Investigating the epidemiology of Bovine Digital Dermatitis: causality, transmission and infection dynamics

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by

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Preface

The research presented in this thesis was conducted at the University of Liverpool as part of a Defra-funded project entitled *Control of digital dermatitis in cattle: understanding transmission and spread of disease*.

The stated objective of this project, which ran from October 2002 to September 2006, was to investigate the origin and route of transmission of pathogenic, bovine digital dermatitis-associated *Treponema* spp. bacteria on UK dairy farms. The project took a multidisciplinary approach, combining epidemiology with microbiology as a means of advancing our understanding of the disease.

A. Epidemiological studies

1. Perform field studies on representative dairy farms in Cheshire, namely a cross-sectional study followed by a longitudinal study.

2. Improve case definition, primarily through investigation of the diagnostic and screening properties of a treponemal serological test (ELISA).

3. Use serology to assess the distribution of antibodies in the farm populations, determine seropositivity rates and estimate the true prevalence of disease.

4. Identify cow-, group- and farm-level risk factors.

5. Formulate and parameterize appropriate statistical and mathematical models of infection dynamics, and use these models to explore putative control strategies.

B. Microbiological studies

1. Further develop and refine techniques for culture and isolation of *Treponema* spp. from the dairy farm environment.

2. Identify and characterize these and other bacterial species isolated from bovine digital dermatitis lesions.

3. Perform phylogenetic analysis to identify spirochaete genotypes associated with the disease, and determine prevalence of pathogenic genotypes in relation to severity.

4. Identify the source(s) of *Treponema* spp. in the farm environment, including which cow tissues harbour these bacteria in normal and diseased cows.

The outputs of the epidemiological studies will be presented in this thesis:

- The *first chapter* consists of a contextual literature review, with specific emphasis on the epidemiology of the disease, and a discussion of putative causal mechanisms of BDD.

- A discussion of the molecular epidemiology in the *second chapter* elucidates the disease determinants associated with bovine digital dermatitis.

- The *third chapter* focuses on detection of disease on the individual animal level through investigation of various diagnostic protocols for case definition, including the development of a *Treponema* spp. ELISA and formulation of a Bayesian model which allows inferences about the predictive probability of infection to be made on the basis of a serological result.

- Exploratory data analysis and statistical modelling of the cross-sectional study dataset (*fourth chapter*) enables investigation of group- and herd-level disease distribution and prevalence, and association of BDD with various risk factors.

- The *fifth chapter* explores temporal trends in BDD and investigates the transmission dynamics in more detail, through exploratory data analysis and statistical modelling of the longitudinal study dataset, including age and lactation stage effects.

- Work on development of a mathematical simulation model, which was formulated and parameterised using the outputs of our field studies, is presented in the *sixth chapter*.

- The *final chapter* reviews and summarizes the findings and contextualizes them to give an oversight of our current understanding and hypotheses.

A note on nomenclature

Historically, the terminology for the description of bovine digital dermatitis has been inconsistent, misleading or even incorrect. I consider Mortellaro's Disease to be non-descriptive; papillomatous digital dermatitis (PDD) is misleading in that a viral aetiology has been excluded and the clinical disease is as often erosive as it is proliferative; and digital dermatitis (DD) is insufficiently accurate in that it does not differentiate from the emerging ovine form of the disease (which is now most commonly referred to a Contagious Ovine Digital Dermatitis, CODD). I therefore consider Bovine Digital Dermatitis (BDD) to be the most accurate term; this nomenclature has been consistently utilized throughout this thesis.

Abstract

Bovine digital dermatitis (BDD) is a painful infectious condition of cattle, which is currently considered to be the leading infectious cause of lameness; hence, it has serious economic and welfare implications. BDD is particularly prevalent in loose-housed Holstein-Friesian dairy cows, where herd prevalences of 30 to 40% are common. The factors precipitating its emergence in 1974 have yet to be clarified; it now has a worldwide distribution. Since the first diagnosis of BDD in the UK in 1987, over 70% of dairy farms are believed to have become infected.

The causal mechanisms of BDD are not well understood. Research progress has been impeded by the multifactorial and polymicrobial nature of the disease. Control measures are based on empirical experience; hence, effectiveness is variable. Fundamental understanding of the aetiology, farm-level distribution of infection and transmission dynamics is required for development of practical and effective intervention strategies.

Multiple species of Gram negative bacteria have been associated with BDD; *Treponema* spp. bacteria are believed to be the aetiological 'necessary cause'. We investigated the molecular epidemiology of BDD-associated *Treponema* spp. by selective culture of lesion tissue biopsies from nine farms in north west UK, yielding 23 isolates. Subsequently, we performed phylogenetic analysis of the 16S rRNA and flagellin genes, assessment of enzyme activity patterns and investigation of serologic reactivity. All microbiological techniques identified clustering of the isolates into three species-level phylotypes. On the farm level, our findings show a large degree of diversity in the treponeme population. On farms where more than one biopsy was taken, multiple species were frequently isolated. On the animal level, mixed infections with two *Treponema* spp. were identified in two cases. Serological evidence for the existence of three serogroups was found, although it was not possible to clearly distinguish between these.

This work led to the development of an ameliorated ELISA test, which was used for routine serological screening in subsequent observational studies. Validation of this ELISA was performed in the absence of a 'Gold Standard' test. Applying Bayesian statistics, a model was formulated which defined infection as a latent variable, and defined disease as presence of BDD lesions. The model did not impose a cut-off but rather estimated the probability of infection given an ELISA test result. Observation

of clinical BDD was performed using a modified borescope, which negated having to lift the feet. The agreement between observation with the borescope and the corresponding lesion inspection of the lifted feet, as determined using Cohen's kappa statistic, was excellent.

A cross-sectional study was performed on eight representative dairy farms in north western England and north Wales, which were stratified into high prevalence, medium prevalence, low prevalence and BDD-free farms. Blood samples were taken for serology of all animals present on the farms on the date of sampling (n=2215); the feet of a randomly selected subset of animals (n=609) were inspected for BDD lesions. Environmental and animal hygiene scores were recorded; management and husbandry practices were recorded by interview, and data on animal records were collected. Appropriate statistical analysis was performed to describe within-farm disease distribution and investigate risk factors for clinical BDD and serology. Almost all animals with clinical BDD belonged to the cow groups. There was substantial inter-farm variability of clinical prevalence in these cow groups, ranging from 0% to 67%, with an overall prevalence of 41%. Higher prevalence farms also showed a higher morbidity of acute lesions. The highest prevalence was observed in animals between 3 and 5 years of age; prevalence decreased with increasing age. Serologically, there was evidence of a bimodal frequency distribution; clinical positives had significantly higher titres than clinical negatives, although there was a large degree of overlap. Titres were low in the young stock groups, and rose significantly in the cow groups; they remained high with increasing age. Body hygiene score was a significant risk factor for clinical BDD; age and body hygiene score were significant risk factors for serology.

A longitudinal study was carried out from February 2004 to December 2005 on four of the cross-sectional study farms. Cohorts of calves, heifers and cows were selected (n=128); these were dynamic, i.e. drop-outs were replaced. Sampling was performed fortnightly during the housing season, and every four weeks during the grazing season (n=1548). At each visit, foot inspection, blood sampling and animal hygiene scoring were carried out. Similar analytic approaches were used as for the cross-sectional study, but with specific emphasis on detection of temporal trends. The incidence of clinical BDD showed a strong seasonal pattern, with a peak between November and January, and lowest incidence in June and July. However, new lesions developed throughout the year. Lesion presentation and development were variable and changed dynamically, and recurrence was common. This complicated investigation of the relationship between the humoral response and clinical disease. First calving heifers did not have a higher incidence of lesions, but the lesions tended to be more severe. Cows older than five years had a higher proportion of regressing lesions. The incidence of lesions increased from the start of lactation to a maximum around the peak of lactation, thereafter decreasing slightly. Associations between clinical disease and serology were comparable to the find-

ings of the cross-sectional study, with the important difference that no seasonal effects were seen for serology. Titres rose after first calving, and with increasing age. Statistical models were formulated to account for the covariance structure and autocorrelation in the data.

A compartmental deterministic mathematical model was developed using a case definition combining clinical inspection and serology. Knowledge of the biology of the disease, derived from the literature, the Bayesian model and the longitudinal study, was used to formulate an appropriate model structure incorporating three infectious states: susceptible, exposed and infected. This model incorporated age- and seasonal effects. Transition parameters, including the transmission parameter β, were estimated or derived from our data.

Considering the growing importance of BDD as a welfare issue, practical and effective measures for control are urgently required. To formulate and test putative interventions, knowledge of the biology of the disease is a fundamental requirement. This knowledge is currently limited. The epidemiological research presented in this thesis concentrated on in-depth, detailed studies to elucidate the farm-level distribution and dynamics of BDD. The combination of microbiological approaches and field-based studies has contributed to an improved science-based understanding of BDD, particularly the aetiology, causal mechanisms and case definition. Although investigation of the effectiveness of interventions was beyond the scope of this project, recommendations for key areas of focus are made.

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Chapter 1

Bovine digital dermatitis (BDD) in context

1.1 Cattle lameness in the United Kingdom dairy industry

1.1.1 Lameness trends: the nature and magnitude of the problem

Lameness is defined as a departure from the normal locomotion, causing an observable deviation in gait. While lameness is usually best observed when the cow is in motion, it is frequently apparent when the cow is standing still, as she often lifts the affected foot and is reluctant to move (Whay, 1997). Clinical lameness in cattle is a manifestation of pain, weakness, deformity, or musculoskeletal defect. It can be attributed to a multitude of causes, and in most cases is the end result of a complex interaction of anatomical, physiological, biomechanical, genetic and management-related factors. The causes of lameness can be classified as infectious and non-infectious; bovine digital dermatitis (BDD) is currently considered to be the most important infectious cause of lameness (Laven, 2003).

