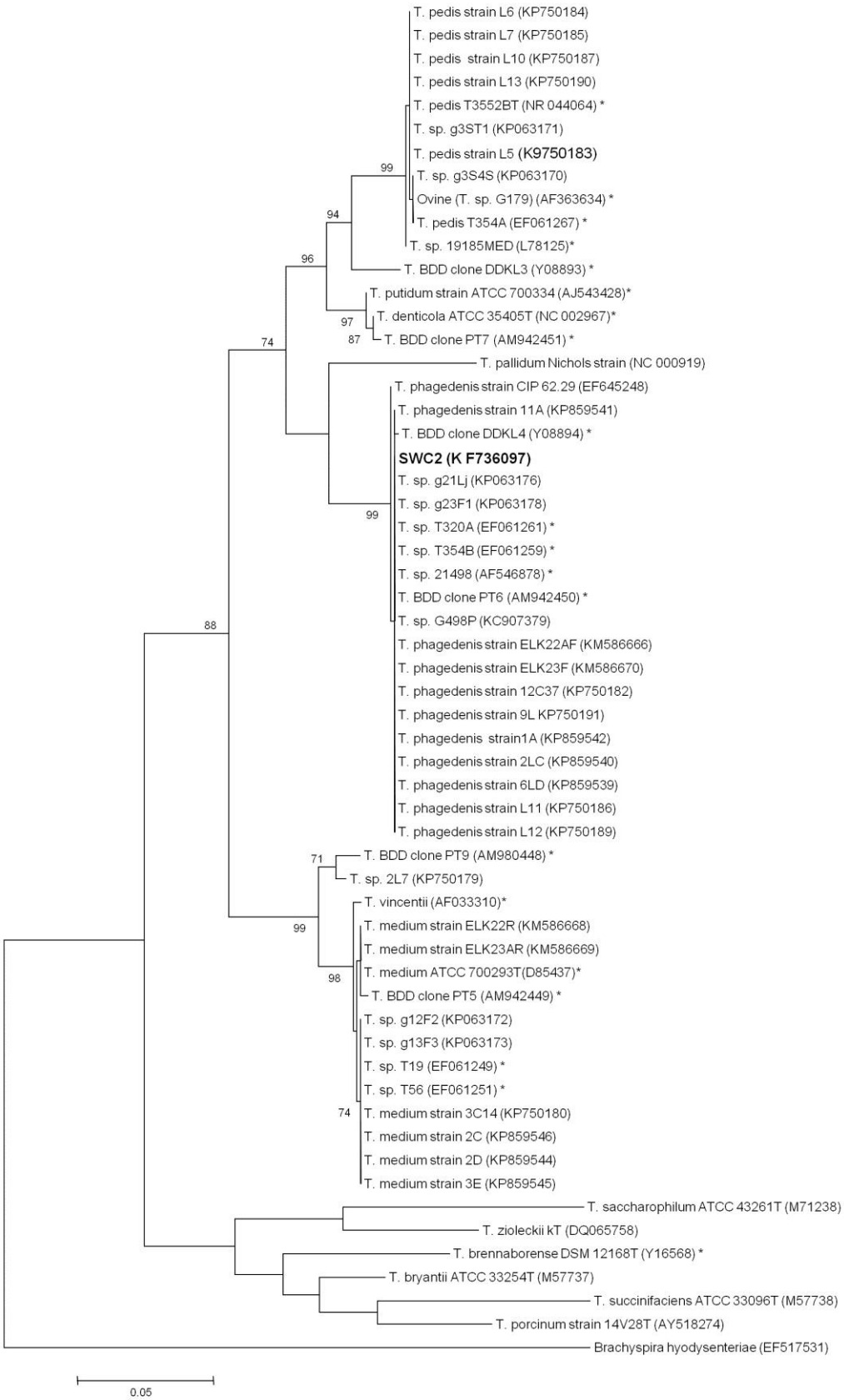
Table 7.4 A summary of the PCR detection rates of DD Treponema phylogroups on trimming equipment after trimming, compared with the detection rates after subsequent disinfection of the equipment.