Reliable nationwide data on the incidence of lameness in the UK are limited but there is a consensus that it has increased over the past 40 years, coinciding with fundamental changes in dairy cattle production including breed, size, nutrition, productivity and housing (Mill and Ward, 1994; Clarkson et al., 1996; FAWC, 1997).

Estimates of the mean annual lameness incidence in the UK have varied widely - this may partly be explained by dissimilar study designs (Hirst et al., 2002). Different sources of data have been used in various studies, including records from veterinarians, farmers and / or foot trimmers (Clarkson et al., 1996). The degree of training and awareness of the observer may have a significant effect, leading to inconsistencies in observational reporting (Mill and Ward, 1994). It is difficult to estimate the magnitude of this observer bias, and this may ultimately make direct comparison between different studies impossible.

The apparent increase in lameness incidence is however evident in various studies.

A 1980 study of cases 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 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. Collick et al. (1989) described an annual incidence of 31% on 17 farms in Somerset, based on a veterinary surgeon collecting individual cow health records. 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 incidence of 54.6% (Clarkson et al., 1996). Also on the basis of farmers' and veterinarians' records for 63 dairy herds, Esslemont and Spincer (1993) found a mean annual incidence of 35.6%. Esslemont and Kossaibati (1996) found an annual incidence of 24.0% in 90 herds; they used computer-based cow records from over 150 herds, and determined an annual average incidence of 38.2%.

There is a greater degree of agreement between these studies regarding the location and nature of the lesions associated with episodes of lameness. Eddy and Scott (1980) found that 92% of lameness occurred in the foot, compared with 84% by Russell et al. (1982) and 92% by Murray et al. (1996). The most common claw horn lesions were white line disease and sole ulcers; foul in the foot was the most common infectious condition in the first two studies, but BDD, which emerged subsequently, had a far higher incidence in the third study. Regional differences and seasonal effects were observed, with the incidence of lameness being highest in winter (Eddy and Scott, 1980; Whitaker et al., 1983; Clarkson et al., 1996).

1.1.2 Welfare aspects

The advent of farm assurance schemes and the increased interest in farm animal welfare have prompted a need for quantitative assessments of welfare. Such assessments have frequently been based on an evaluation of the condition and dimensions of the farm facilities, combined with provision of husbandry and management (Wood et al., 1998; Regula et al., 2004; Cook et al., 2005; Zurbrigg et al., 2005). This has the advantage of being relatively objective and repeatable. Measurement of the welfare of an animal by direct observation is inherently more difficult as it involves all interactions between a cow and the sum of the elements constituting her environment; this multiplicity of factors complicates its inclusion into any system of objective, integrated assessment (Waiblinger et al., 2001). However, it is ultimately at the animal level that welfare is relevant, and animal-based observations should provide a more direct assessment of it (Whay et al., 2003a). Webster (2001) stated that "The welfare of a farm animal depends on its ability to sustain fitness and avoid suffering. The responsibility of the farmer is to make provision for good welfare through good husbandry; he cannot

ensure good welfare." Adequate foot care is an essential element of "good husbandry" (Toussaint Raven, 1985).

The Farm Animal Welfare Council (FAWC, 1993, 2001) defined a framework for a welfare code based on 'Five Freedoms', assessing welfare in terms of an animal's state of nutrition, comfort, health, temperament and behaviour. The identification and scoring of lameness is important in this context (Whay, 2002). The avoidance of full weight-bearing on one or more limbs, as an expression of pain and discomfort, indicates suffering. Signs of pain include depression, inappetence, weight loss, milk yield drop, rigid and abnormal posture, increased reactivity and evasive behaviour (Whay, 1997; Blowey, 1998). Other impacts on behaviour include restlessness, prolonged lying times, reduced feeding times and later entry into the milking parlour (Hassall et al., 1993; Singh et al., 1993; Galindo et al., 2000; Galindo and Broom, 2002). By restricting mobility, lameness reduces the physical and social interactivity between the cow and her environment. Each of these parameters directly compromises the cow's welfare, and lameness must be considered a leading welfare concern (FAWC, 1997).

Whay et al. (2003b) developed a protocol of animal-based measurements to assess the welfare of dairy cows, founded on the 'Five Freedoms'. Using a Delphi technique, they gathered consensual opinions of animal welfare experts on the most appropriate measures for assessing farm animal welfare. The results suggested measures based upon observations of health status, behaviour, and examination of records; the highest ranking of importance was given to observation of lameness in dairy cattle. Whay et al. (2003a) subsequently applied this protocol to establish a profile of the welfare on 53 dairy farms in England, which constitutes the largest and most recent independently observed assessment carried out in the UK. When the welfare assessment had been compiled it was returned to the same panel of experts who had originally been consulted, as well as to 166 veterinary cattle practitioners. Over 75% of the respondents considered that action should be taken on over 80% of the farms to reduce the prevalence of lameness, overgrown claws, swollen and ulcerated hocks and non-hock injuries. The farmers' recording of lameness cases and treatments was intermittent and their estimates of lameness incidence were higher than that shown in their records; this implies that either some treated cases were not recorded, or some observed cases were not treated, or both. The mean annual incidence estimated by these farmers was 22 cases per 100 cows per year, which is less than half the 50.6 cases per 100 cows per year reported by Clarkson et al. (1996). The farmers' mean estimate of the prevalence of lameness at the time of the visit was 5 – 7%, but the mean prevalence recorded by the first author was 21 – 22% (Whay et al., 2002), which is very similar to the 21% reported by Clarkson et al. (1996). The authors concluded that the prevalence of lameness has not decreased compared to earlier studies and that the farmers failed to identify 75% of cases.

Earlier studies indicated that lameness is treated more frequently by farmers or stockmen than by veterinarians (Whitaker et al., 1983; Murray et al., 1996; Whay et al., 2002); moreover, treatment is often deferred, so that acute cases become chronic, or are not treated at all. A study evaluating the relationship between the lameness prevalence on 15 farms and an assessment of the level of the farmers' knowledge, training and awareness found a significant inverse correlation (Mill and Ward, 1994). Sixty percent of the farmers underestimated the lameness prevalence; none overestimated it, thus indicating that the severity and extent of the problem is frequently underrated. Also, 20% of respondents never called a veterinary practitioner to treat cases of lameness in their herds, 60% sometimes called a veterinary practitioner and 20% always called a veterinary practitioner. The conclusion that lameness is an under-perceived problem has been confirmed by Whay et al. (2002), who inferred that this could be a result of a lack of knowledge or training, a desensitization brought about by constant confrontation with lame cows, or a reluctance of farmers to admit the extent of the problem.

A possible explanation for UK dairy farmers' perceived neglect of care and inadequate response to cattle lameness could be the low milk prices, which have been falling across the EU for a number of years; as of June 2006, the reported EU average milk price was at its lowest since the Euro was introduced in January 2001. UK producers receive among the lowest average prices within the EU; the milk prices are currently below the cost of production for most farmers (MDC Datum, http://www.mdcdatum.org.uk). This may constrain the inputs required to address lameness problems on the farm level.

While lameness in dairy cattle is now a recognized welfare concern, the interest and involvement of the veterinary profession in cattle lameness is relatively limited. This has been ascribed to the time-consuming and dirty nature of the work; a general lack of time, interest and experience; inadequate equipment; and the associated costs for the farmers (Weaver, 2000; Manske, 2002).

1.1.3 Economic impacts of cattle lameness

The economic losses attributable to lameness include the following (FAWC, 1997; Greenough and Weaver, 1997; Booth et al., 2004):

- Reduced feed intake, possibly due to unwillingness or inability to compete for feed, resulting in weight loss;
- Reduction in milk yield as a consequence of reduced feed intake;
- Reproductive problems, due to incomplete manifestation of oestrus behaviour, resulting in more services per conception and an extended calving interval;
- Veterinary costs of treatments (including labour and medication);

- Discarded milk due to antibiotic residues;
- The farmer or herdsman's time;
- Premature culling of chronically lame or frequently recurrently lame cows;
- Increased susceptibility of lame cows to other diseases;
- Costs of purchase of replacement animals;
- Costs of prevention and control, e.g. footbathing or increased frequency of foot trimming.

The economic cost of lameness appears to follow the apparent trend of increasing incidence. In 1981, this cost was estimated to be £15.5 million (Baggott and Russell, 1981). Whitaker et al. (1983) concluded that the average annual cost in a 100 cow herd was £1175 with an overall cost to British farmers of more than £35 million. Booth (1989) estimated the annual cost to the industry was £44 million, but Esslemont (1990) put it as high as £89.2 million. Using reported rates of disease incidence and applying herd health economic indices, Kossaibati and Esslemont (1997) ascribed an average annual cost of £1715 per 100 cow herd to lameness, which accounted for 27% of all economic losses due to common dairy cow diseases. These estimates are as reported in the original publications, and have not been adjusted for inflation; more recent estimates are not available.

Whitaker et al. (1983) found that 1.4% of dairy cows were culled because of lameness, with a range from 0% to 5% on individual farms. A similar rate of 1.2% was determined by Collick et al. (1989). Later, Esslemont and Kossaibati (1997) gave a figure of 5.6%. Booth et al. (2004) found that for all lameness diagnoses combined, survival decreased for those cows becoming lame during the first half of lactation, with a hazard ratio of up to two times that of a sound cow.

1.2 Bovine digital dermatitis (BDD)

1.2.1 Emergence and spread

BDD appears to be a true emerging disease, rather than a re-emerging one. Its rapid spread both within infected farms and between countries attests to its highly contagious nature. Furthermore, as BDD is a highly painful condition (Blowey, 1992; Brizzi, 1993; Murray et al., 2002), it has obvious welfare implications.