Trimming equipment[a]	***Treponema* group**[b]			***Treponema* (genus-wide)**
	1	**2**	**3**	
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

4.6. Beef cattle serology studies Results

4.6.1. 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	+	+	+	+	+	+	+	+	+								+	+
2	+	+	+	+	+	+	+	+	+								+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
5				+	+	+	+	+	+							+	+	+
6													+	+	+			
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+
9	+	+	+	+	+	+	+		+								+	+
10	+	+	+															
12																		
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14				+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15						+							+	+	+	+	+	+
16																		
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+				+	+	+	+	+	+				+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+											+				
25	+	+	+			+								+				
26																		
27	+	+	+	+										+				
28	+	+	+	+	+	+	+	+	+							+		+
29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30					+		+	+	+							+	+	+
A1	+	+	+	+	+	+	+	+	+									

A2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A3	+	+	+	+	+	+	+	+	+				+	+	+	+	+	+
A4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A5	+	+	+	+	+	+	+	+	+				+	+	+	+	+	+
A6	+	+	+	+	+	+	+	+	+									
A7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A8	+	+		+	+	+			+	+				+	+	+	+	+
A9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A10	+	+		+	+	+	+	+		+	+	+		+	+	+		+
A11	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
A12	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
A13	+	+	+	+	+	+	+	+	+				+	+				
A14					+													
A15	+	+	+															
A16	+	+	+	+	+	+	+	+	+				+	+	+	+		
A17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A19						+												
A20						+	+	+	+									
A21									+									
A22							+											
A23	+	+	+	+	+	+	+	+	+									
A24																		
A25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A26	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+
A27	+	+	+	+	+	+		+	+				+	+	+	+	+	+
A28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+
A29																		
A30	+	+	+	+	+	+	+	+	+	+	+	+						
A31	+	+	+	+	+	+	+	+	+	+	+	+		+	+			+
A32	+	+	+	+	+	+		+	+									
A33																		
A34	+	+	+	+	+	+	+	+	+	+	+	+						
A35	+	+	+	+	+	+	+	+	+	+	+	+			+			
A36	+	+		+	+	+									+			
A54	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A65																		
J1																		
J2																		
J3																		
J4																		
J5																		
J6																		
J7																		
J8	+	+	+			+	+	+	+									
J9																		
J10																		
1																		

2																		
3	+	+	+	+	+	+	+	+	+					+				
4																		
5																		
6																		
7																		
8																		
9																		
10																		
11																		
12																		
13																		
14																		
15																		
16																		
17	+	+	+	+	+	+	+	+	+									
18	+	+	+			+	+	+	+									
19																		
20																		
21																		
22																		
23																		
24	+	+	+				+	+	+									
25																		

[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.

+; indicates seropositivity, blank indicates seronegativity. .

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 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.

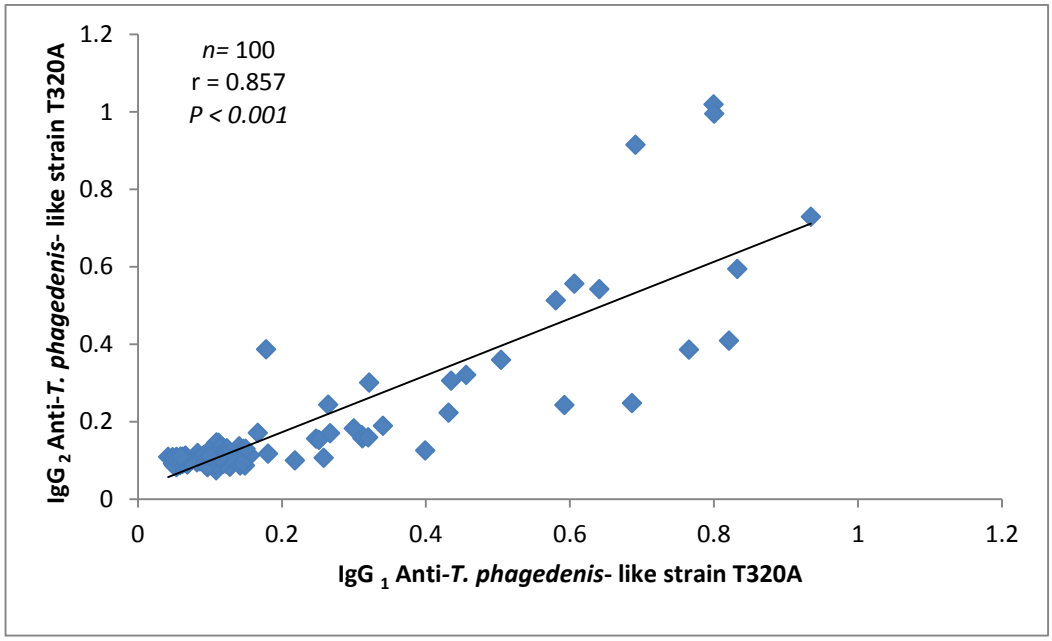


Figure 8.2: The linear regression analysis of IgG_1 and IgG_2 antibodies against T. pedis strain g3S4S.