It was first described in Italy (Cheli and Mortellaro, 1974). The Netherlands was the second European country to report BDD (Cornelisse et al., 1981). It has since been identified in most European countries (Kyllar et al., 1985; Gunther et al., 1988; Roztocil et al., 1988; Bassett et al., 1990; Axberg, 1991; Gourreau et al., 1992; Koniarova et al., 1993; Zemljic and Trenti, 1994). It was first reported in the UK

by Blowey (1987) and was first documented in the US by Rebhun et al. (1980). It has spread worldwide, with reports from countries as diverse as Argentina (Rutter, 1989), Australia (McLennan and McKenzie, 1996), Brazil (Borges et al., 1992), Canada (Hanna et al., 1994), Chile (Rodriguez-Lainz et al., 1998), Iran (Nowrouzian, 1994), Israel, Japan (Kimura et al., 1993), Mexico (Argáez Rodríguez et al., 1997), South Africa (Van Amstel et al., 1995), and Turkey (Demirkan, 1997).

1.2.2 Clinical diagnosis

Weaver et al. (1981) define BDD as a diffuse or circumscribed superficial epidermitis of the digit at the coronary margin. Affected cows are often severely lame and may walk on their toes (Blowey and Sharp, 1988; Read and Walker, 1998). They sometimes shake the foot presenting the lesion or shift their weight from one foot to another (Bassett et al., 1990). However, in many cases the presence of a lesion is not accompanied by obvious lameness.

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; Cornelisse et al., 1981; Blowey and Sharp, 1988; Brentrup and Adams, 1990; Read et al., 1992; Kimura et al., 1993; Sauvageau et al., 1994; Sheldon, 1994; Mortellaro et al., 1985). 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). Lesions have also been described in the interdigital space and around the dew claws (Döpfer and Willemen, 1998). Approximately 80-90% of lesions occur in the hind feet. Many 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 all affected cows presented lesions bilaterally.

The diameter of the lesions varies in size from <1 cm to >6 cm. They are frequently seen as an irregular circular area, but the shape is variable depending on location.

The presentation of the lesions changes during their development and regression, and is therefore a useful marker to describe the stage of the infection (Figure 1.1):

- *Erosive*. Initially, hyperaemia and swelling with wet eczema and matting of superficial hairs of the affected digital skin is seen (Blowey, 1993; Mortellaro et al., 1985). This develops into an erosive dermatitis. The surface is usually flat or lower than the epithelial level, although some investigators report an elevated level of altered tissue (Döpfer and Willemen, 1998; Read and Walker, 1998); the lesion is red, moist and bleeds easily (Walker, 1995). These lesions are usually small, but intensely painful.

- *Granulomatous.* Granulation is marked by an ingrowth of keratin pins on the surface of the erosions, giving the lesions a stippled ('strawberry-like') appearance (Toussaint Raven, 1989). As keratinization progresses, the lesions take on a more greyish colour, and become raised above the epithelial level; their diameter typically increases. They are less prone to bleeding, and are commonly covered by grey, adherent debris (Blowey and Sharp, 1988; Blowey, 1993). Lesions have a characteristic pungent smell.

- *Proliferative.* Characterized by progressive hyperkeratosis, the keratin filaments proliferate and may become several centimetres long. Granulation at the margins of the lesions is more solid, presenting as a dense, paler border. The lesions become more solid and papillomatous; they may become very prominent. In many cases typical, long hairs grow around the lesion.

- *Regressing.* When lesions are treated, they regress into a dark, rubbery, firm scab (Döpfer and Willemen, 1998). Depending on the duration of this lesion, this scab may be solid or have ragged fronds which eventually sloughs off. Depending on the stage of the lesion prior to treatment, the skin underneath can be smooth, show a remnant scar of the lesion, or even be hyperkeratotic. This may suggest that regression is incomplete / superficial, in which case reactivation of lesions may occur.

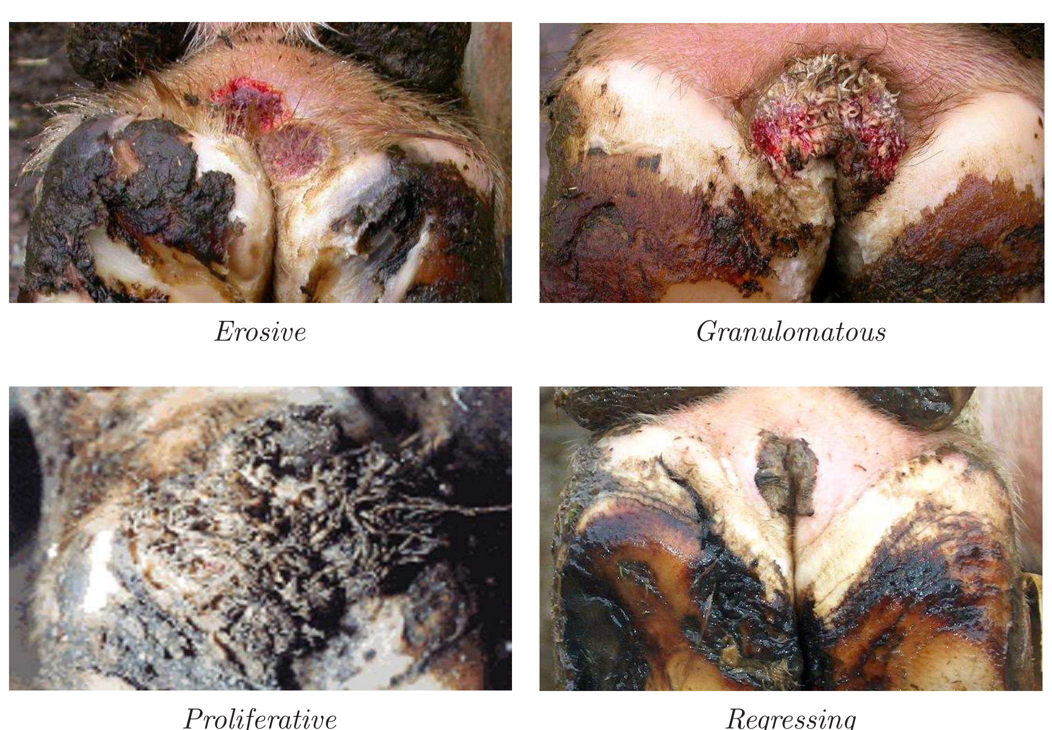


Figure 1.1: Different presentation BDD lesions; detailed descriptions given in the text

There is a substantial degree of variability in presentation. New lesions may develop immediately after regression; multiple lesions at different stages of development have been observed on the same foot (Döpfer and Willemen, 1998). Geographical variability in clinical presentation has also been noted. In Europe, the clinical expression tends to be relatively mild. In the United States, the course of the disease tends to be more chronic and proliferative, hence the name 'papillomatous digital dermatitis' or 'hairy heel warts'. BDD in the UK appears to occupy the middle ground between these manifestations (Laven, 2003).

1.2.3 Differential diagnosis, aetiology and pathogenesis

Differential diagnosis

The infectious skin conditions of the bovine foot are complex, dynamic polymicrobial processes. It is likely that the bacterial ecology changes in time: this influences the clinical expression, resulting in a range of interrelated symptoms.

Discrete diseases have been defined on the basis of symptomatology and bacterial aetiology (see Table 1.1). However, systematic classification and case definition have been impeded by a number of common symptoms associated with this polymicrobial aetiology. Furthermore, the terminology and nomenclature used has been inconsistent and sometimes conflicting. Many lay terms have been coined. This has led to the usage of an extensive array of definitions, both scientific and colloquial. Although Döpfer (1994) highlighted the importance of a reproducible classification system (in the absence of a 'Gold Standard' test) and Döpfer and Willemen (1998) proposed a standardized protocol, most studies reporting on clinical BDD apply an *ad hoc* scoring protocol (e.g. Laven and Hunt (2002); Shearer and Hernandez (2000)), and no internationally recognized classification and nomenclature currently exist. Ultimately, this has made differential diagnosis and comparison of bovine infectious foot conditions problematic.

Using conventional terminology, interdigital dermatitis is the most important differential diagnosis: it shares several clinical characteristics with digital dermatitis such as hyperkeratosis and exudation (Döpfer and Willemen, 1998). It is frequently seen concurrently with BDD. There has been much debate and disagreement on whether digital dermatitis and interdigital dermatitis are in fact the same, with some investigators believing them to be different stages of the same disease (Zemljic, 2002). An association with similar organisms (particularly *Dichelobacter nodosus*) has been suggested (Blowey et al., 1994).

In addition, both are frequently seen in conjunction with heel horn erosion; it is unclear whether this is a related symptom or whether it is an unconnected condition – and if the latter, whether it is infectious or not.

Site	*Disease*	*Synonyms*
Digital	Bovine digital dermatitis	Mortellaro's disease
		Papillomatous digital dermatitis
		Digital papillomatosis
		Strawberry foot rot
		Raspberry heel
		Hairy heel warts / foot warts
		Verrucose dermatitis
	Heel horn erosion	Slurry heel
Interdigital	Interdigital dermatitis	Stable footrot
		Scald
	Interdigital phlegmon	Interdigital necrobacillosis
		Interdigital pododermatitis
		Foul-in-the-foot
		Footrot
		Hoof-rot
		Clit-ill
		Peracute foul / super foul
		Blind foul

Table 1.1: Synonyms used to define gross clinical lesions of the bovine digit associated with lameness

Microbial aetiology

The precise aetiology is complex and has not been well elucidated. The rapid spread of BDD attests to its contagious nature. Bacteria have been consistently identified in histological examination of lesions, and lesions respond well to antimicrobial agents, regressing rapidly (Read et al., 1992). It is therefore highly likely to include a bacterial component as a 'necessary' cause (see 1.3).