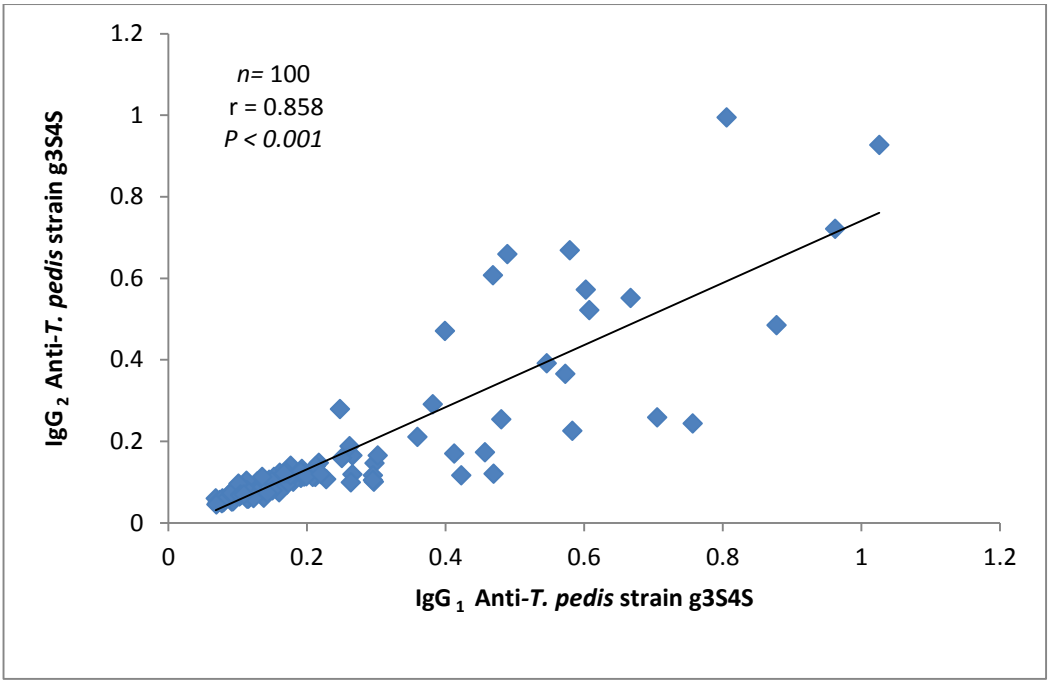


Figure 8.3: The linear regression analysis of IgG_1 antibodies against T. phagedenis-like strain 6LD versus T. phagedenis- like strain T320A.