Histological examination of lesions shows a wide spectrum of bacterial morphotypes, and it is probable that BDD is a polymicrobial disease process (Edwards et al., 2003b). Superficial epidermal debris contains many Gram-negative bacterial species (Choi et al., 1997; Moter et al., 1998), the aetiological significance of which has yet to be established. A consistent finding is a profusion of spirochaetes in the deeper layers of the dermis; they may be the predominant bacterial morphotype (Blowey and Sharp, 1988; Read et al., 1992; Scavia et al., 1994; Grund et al., 1995; Demirkan et al., 1998). These appear to be associated with necrotic changes in the lesions, which suggests that they may be implicitly implicated in the infection. These spirochaetes have been identified using electron microscopy to be *Treponema* spp. Phylogenetic analysis has

indicated close relatedness with the human oral spirochaetes *T. phagedenis*, *T. denticola* and *T. vincentii* (Rijpkema et al., 1997; Choi et al., 1997; Demirkan et al., 1998; Edwards et al., 2003; Trott et al., 2003), or divergence from other known treponemes (Choi et al., 1997; Schrank et al., 1999). Culture of these organisms has been problematic due to their fastidious anaerobic nature, and was first successfully performed by Walker et al. (1995).

Due to the difficulties of bacterial isolation and culture, nothing is known of the distribution of the BDD-associated *Treponema* spp. in the farm environment. It is possible that transmission of infection is achieved by dissipation of infectious material from lesions into the environment, thus infecting other animals by indirect contact. However, the fastidious anaerobic nature makes persistence of a population of free-living environmental treponemes unlikely. If the reservoir is not primarily environmental, it follows that it must be an animal-related source. Treponemes make up part of the symbiotic ruminal flora (Ziolecki, 1979; Ziolecki and Wojciechowicz, 1980; Paster and Canale-Parola, 1982; Stanton and Canale-Parola, 1979), so a gastrointestinal reservoir is a possibility. A Japanese group have provided evidence to this effect (Shibahara et al., 2000, 2002), but their results have not been replicated or confirmed by other workers. Another possibility is that these bacteria make up part of the epidermal flora, only causing infections when predisposing conditions, as described below, are met. Thirdly, considering their close relatedness with human oral treponemes, a buccal origin is possible. In the absence of such basic knowledge, the origin and transmission dynamics of the infection, i.e. how it is transmitted and propagated, cannot easily be clarified.

Pathogenesis

Considering the close relatedness of the BDD associated treponemes with human oral treponemes, an interesting comparison can be made between the bovine infectious foot diseases and human oral periodontal disease. Both are collective terms for describing a number of different conditions; both are complex and multifactorial, with no single bacterial species responsible for the initiation or progression of disease (Edwards et al., 2003b). Periodontitis in humans is preceded by, and develops from, a mild non-specific bacterial inflammation of the gums (gingivitis). This facilitates the formation of periodontal pockets, within which a shift in bacterial ecology takes place to become progressively more anaerobic. This favours growth of proteolytic Gram-negative microorganisms including *Treponema* spp. The epithelial degeneration and necrosis caused by these bacteria is aggravated by the host immune response, which is manifested by infiltration of neutrophils and release of inflammatory mediators and cytokines. Without therapeutic intervention, this process continues unabated.

By analogy, BDD may be preceded by a mild bacterial epidermitis. This is bio-

logically plausible as the natural environment surrounding the bovine foot contains a large number of micro-organisms; in conjunction with other risk factors which cause microtrauma to the skin (see 1.2.4), treponemes may subsequently penetrate the deeper epithelial strata. It is likely that the characteristic lesions develop secondarily to these initiating factors; experimental intracutaneous inoculation of suspended lesion debris in healthy digital skin failed to induce the disease (Rebhun et al., 1980; Bassett et al., 1990), or only accomplished this after scarifying the skin and keeping it moist (Read and Walker, 1996).

Histologically, BDD lesions are characterized by superficial loss of keratin and damage of keratinocytes, inducing reactive epidermal proliferation, including the production of elastic filaments (Peterse et al., 1992). Production of a keratolytic toxin by the treponemes has been proposed (Blowey et al., 1994); as a compensatory mechanism, epidermal proliferation and hyperplasia takes place. There is a large influx of monocytes, neutrophils and lymphocytes (Bassett et al., 1990; Brizzi, 1993; Dopfer and Willemen, 1998; Dopfer et al., 1997; Edwards et al., 2003; Borgmann et al., 1996; Leist et al., 1998). In advanced cases, the epidermis is completely destroyed, and the dermis is invaded (Blowey et al., 1994).

1.2.4 Epidemiology

Introduction onto farms, spread and morbidity

The infection may be introduced onto previously disease-free farms through a breach in biosecurity. Buying in stock seems to be particularly important; quarantine and treatment are seldom practised and even though the purchased animals may not present with clinical lesions, an epidemic outbreak may occur some weeks later. Cross-contamination is another possibility: veterinarians and foot trimmers who visit multiple farms without cleaning and disinfecting their materials and instruments have been implicated in spreading the infection (Wells et al., 1997; Losinger, 2006). In a naive herd, the disease initially spreads rapidly throughout the herd: there is a high incidence of acute lesions. All cattle, irrespective of age, are susceptible to contracting BDD, although the lactating and dry cow groups are usually the worst affected. 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. These may be related to the introduction of new stock.

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

The disease has spread remarkably rapidly throughout the UK since first being reported in 1987; however, there is a paucity of reliable epidemiological data. From

1989 to 1992, Murray et al. (1996) studied lameness on 37 dairy farms in four regions of England and Wales; BDD was the primary cause of lameness, accounting for 8% of all cases, and was the most commonly observed lesion affecting the skin. Ten years later, Laven (2003) suggested there was evidence of increased morbidity, with BDD accounting for approximately 25% of all lameness cases in dairy cattle, and over 70% of dairy herds being infected. Data collected from 53 dairy farms by Whay et al. (2002) showed that BDD occurred on 39 farms, and affected farms had a significantly higher lameness prevalence than those without. As part of a longitudinal study on the effect of biotin supplementation on the incidence of lameness, Hedges et al. (2001) reported that BDD was the third most common cause of lameness, after sole ulcer and white line separation, with an annual incidence rate per 100 cows of 12.

In a cross-sectional study undertaken by in the Netherlands by Frankena et al. (1991), BDD was found to have a prevalence of 13.8% at the cow level. The between herd variation was large, suggesting that risk factors have a large influence on disease prevalence. Another observational study in the Netherlands (Somers et al., 2003) showed that all study herds housed on concrete floors had BDD problems with an average cow prevalence of 30%; they conclude that the morbidity of the disease had increased substantially. Later, Holzhauer et al. (2006) performed another cross-sectional study and reported a study population prevalence of 21.2%; the herd prevalence ranged from 0 to 83%. Only 9% of the 383 herds investigated were unaffected. As the study design was similar to that of Frankena et al. (1991), they concluded that BDD prevalence appeared to have truly increased in the intervening period; they suggested this might be associated with prolonged housing periods.

A large-scale population-based cross-sectional survey representing 79% of US dairy cows used on-farm questionnaires administered by veterinary medical officers or animal-health technicians to dairy managers; in the previous 12 months, BDD had occurred in dairy cows on 43.5% of all US dairy herds (Wells et al., 1999).

An observational study from Chile (Rodriguez-Lainz et al., 1998) found that 70% of 119 dairy managers responding to a questionnaire had observed BDD on their farms. Subsequently, a screening process was carried out on 43 randomly chosen farms, where cows' feet were inspected for lesions at milking. 91% of these farms were found to be infected, with a median prevalence for milking cows of 6.1%.

Seasonality

A seasonal effect has commonly been reported, with peak morbidity during the housing period – this is probably associated with poor hygiene, poor cubicle design, and over-crowded conditions within the building (Blowey and Sharp, 1988; Blowey, 1993; Nutter and Moffitt, 1990; Peterse et al., 1992).

Frankena et al. (1991) found a population prevalence was 8.1% during the grazing period and 13.8% during the housing period. Somers et al. (2005) reported a slightly higher prevalence during the housing period (28.5%) compared to the pasture period (27.3%).

Risk factors

BDD is highly multifactorial, and many risk factors have been identified.

Environment-related

- Poor hygiene in the housing environment is considered the most important risk factor. Prolonged contact of the skin with slurry, or moist underfoot conditions, soften the skin barrier; faecal ammonium may further erode the skin. In one case-control study performed in southern California, Rodriguez-Lainz et al. (1996) compared 37 high incidence dairy farms (cases) with 20 low incidence dairy farms (controls); poor hygiene was identified as a significant risk factor.

- The housing system is a risk factor, which might be attributed to poorer hygiene and increased animal contacts in cubicle systems. Loose (cubicle) housing systems are particularly associated with BDD (Laven, 1999); in a Danish study, Capion (2004) reported that the prevalence of Holstein Friesian herds affected with BDD has increased dramatically since a transition in the predominant housing system, from tie-stall systems to loose housing. Within farms, the most affected groups tend to be cubicle housed. Somers et al. (2003) reported that all study herds on concrete flooring were affected by BDD, with a lower prevalence for herds on slatted floors with manure scrapers. In the US, Rodriguez-Lainz et al. (1999) found that loose-housed cows had the highest risk of contracting BDD, followed by cows in free stalls or in open corrals, compared to cows on pasture all year.

- Factors which cause mechanical (micro)trauma to the skin of the feet may facilitate infection. Poor quality walkway surfaces and tracks (i.e. uneven, broken up, too rough or slippery) can cause foot trauma. Prolonged standing times increase strain on the feet, which may facilitate micro-trauma. This may be partly attributed to the housing type: Singh et al. (1993) showed that cows in loose housing systems stand for longer periods than in straw yard systems. The housing quality (poor cow comfort and inappropriate use of the housing) may also lead to prolonged standing times.

Management-related

- Poor biosecurity is considered to be a key risk factor. The BDD infection is thought to have been introduced onto many farms by buying in stock

(Nutter and Moffitt, 1990; Brizzi, 1993; Walker et al., 1995). A large-scale US survey identified the percentage of replacement cows born off the farm as a significant risk factor (Wells et al., 1999). Rodriguez-Lainz et al. (1999) found that farms that bought in heifers in the past 10 years had a threefold increase in the odds of acquiring BDD compared to those on farms that used exclusively home-reared heifers. The emergence of an apparently closely related ovine form of BDD has raised the question whether cross-species transmission is possible.