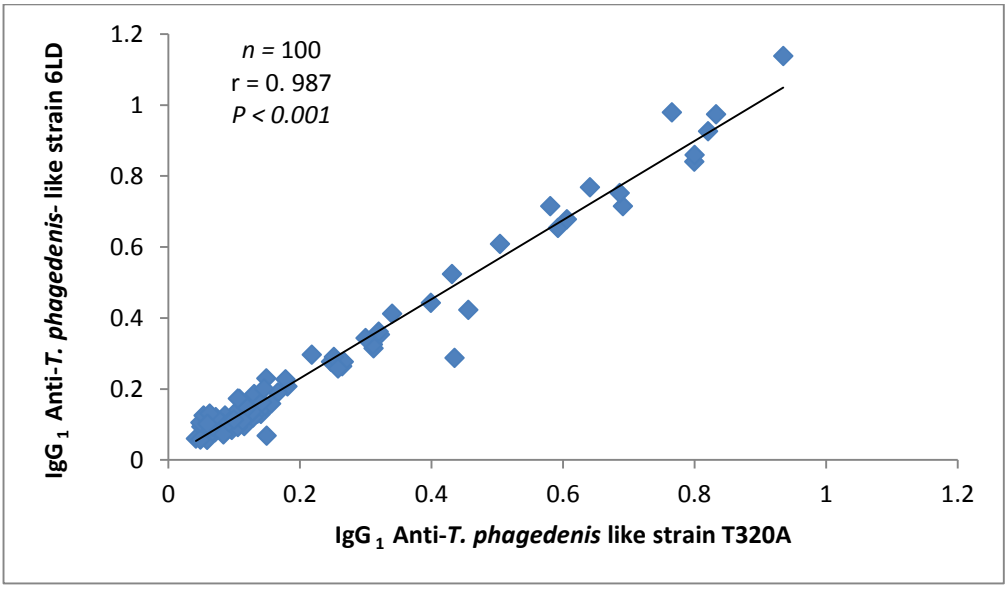


Figure 8.4: The linear regression analysis of IgG_2 antibodies against T. phagedenis-like strain g2F9 versus T. phagedenis- like strain 6LD.

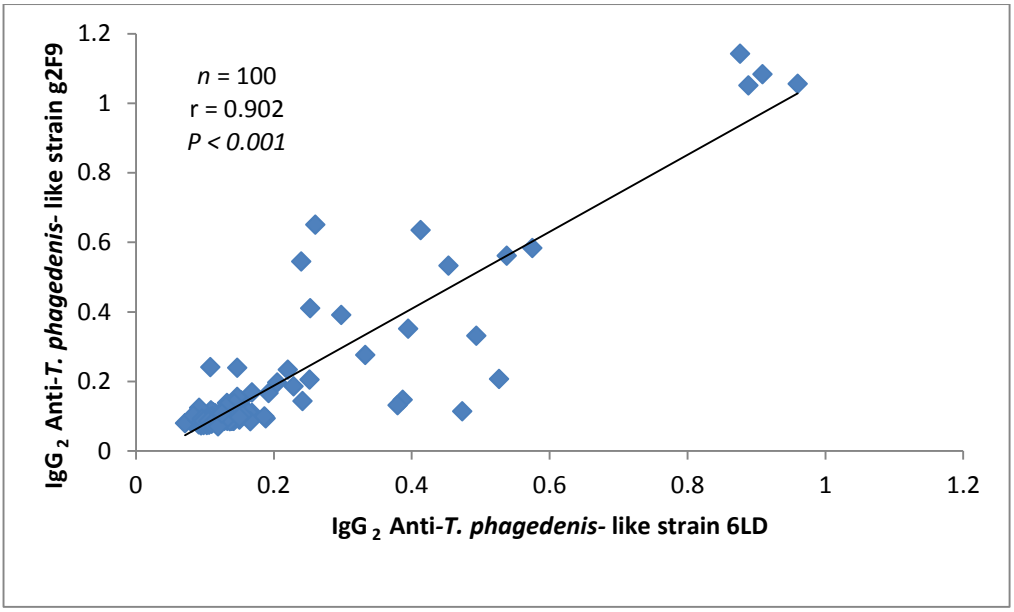


Figure 8.5: The linear regression analysis of IgG_1 antibodies against T. pedis strain T320A versus T. medium- like strain T19.

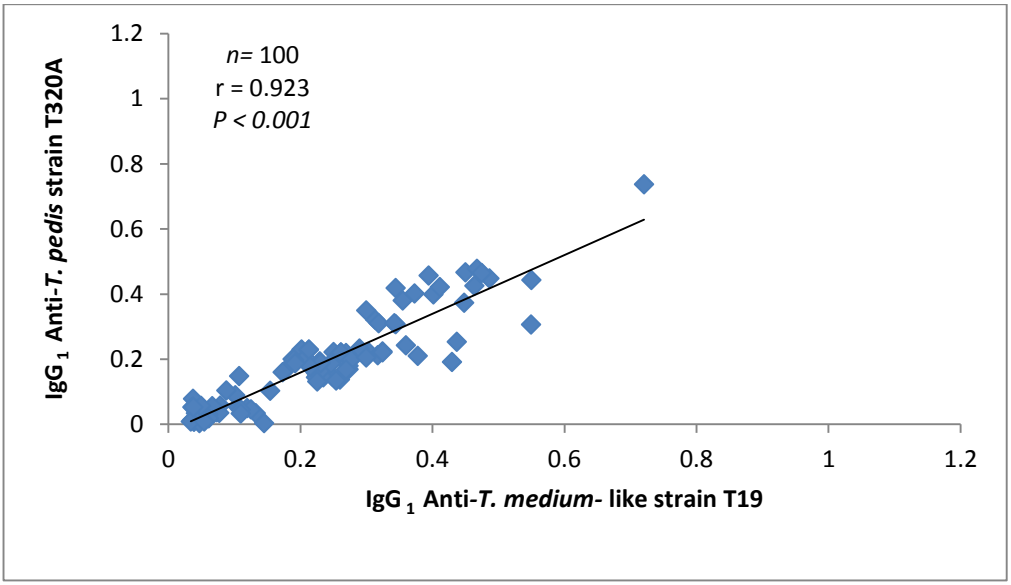


Figure 8.6: The linear regression analysis of IgG_1 antibodies against T. pedis strain g3S4S versus T. phagedenis- like strain g2F9.

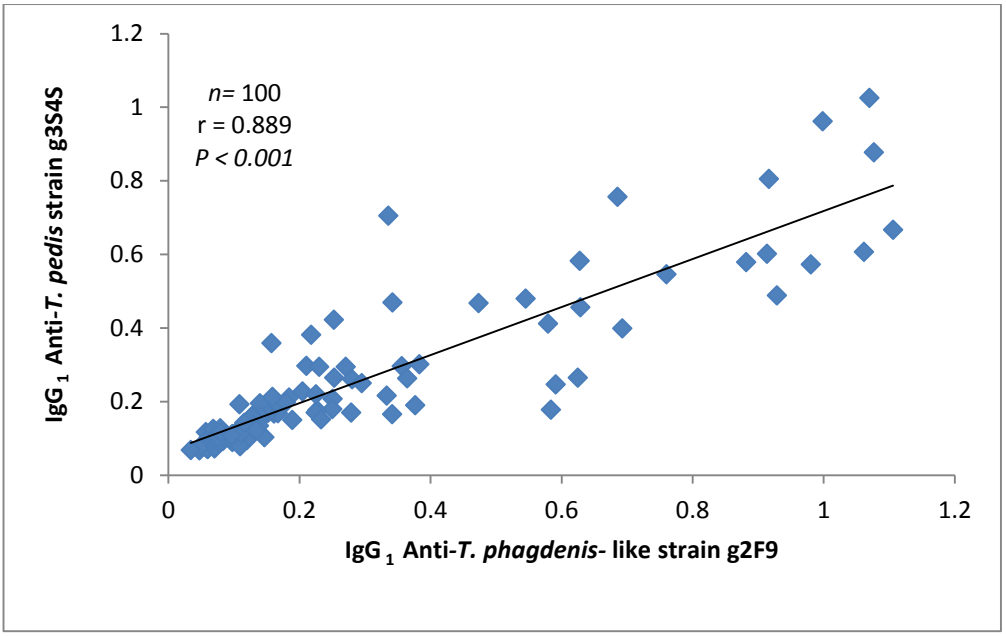


Table 8.5: (a)- (c); Correlation coefficients (r values) and associated probability (P values) for sera IgG_1 antibody response to each treponeme strain versus sera IgG_1 antibody response to each other treponeme isolate from the same phylogroup. (d)- (f); Correlation coefficients (r values) and associated 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

4.6.2. 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.