- Husbandry factors are thought to influence BDD. Social unrest and stress brought about by various causes – too high stocking density, abrupt introduction of heifers into the cow herd, lack of training heifers in cubicle use – results in restlessness and increased standing times.

- Inadequate lameness management, or an under appreciation of its morbidity, is undoubtedly important from a disease and welfare point of view. Factors include poor foot care (infrequent trimming and bad trimming technique), insufficient time spent observing lameness, lack of prompt treatment or failure to consult a veterinarian, ineffective treatments (footbath regimen and individual treatments), and lack of disinfection of foot trimming equipment between cows (and, in the case of professional foot trimmers and veterinarians, between farms) (Wells et al., 1999). In one US study, cows on dairies that used a footbath were found to be less likely to have BDD than those in dairies not using one (Rodriguez-Lainz et al., 1999).

Anatomical, genetic and production-related factors

- It is possible that foot conformation has a bearing on the incidence of digital dermatitis. For instance, feet with a low heel height may have greater contact with slurry, and be predisposed to infection.

- Other genetic parameters may be relevant, for example those influencing growth and production. The Holstein-Friesian breed is more susceptible than other dairy and beef breeds (Rodriguez-Lainz et al., 1999; Frankena et al., 1991). A comparative study of dairy versus beef cattle was conducted over four visits to a slaughterhouse (Brown et al., 2000); the left hind feet of 22 of 76 (29%) dairy cattle and 29 of 739 (4%) beef cattle were found to have gross BDD lesions. Blowey (1998) postulates that increased genetic potential for production has led to high nutritional demands which could compromise immunity, especially during periods of negative energy balance (such as in the first 3 months of lactation); this in turn might increase the susceptibility to incidence of BDD. However, no evidence to support this has been produced.

- The effects of lactation stage and parity are not clear cut. A retrospective study (Argáez Rodríguez et al., 1997) found that 33% of cows were affected during lactation and 1% during the dry period. Of all cows affected in the previous lactation and still present on the farm, 68% of had lesions in their current lactation; the yearly estimated cumulative incidence risk was 35%. The highest risk for BDD occurred during the first month of lactation. A cross-sectional study by Rodriguez-Lainz et al. (1999) included 3,265 cows on 22 farms, which were examined for lesions. First-parity cows had the highest odds of BDD; the odds decreased as parity increased. Odds of BDD increased with increasing days in lactation. Frankena et al. (1991) found that first and second parities were more susceptible to BDD; however, there was a negative correlation between lactation stage and BDD prevalence. Herd size and milk production were also negatively correlated with the disease, but this was possibly attributed to the associated higher level of management.

- Concurrent infectious foot conditions may act as a precursor to infection by compromising the epidermal barrier. Frankena et al. (1991) found that BDD was strongly associated with other conditions, particularly interdigital hyperplasia.

1.2.5 Economic impacts

An accurate calculation of the specific economic cost of BDD to the UK dairy herd is problematic, as many of these costs are hidden and therefore difficult to quantify.

The costs of clinical lameness of combined etiologies (see 1.1.3) have been relatively better documented. However, it is important to differentiate between clinical lameness as a disease complex and BDD, which is just one of several diseases causing clinical lameness. Furthermore, the individual cost of several other conditions causing lameness will exceed that of a case of BDD. Although BDD does not always lead to appreciable lameness, it is likely that BDD has a considerable economic impact, both in terms of production losses, which are frequently not acknowledged, as well as input costs such as veterinary drugs, consultation fees, labour, etc. No specific economic study or reliable cost-benefit study has been performed on BDD in the UK.

Nutter and Moffitt (1990) estimated that BDD accounted for a loss of 57 litres per cow per lactation, which was equivalent to a loss in income of approximately £10 per cow at the then current milk prices. Direct milk loss of affected cows was also reported by Green et al. (2002). Kossaibati and Esslemont (1997) estimated the cost of each recorded case of digital skin disease, 56% of which was BDD-associated, as being £59. However, as a high percentage of cattle are affected but not treated (and therefore not recorded), it is probable that the actual cost of BDD is considerably higher.

Laven (2003) considered that the clinical severity of the disease, which appears to vary geographically, will inevitably influence the nature and effectiveness of treatment

and control programmes, and hence the associated costs. The consequence of this is that the economic impacts of BDD in different countries will not be directly comparable.

In the Netherlands, Enting et al. (1997) calculated the average losses from clinical digital diseases per foot-lame cow to be approximately £75 per year. The incidence in these herds averaged 21%, resulting in a loss of almost £16 per average cow present in the herd. This ranked third after mastitis (£49 per cow) and fertility problems (£41 per cow). However, this study does not consider BDD separately, and Barkema et al. (1994) reported that although BDD was responsible for 21% of lameness in Dutch dairy cows, it did not result in a significant economic loss. In Mexico, Argáez Rodríguez et al. (1997) found no significant differences in milk yield between cows with BDD and those that were unaffected. However, they did show that mean calving to conception intervals of 93 days for healthy cows were significantly lower than the 113 days for lame cows. In an Israeli study of three dairy herds, the culling rate of BDD-affected cows was 8%, the bulk tank milk somatic cell count was doubled and the average milk production loss was 1.7% per animal affected with BDD per day (Yeruham et al., 2000).

1.2.6 Treatment

Despite the high morbidity of BDD, few peer-reviewed studies of the effectiveness of treatments have been published; furthermore, the scope and design of the clinical trials that have been performed were not rigorously controlled. A recent review by Laven and Logue (2006) concisely covers all the available literature.

Spontaneous recovery from BDD seldom occurs (Cornelisse et al., 1981; Kyllar et al., 1985; Peterse et al., 1992). Regular and correct claw trimming is strongly advocated as an adjunct to all treatments (Toussaint Raven, 1989). Surgical removal or debridement of lesions may be attempted, especially for cases with an advanced degree of proliferation (Cornelisse et al., 1981; Mortellaro et al., 1985); if the affected skin is kept clean, epithelization of the lesion occurs with complete healing (Greenough and Weaver, 1997). However, this is seldom feasible under farm conditions. As BDD is widely considered to be bacterial in aetiology, most products used for treatment are antibacterial agents.

Systemic antibiotics

In vitro studies have shown that the microorganisms associated with the disease are susceptible to most antibiotics, and particularly to penicillin. Nevertheless, only a few investigators have found systemic treatments to be effective (Read and Walker, 1998; Rutter et al., 2001); others disagree (Blowey and Sharp, 1988; Guard and Williams, 1995; Britt et al., 1996; Borgmann et al., 1996; Watson, 1999; Laven and Hunt, 2000; Silva et al., 2005). The perceived lack of effectiveness, high cost and associated milk withdrawal times of injectable antibiotics make them an unattractive option for routine

treatment.

Individual topical treatment

Antibiotics Most of these are applied topically per aerosol. One oxytetracycline aerosol is licensed in the UK specifically for the treatment of BDD; other similar aerosols are licensed for topical infections sensitive to oxytetracycline in cattle, and may also be used. These aerosols are widely used, and are generally considered to be effective; repeated application shows better efficacy than a single treatment. There is a large variability in the treatment regimes used by veterinarians in the field: the results have seldom been published and are mostly anecdotal. Other topical antibiotic sprays have been used, including lincomycin (usually in combination with spectinomycin), chloramphenicol (Ledecky et al., 1997) and valnemulin (Laven and Hunt, 2001).

Other treatments include oxytetracycline aerosols combined with products such as gentian violet (Cornelisse et al., 1981; Toussaint Raven, 1989; Nutter and Moffitt, 1990; Brentrup and Adams, 1990; Brizzi and Salomoni, 1993; Suichies et al., 1993). Bandages may also be applied to prolong the contact time of the antibiotic formulation with the lesion; they may be soaked in an antibiotic solution (Britt et al., 1999), or applied in powder form or oily suspension such as used for mastitis dry cow therapy.

It is difficult to objectively ascertain the efficacy of these treatments. Interventions such as precleaning of the foot and contact time with the antibiotic will influence the cure rate. In addition, the efficacy may vary between farms, depending on the presentation of BDD on that farm.

Non-antibiotics Medical concerns regarding antibiotic resistance, environmental contamination, milk withdrawal and cost have prompted investigation into many non-antibiotic treatments for BDD. The organic dairy sector has no other recourse for treatment. However, the published data on the efficacy of alternative treatments are limited.

Non-antibiotic bacteriostatic and bactericidal products used include PVP-iodine, hydrogen peroxide / peroxyacetic acid, glutaraldehyde, acidified sodium chlorite solution, copper sulphate or acidified ionized copper solution. A number of non-antibiotic products are commercially available which are alleged to be beneficial for treating hoof conditions, and may be used for BDD, because they do not fall under medicines legislation; however, the efficacy of these products has not been verified in controlled clinical trials. Various other substances have been used, including antiseptics such as benzalkonium chloride, acidified copper salts, organic acids with 'essential oils', 'effective enzymes' and 'specific trace elements'.

Group topical therapy

Collective treatments can be an effective control measure on the group level; such treatments are advantageous for several reasons:

- they negate the need for individual identification of affected animals, which is time and labour intensive;
- as BDD prevalence increases, specifically in lactating and dry cows, the individual treatments become a time-consuming chore;
- routine group treatments may limit the further growth of small early lesions, which are easily missed by casual foot inspection. Such treatments may also be effective if a preclinical disease state exists, where the animal has been infected but has yet to develop clinical signs. Also, they can prevent acute cases from developing into more chronic, proliferative lesions, which are more resistant to treatment, thereby reducing the force of infection in the group as a whole.

Such treatments are generally administered by footbathing; a practice which has been adopted widely. There is a large variability in design of such footbaths, the most common being of the walk-through variety – sometimes, the animals stand side by side in a footbath for 15 – 20 minutes. The solutions used and the protocols followed are inconstant. These factors influence their efficacy for the control of BDD. Evidence suggests that individual lesions respond better to topical treatment than to footbathing (Nowrouzian and Zareii, 1998). Limitations include:

- cows frequently defecate and urinate as they walk through the bath, which dilutes and inactivates the active ingredients in the solution;
- topping up may be required due to spillage, reducing the concentration;
- the feet, especially of loose-housed cows, tend to be encrusted with faeces, which limits contact of the active ingredients with digital skin. Tandem foot baths have been designed, the first of which contains water and is designed for washing the feet, and the second of which contains the active solution (Roztocil et al., 1988; Blowey and Sharp, 1988; Sheldon, 1994; Nutter and Moffitt, 1990); however, the short duration of walking through does little to remove the faeces;
- the short contact time of the feet with the active ingredients (in walk-through footbaths);
- cost, especially of antibiotic footbaths. As a result, farmers often reduce the concentration of the active ingredient. This has repercussions for possible development of resistance, and the effectiveness is consequently lower than topical administration of the product;

- poor footbathing protocols, such as timing and frequency of treatment. These lead to disappointing results, and discourage the farmer from initiating further disease control measures.

Antibiotic footbaths As with individual antibiotic topical treatments, the use of antibiotic footbaths is widespread, yet under-researched. Different antibiotics have been used (Laven and Logue, 2006; Laven and Proven, 2000; Laven and Hunt, 2001), but no work on comparative efficacy has been performed. The optimal concentrations and intervals between treatment have not been established. The number of cows that can be treated before needing to refresh the solution, due to inactivation, is unknown. Lincomycin / spectinomycin and oxytetracycline seem to be the most commonly used products.

No antibiotic is currently authorized in the EU as a footbath solution for treating BDD. If such a treatment is administered, a statutory milk withdrawal of at least seven days is required, even though research has shown that the systemic bioavailability of antibiotics administered by this route is extremely low, making it unlikely that antibiotic contamination of the milk will occur (Blowey et al., 1994; Hartog et al., 2001; Britt et al., 1999). This implies that the veterinarian must be involved in the decision-making process to prescribe antibiotics for use in footbaths. Concerns related to disposal, environmental contamination, and the implications for bacterial resistance development has led to a recommendation being made to make use of a 'minimal solution' footbath. While use of antibiotic footbaths is legally permitted in the UK, the situation is far from clear; there are no clear guidelines on disposal of the leftover solution.

Non-antibiotic alternatives Products that are used include formalin, copper sulphate, peroxide-based products, peracetic acid, and others such as zinc sulphate and sodium hypochlorite; several 'homespun' alternatives such as slaked lime, milking machine wash water and teat dips / sprays have been used, but no formal research has been performed regarding their efficacy. A foam product (Kovex®) containing peracetic acid, glycerin and a skin toner has been marketed for disinfecting the feet of cattle while they stand in the collecting yard (Laven and Logue, 2006).

Ambiguous results have been published on the use of formalin footbaths for the treatment of BDD. There appears to be a dose-response relationship, with the efficacy of such footbaths depending on the concentration and frequency of use (Blowey and Sharp, 1988; Clark, 1990; Nutter and Moffitt, 1990; Blowey, 2000). However, adverse effects were also observed, which may be ascribed to the painfulness caused by the formalin (Blowey, 1990; Nutter and Moffitt, 1990; Peterse et al., 1992); hence this product is less appealing from a welfare perspective. Another drawback of formalin is its carcinogenic

potential.

Similarly, contradictory reports on the efficacy of copper sulphate in footbaths have been published, with most authors finding them to be ineffective for the control of BDD (Blowey and Sharp, 1988; Rodriguez-Lainz et al., 1996; Nutter and Moffitt, 1990), but others suggesting that they may be effective (Laven and Hunt, 2002). A serious negative aspect of copper sulphate is its environmental toxicity, which necessitates responsible disposal.

A recent trial demonstrated the effectiveness of sodium hypochlorite, when used alone as well as in support of systemic oxytetracycline treatment (Silva et al., 2005).

Anecdotal reports claim that the Kovex® foam product is effective for the treatment of BDD, particularly when used intensively. However, no controlled trials have been published and it cannot be determined whether the product has an active effect, or whether the reduction in BDD is biased by the mechanical effect of frequent cleaning of the feet, or alerting the farmer to the incidence of BDD.

1.3 Causation of BDD

BDD is a relatively poorly understood condition, which is remarkable considering its rapid spread, high morbidity, and economic and welfare impacts. It is a matter for debate whether this should be attributed to the complex multifactorial and polymicrobial nature of the condition, or to the dearth of fundamental disease research that has been performed, or both (Figure 1.2). Whatever the case, the causality of the condition has not been well elaborated, and no attempt has yet been made to systematically apply Hill's criteria of causation (Table 1.2)(Hofler, 2005; Thygesen et al., 2005).

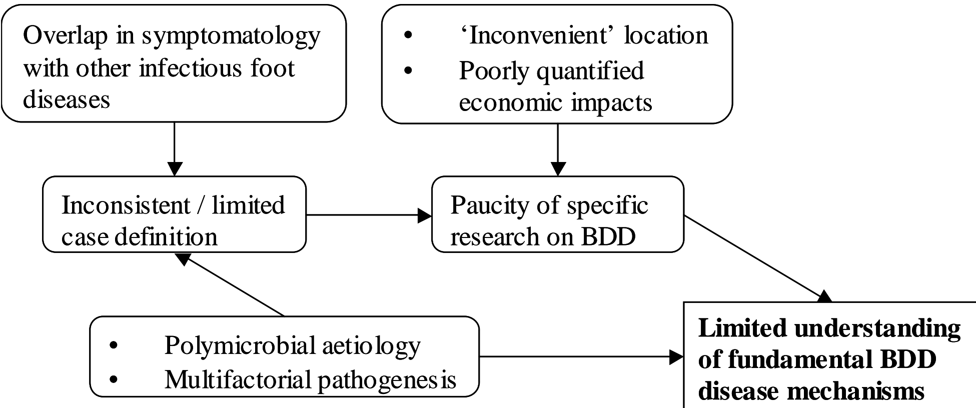


Figure 1.2: Possible reasons for the limited understanding of BDD disease mechanisms

1. *Strength of association.* The stronger the relationship between the independent variable and the dependent variable, the less likely it is that the relationship is due to an extraneous variable.
2. *Temporality.* It is logically necessary for a cause to precede an effect in time.
3. *Consistency.* Multiple observations of an association, with different people under different circumstances and with different measurement instruments, increase the credibility of a finding.
4. *Theoretical plausibility.* It is easier to accept an association as causal when there is a rational and theoretical basis for such a conclusion.
5. *Coherence.* A cause-and-effect interpretation for an association is clearest when it does not conflict with what is known about the variables under study and when there are no plausible competing theories or rival hypotheses.
6. *Specificity in the causes.* In the ideal situation, the effect has only one cause. In other words, showing that an outcome is best predicted by one primary factor adds credibility to a causal claim.
7. *Dose response relationship.* There should be a direct relationship between the risk factor (i.e. the independent variable) and the individual's status on the disease variable (i.e. the dependent variable).
8. *Experimental evidence.* Any related research that is based on experiments will make a causal inference more plausible.
9. *Analogy.* Sometimes a commonly accepted phenomenon in one area can be applied to another area.

Table 1.2: Bradford Hill's criteria of causation

A discussion of the causal mechanisms is of fundamental relevance as it assists us in the formulation of hypotheses regarding aetiology, pathogenesis and transmission. Although such a discussion is by definition largely theoretical, it is an essential precursor to study design, and the subsequent modelling of the intricate and complex relationships between risk factors (Krieger, 1994; Victora et al., 1997; Dohoo et al., 2003), including the definition of variables of interest, and identifying possible confounders and sources of interaction.

Amalgamating the information presented in this review, it is possible to classify the plausible risk factors and causative mechanisms of BDD (see 1.2.4) in accordance with the component — cause model, a conceptual framework originally described in the seminal paper of Rothman (1976) which was developed to provide "an intuitive base for causal thinking". Each risk factor is regarded as a cause, which contributes to bringing about an eventual effect, which in this case is BDD. For a multifactorial condition such as BDD, no single cause is likely to bring about this effect; rather, a complex of causes is involved. These causes are defined as components. A minimum set of component causes which inevitably brings about disease is defined as *sufficient*; this implies that the lack of any component cause renders the remaining component causes insufficient. The effect may be brought about by a variety of sufficient causes, which may or may not have component causes in common. A component cause which is part of every sufficient cause is defined as a *necessary* cause.

A discussion of causality obviously implies an understanding of the individual component causes, i.e. biological determinants, disease mechanisms and management practices. As noted above, in the case of BDD this is incomplete or speculative; this necessitates making assumptions, which may result in spurious hypotheses. The factors in 1.2.4 can be used to define a set of sufficient causes; three examples are given in Figure 1.3. In analogy with the example given by (Rothman, 1976), five component causes are included in each sufficient cause; this is arbitrary and has not been determined. The body of evidence identifying exposure to *Treponema* spp. as the necessary cause is, however, growing.

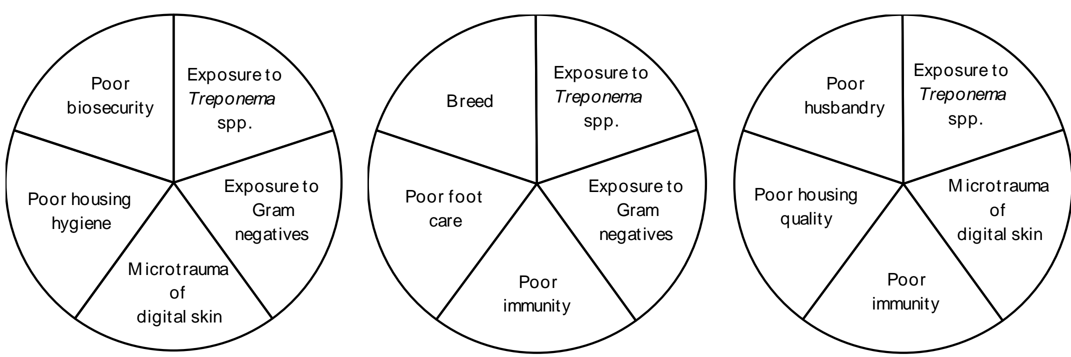


Figure 1.3: A conceptual scheme of three putative 'sufficient causes' for BDD, with exposure to *Treponema* spp. as the 'necessary cause'. The 'sufficient causes' contain component causes thought to be associated with BDD; they are hypothetical and have been included for illustration only

The limitation of this exercise is that it is difficult to determine with certainty the minimum combination(s) of component causes which will bring about disease (i.e. the permutations making up the sufficient causes), and quantify the relative contribution of each component cause to the development of disease. Besides, it does not explicitly allow for hierarchical structures and temporal sequences or ordering of the causes.