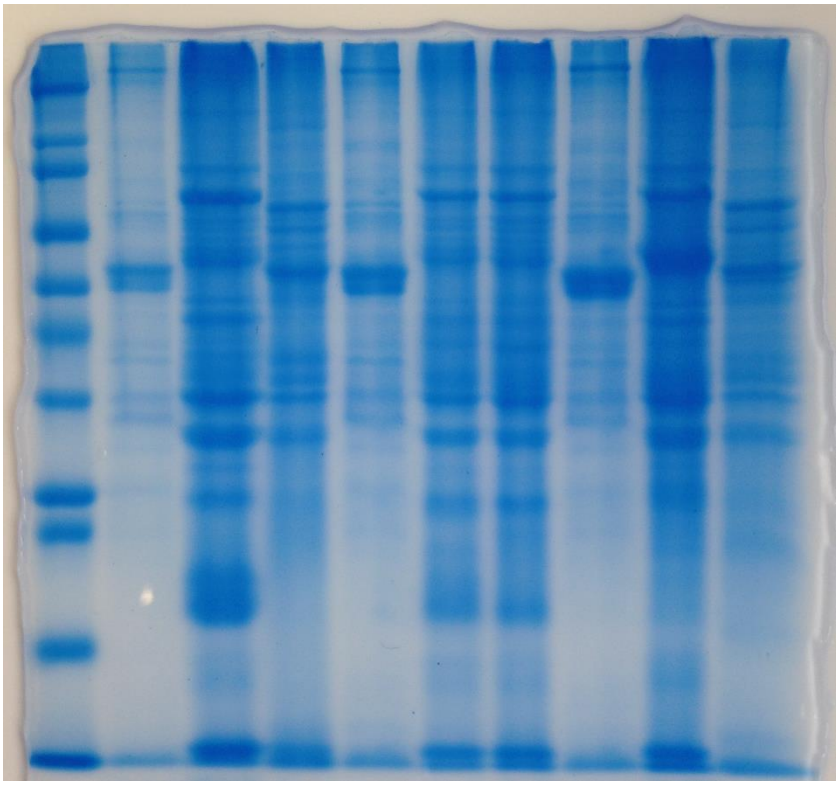
Against *T. medium*- like treponemes, the most common IgG_1 and IgG_2 band staining was detected at ~53kDa (11/12 ELISA positive sera for IgG_1 and IgG_2, 8/12 of negative sera for both IgG_1 and IgG_2), 30-32 kDa (5/12, 4/12 ELISA positive sera for IgG_1 and IgG_2, respectively, and 4/12 of negative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