A more effective way of conceptualizing the mechanisms which exert an influence on the causation of disease is to construct a causal web (Dohoo et al., 2003). Causal mechanisms can be integrated into a framework which reflects the hierarchical relationships between them (Victora et al., 1997). They may contribute directly to the development of disease (referred to as *direct* causes), or exert an *indirect* (or *distal*) effect; the more distal the determinant, the smaller the effect it can be assumed to exert. The most proximate causes are therefore physiological in nature, and more distal effects are exerted by relevant management (and even sectoral) factors. An additional advantage of a causal web is that it readily lends itself to application of multivariable statistical techniques, in this case hierarchical or multilevel analysis (Krieger, 1994). The current

state of knowledge is summarized in Figure 1.4. Note that, while most of the component causes from Figure 1.3 are included, the causal web has an implicit hierarchical structure and exhibits a greater degree of complexity in the interrelationships between factors. Repeated exposure to BDD appears to engender a degree of resistance, which has been included as a protective effect.

As mentioned previously, an inherent problem with BDD investigation is that the proximate determinants of infection (i.e. the physiological factors) have not yet been ascertained with certainty, and are difficult quantify. Thus the variables that can be observed or measured are mostly indirect in nature, whether on the animal level (e.g. foot hygiene score and body hygiene score), group level (e.g. housing hygiene and comfort) or farm level (e.g. factors relating to foot care, BDD management and purchase of stock). Obviously, inclusion of the physiological factors would be highly desirable; the development of diagnostic tools to facilitate measurement of these physiological factors (and hence assist epidemiological study of the condition) is a priority for future research.

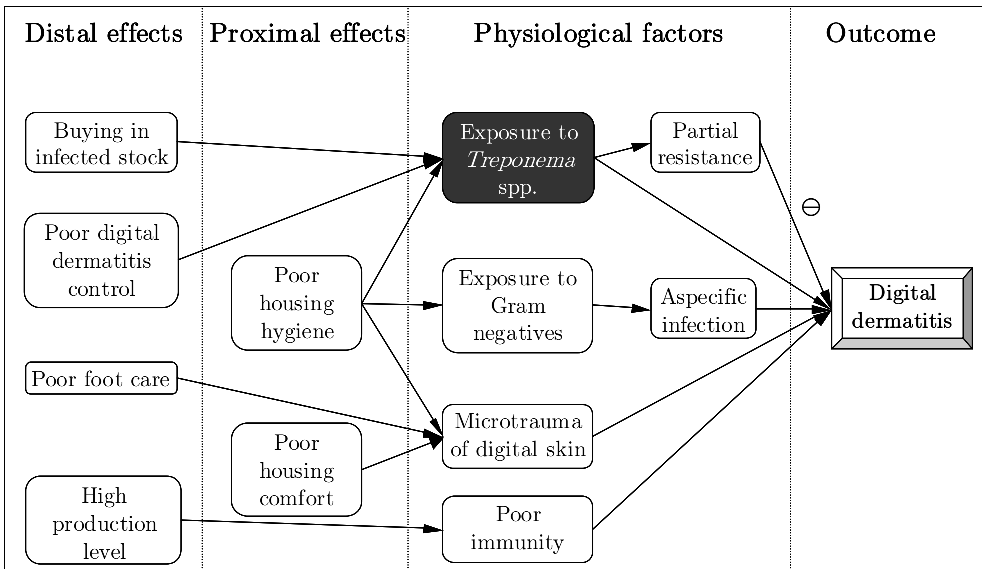


Figure 1.4: A causal diagram showing the hierarchical relationship between the development of BDD lesions and physiological factors (animal level), proximal effects (management group level) and distal effects (farm and sectorial levels). The 'necessary cause' has been shaded

Chapter 2

Molecular epidemiology of treponemes associated with BDD: tip of the iceberg?

2.1 Introduction

2.1.1 The emergence of BDD

BDD was first described as a clinical condition in 1974 (Cheli and Mortellaro, 1974); it appears to have been a 'true' emerging disease (rather than a re-emerging one) as no reference has been made to the clinical condition before this time. BDD now has a worldwide distribution. Empirical information from dairy farmers, veterinary practitioners and researchers indicates that the disease frequency and severity is variable between farms and regions, as well as temporally. Many management and nutritional factors (see 1.2.4) have been linked to the disease, possibly reflecting the fundamental changes that have occurred in dairy production over the past 40 years. It is possible that the highly-productive Holstein-Friesian breed of the present day is predilected to the disease.

The rapidity of the spread of BDD attests to its contagious nature. Current evidence supports a bacterial aetiology, which furthermore is probably polymicrobial; multiple species of Gram negative bacteria have been associated with the characteristic lesions (Koniarova et al., 1993). A consistent finding has been the presence of numerous spirochaetes in lesion tissues, which appear to be associated with necrotic changes in the lesions. These were demonstrated by microscopy and immunohistochemistry to be *Treponema* spp. (Grund et al., 1995; Demirkan et al., 1998; Demirkan et al., 1999a). Treponemal IgG antibody titres were found to be significantly higher in diseased animals (Walker et al., 1997; Demirkan et al., 1999b; Laven et al., 2000). Döpfer et al. (1997) observed three treponemal morphotypes from BDD lesion tissues by microscopy, whereas no treponemes were seen in sections from unaffected feet. The spatial distribution in the lesions of three different phylotypes was visualized by Moter et al. (1998).

On the basis of this evidence, the BDD-associated treponemes are currently considered to be the primary microbiological agents involved in the aetiology of BDD (the 'necessary cause' in causal terms – see Chapter 1.3).

There is evidence that spirochaetes have been present in the farm environment prior to the emergence of disease in 1974. In 1936, Beveridge isolated spirochaetes from the lesions of sheep footrot in Australia, which he named *Spirochaeta penortha* (Beveridge, 1936). Egerton and Parsonson (1966) investigated an outbreak of foot infection in a herd of dairy cattle in Australia. Smears and microscopic examination of infected tissues showed the presence of multiple bacterial species, particularly *Dichelobacter* spp., and a remarkable abundance of spirochaetes. As the spirochaetes could not be cultured, their aetiological relevance was not considered, but the clinical description and photographs bear a strong resemblance to BDD.

It is unclear what precipitated the emergence of BDD. Compared to other infectious diseases of dairy cattle as well as human treponemal diseases, there has been little research on the microbiology of BDD-associated *Treponema* spp. to date. Due in part to the slow-growing and fastidious anaerobic nature of these micro-organisms, it is only since 1995 that *in vitro* microbial culture has been successful (Walker et al., 1995). Advances have been made in this respect, as well as in the application of other microbiological techniques such as PCR and serology.

The characterization of bacterial isolates is important for an understanding of the bacterial epidemiology: bacterial populations exhibit varying degrees of virulence, and differ from each other in their reproductive processes – from the strictly clonal (i.e. asexual, with no recombination of genetic material) to the panmictic. Highly clonal populations are conserved (so that they form a collection of stable lineages), whereas non-clonal populations diversify rapidly. It is increasingly clear that there is also intra-species variability in the degree of clonality. An understanding of the extent of recombination will determine the most appropriate methods for studying the molecular epidemiology (Spratt and Maiden, 1999). The integration of population genetic and epidemiological studies can provide important insights into the origins and spread of bacterial diseases (Musser, 1996; Tibayrenc, 1998).

Although microbiological research over the past 10 years has substantially enhanced our knowledge of specific treponemes associated with BDD (see 2.1.3), our understanding of the characteristics of the populations of BDD-associated bacterial pathogens is still limited, and no evolutionary studies have been performed. We do not yet have an understanding of the degree of clonality of BDD-associated *Treponema* spp.

In this chapter, several molecular approaches will be described that were applied to characterize BDD-associated *Treponema* spp. cultured from lesion tissue biopsies, and explore issues such as antigenic heterogeneity and serotyping; the findings will be linked to existing knowledge of the microbiology and epidemiology of BDD to discuss

our current understanding.

2.1.2 An overview of spirochaetal diseases in different host species

In the absence of specific and detailed knowledge of the aetiology and pathogenesis of BDD, it is useful to study other, better elucidated spirochaetal diseases for possible analogies which may inform our understanding, and on which we may base our working hypotheses.

These bacteria infect the digestive tract and the skin, and particularly in the latter instance may be highly tissue-destructive. Very little is understood of their virulence determinants. Infections tend to be characterized by long-term persistence of the organisms in the host; the strong host immune response contributes to the pathology of these diseases (Edwards et al., 2003b).

Intestinal spirochaetes in production animals

Spirochaetes are commonly present in the rumen, caecum and colon of ruminants and other production animals (particularly in swine). The many species descend from a common ancestor. They can be subdivided into *Treponema* spp., *Serpulina* spp. and *Brachyspira* spp. 16S rRNA sequence analysis of 1438 base pairs has shown that the spirochaetes fall into 2 distinct clusters, the *Serpulina* spp. and *Brachyspira* spp. in one and the *Treponema* spp. in the other (Paster et al., 1991).