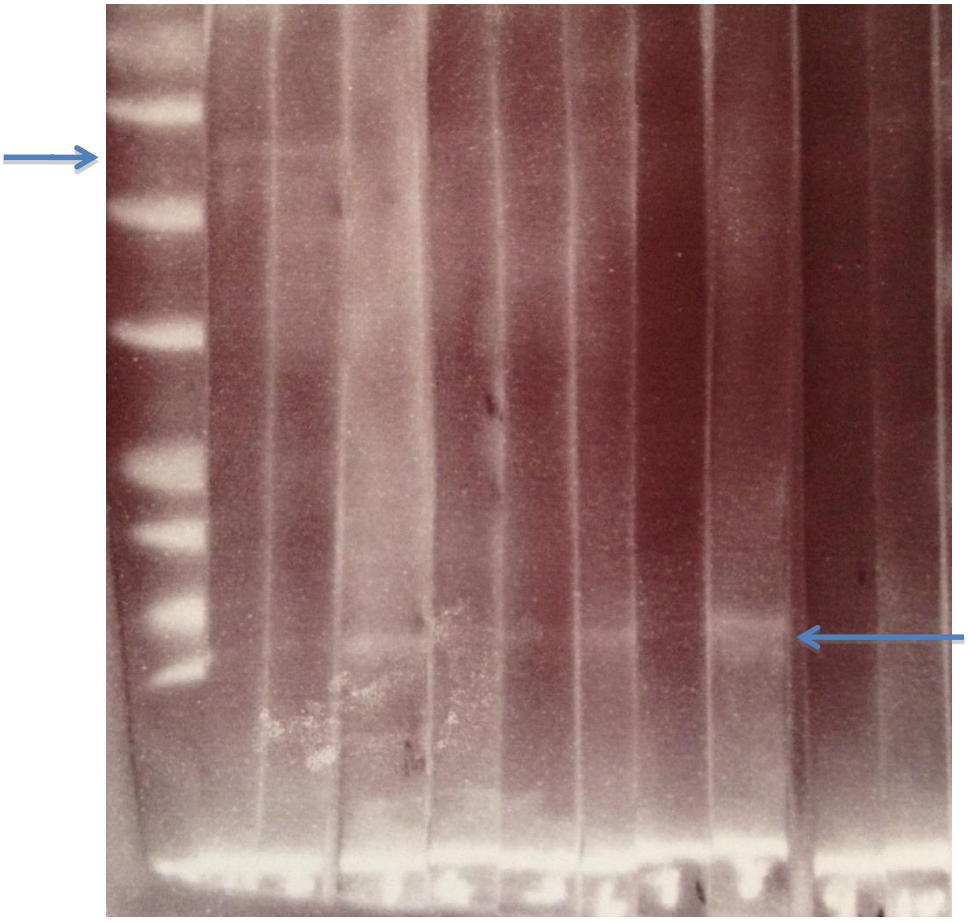
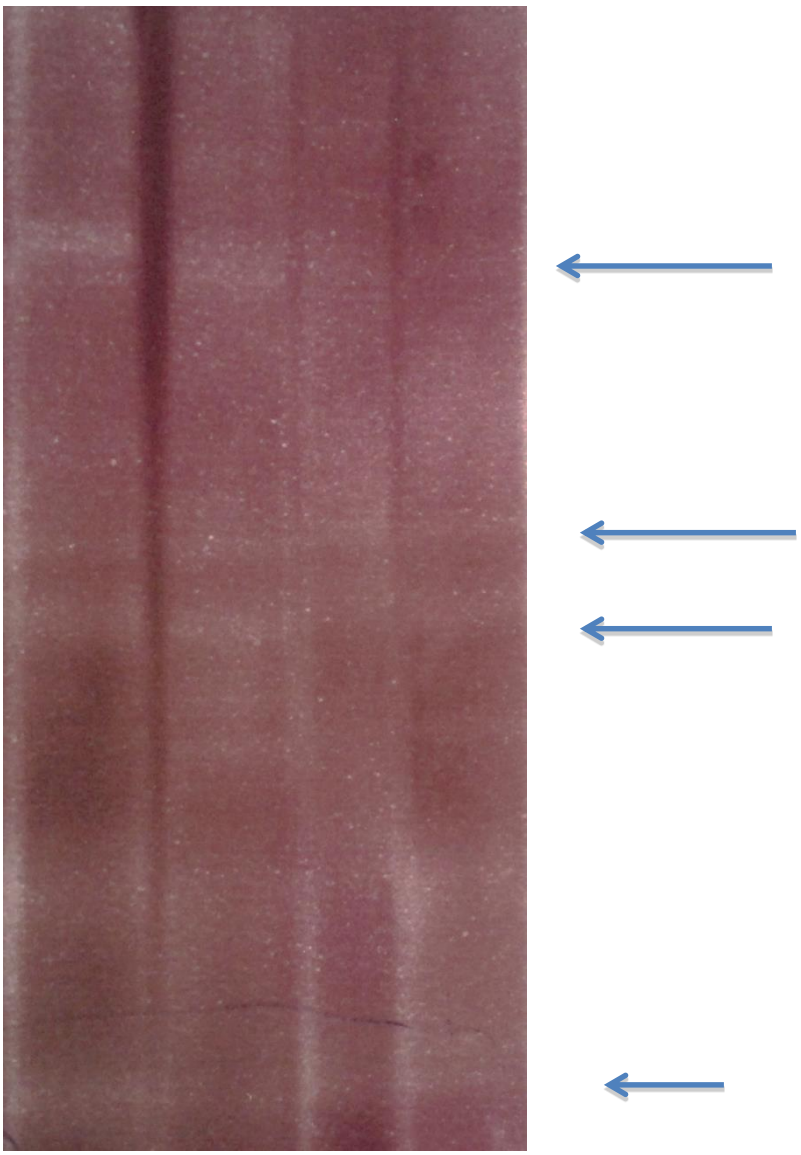


Figure 8.9: IgG_2 antibody reaction of seropositive sera samples against T. phagedenis- like strain T320A. Colour has been inverted to show protein bands more clearly. Lane numbers are listed along the bottom of the NCS picture. Bands of interest at ~55kDa, 12-14 kDa, 30-32 kDa and 32-34 kDa can be seen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



1 *2* *3* *4*

5. Industry messages and discussion

5.1. 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 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.

5.2. 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.

5.3. Immunological response and potential for vaccine development

Most beef cattle from BDD positive farms showed an immunological responses 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, ifB DD 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.

5.4. 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.

5.5. 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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7. Supporting Papers

Please find below all supporting papers for this thesis.

SULLIVAN L. E., CARTER S. D., BLOWEY R., DUNCAN J. S., GROVE-WHITE D. & EVANS N. J. (2013) Digital dermatitis in beef cattle. The Veterinary Record 173, 582

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