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 ruminal digesta contents (they are cellulolytic), and occur in high population densities (10^8 – 10^9 spirochaetes ml^{-1}) (Ziolecki, 1979; Ziolecki and Wojciechowicz, 1980; Paster and Canale-Parola, 1982; Stanton and Canale-Parola, 1979). These treponemes have not been associated with gastrointestinal disease; it is likely that they play a substantial role in the degradation of ingested plant materials (Paster and Canale-Parola, 1982).

A Japanese paper reports a case of a Holstein-Friesian cow with dysentery, which was attributed to a colitis caused by spirochaetes (Shibahara et al., 2000). Subsequently, a report was published in which tissues from cattle with clinical BDD were examined by histology, immunohistochemistry, electron microscopy and bacteriology (Shibahara et al., 2002). The authors claimed to have isolated pathogenic spirochaetes from the colon lining which caused colitis. Ultrastructurally, these spirochaetes were similar to the spirochaetes in BDD. The authors suggest that concurrent infections of feet and colon may occur. However, their results have not been replicated or confirmed by other workers.

Swine Different clinical diseases have been described: Swine Dysentery (SD) which is caused by *Brachyspira hyodysenteriae*; Porcine Intestinal Spirochaetosis (PIS) and Porcine Spirochaetal Colitis (PSC) which are caused by multiple *Brachyspira* spp. (Hampson et al., 1997).

These spirochaetes are associated with the lining of the large intestine and are not obligatory anaerobes. Immunity after recovery from SD is variable – reports are contradictory. Protective immunity up to 17 weeks post infection has been described. As with BDD, repeated re-infection is possible, although there does appear to be a degree of development of immunity after repeated infection. Diagnosis is typically assumptive (based on history and clinical presentation).

The serology of SD has been well described, and could provide clues for the serology of BDD. Many different serogroups (> 9) are know to exist and many different serotypes (> 30) have been described on the basis of DNA analysis. Antibody detection tests are more useful for detecting infected herds than infected individuals. Titres rise post infection, but there is no clear correlation with the level of the titre and protective immunity. The IgG titre is said to correlate with the duration of clinical signs, the IgA titre is indicative of recent exposure. Cell-mediated immunity is possibly important in the immunology, but has been poorly characterized to date.

Laboratory techniques are usually based on the demonstration of antigen rather than on antibody detection. Polyclonal sera raised against *B. Hyodysenteriae* have limited applicability because they detect many other spirochaetes besides *B. Hyodysenteriae*, so that a high degree of false positives may result (i.e. low specificity)(Jensen, 1997). Monoclonal antibodies have limited use; while they identify specific strains or serotypes, they do not identify others.

Oral treponemes in humans

Development of human periodontal disease Human periodontal disease and the bovine infectious foot conditions (recently described as 'bovine digital epidermitis' by Cruz et al. (2005)) are collective terms for describing various different clinical conditions; both are complex and multifactorial, with no single bacterial species responsible for the initiation or progression of disease. Treponemes have been implicated in both processes, leading to periodontitis and BDD respectively. Furthermore, the human treponemal species *T. denticola*, *T. vincentii* and *T. phagedenis* are closely related to BDD-associated treponemes (Trott et al., 2003; Choi et al., 1997; Collighan and Woodward, 1997; Woodward et al., 1998). There is very high phylogenetic diversity within these oral spirochaete populations (Dewhirst et al., 2000).

As with BDD, culture of oral *Treponema* spp. has been problematic, and it has been estimated that 75% of the total number of species still needs to be cultured (Dewhirst et al., 2000). However, PCR amplification and sequence comparison of 16S

rRNA genes, immunohistology and immunocytochemistry, and electron microscopy have shown that these treponemes are associated with the disease (Choi et al., 1994, 1996; Dewhirst et al., 2000; Riviere et al., 1999; Paster et al., 1998; Edwards et al., 2003a).

There are notable similarities in the clinical progression of disease, which may reflect parallels in the pathogenesis. Periodontitis is preceded by, and develops from, a mild inflammation of the gums (gingivitis) associated with multiple non-specific bacterial species; this has been linked to an overgrowth of plaque. Advancement of plaque formation facilitates the formation of periodontal pockets, which brings the bacteria into contact with the gingival tissues. Another major risk factor for the development of gingivitis and periodontitis is undermining of the epithelial barrier of the gingiva, for instance caused by smoking. A shift in bacterial ecology takes place within the pocket; conditions become progressively more anaerobic, favouring growth of proteolytic Gram-negatives including *Treponema* spp. The tissue damage caused by these bacteria is aggravated by the host immune response (infiltration of neutrophils and release of inflammatory mediators and cytokines). The pockets become deeper and the infection progresses. Without intervention (surgery or treatment), this process continues unabated, until the tooth falls out. *Treponema* spp. are rarely found in healthy oral flora but constitute a significant proportion of the total microflora in active periodontitis lesions, in some cases constituting over 50% of the total bacterial flora, particularly in the deeper regions of the pockets. A dose-response relationship has been shown for the number of *Treponema* spp. present and the severity of clinical symptoms (Edwards et al., 2003b). Treatment is by mechanical debridement and local antibiotics (tetracycline); which is comparable to BDD.

The evidence that *Treponema* spp. are the primary aetiological agents in BDD is not yet equally conclusive. The origins and distribution of the organisms are unknown. The factors involved in the initiation of the disease are likewise obscure, and little is known of the transmission dynamics. Numerous Gram negatives have been cultured from lesion tissues, although their pathological significance has not been determined; it is therefore likely that BDD is a polymicrobial infection. Collighan and Woodward (1997) suggest that the bovine underfoot environment may mimic the periodontal microenvironment, i.e. humid and poorly oxygenated. In analogy with periodontal disease, a non-specific epidermitis may be required as a precursor to BDD; several authors have reported treponemes in lesions of interdigital necrobacillosis and interdigital dermatitis (Cruz et al., 2005). In the superficial infection, *Treponema* spp. may colonize host tissues, becoming more invasive as tissue damage leads to an ecological shift which promotes the development of anaerobic conditions. Treponemal motility enables migration of the treponemes to deeper layers of the epithelium; cytopathic effects facilitate the penetration of basal cell layers; and further degradation opens the way for endothelial

tissue invasion (Edwards et al., 2003b). It is currently unknown whether *Treponema* spp. may exist as commensal organisms on the healthy digital skin.

The isolation of closely related treponemal strains from human periodontitis cases and BDD lesions suggests that these bacteria may have a relatively non-specific host range. It is not yet known to what extent the large strain variability also applies to BDD - but different phylotypes / morphotypes have been consistently described (Walker et al., 1995; Choi et al., 1997; Döpfer et al., 1997; Stamm et al., 2002; Trott et al., 2003).

Humoral response to oral treponemes Due to the large number of bacterial species involved in human oral periodontitis, the immune response is considered to be complex. Consequently, limited specific work has been performed on the serology of treponemes. In a case-control study performed by Takahashi et al. (2001), IgG antibody titres to *T. denticola* (among 12 other antigens) were found to be significantly elevated for the case population, as tested by ELISA. They reported substantial variability of antibody titres, between cases and controls as well as within the case group; although they did not specifically state that this was the case for *T. denticola*. In a similar study, Papapanou et al. (2000) investigated associations between subgingival microbial profiles and serum IgG responses. *T. denticola* was among the bacteria found at higher levels in cases; however, IgG titres were lower in cases than controls (a 'checkerboard' immunoassay was used, which may make comparison with ELISA results difficult). In a longitudinal study carried out over a 30-month period, cases showed elevated IgG antibodies to 19 periodontal species, including *T. denticola* (also by 'checkerboard' immunoassay)(Papapanou et al., 2004). Over the study duration, antibody titres remained low for controls, and tended to remain elevated for cases, despite an improvement in clinical status. This indicates that the antibody half-life appears to be longer than for BDD-associated *Treponema* spp., which current evidence suggests is short (Walker et al., 1997; Trott et al., 2003).

In an experimental infection study, Kesavalu et al. (1999) elicited a humoral response in mice by primary infection with *T. denticola*; they found a significant increase in IgG titres, which rose further after reinfection. They immunized mice by inoculation with inactivated treponemes. However, although serum antibody was effective *in vitro* in immobilizing and clumping the bacteria, the size and severity of lesions after reinfection was not reduced for either primarily infected or immunized mice; they concluded that the humoral response is not capable of resolving a *T. denticola* infection. Similarly, high treponemal IgG titres in cows do not appear to reduce the severity of BDD.

Yaws

Yaws is a 're-emerging', endemic, non-venereal treponematosis caused by *T. pallidum* subsp. *pertenue*, which shares many similarities with BDD. It has a relapsing clinical course manifested by prominent cutaneous lesions. Yaws is transmitted by direct skin contact with the exudate or serum from an infectious lesion; the entry of treponemes is facilitated by breaks in the skin from scratches, scabies, etc. The disease is associated with humidity, poor hygienic conditions, poor health care and close interpersonal contact. Its distribution is associated with poverty and isolation from organized social and health services ("diseases at the end of the road"), hence it is in rural communities in developing countries in the humid tropics that it is endemic. Yaws predominantly affects children under 15 years of age (Engelkens et al., 1999; Antal et al., 2002).

Like the other endemic treponematoses, the clinical manifestations are divided into early (which includes primary and secondary lesions) and late stages of the disease. The incubation period varies from 10 days to 3 months. The primary lesion, a small papule (or 'mother yaw'), develops at the site of treponemal infection. This itchy erythematous, scaly papule then proliferates into a papilloma which may become ulcerated, and contains numerous treponemes. The lower extremities are predilected lesion sites. The lesions may persist for 3 to 6 months, resolving spontaneously. Disseminated secondary stage lesions may develop before disappearance of the initial lesion, but may not appear for 1 to 2 years. These lesions resemble the initial lesions, but may become larger; exudation of highly infectious serum is a feature. In addition, a variety of squamous lesions (i.e. macules, papules and nodules) may appear. Hyperkeratotic changes of the palms and soles are common. The secondary lesions resolve spontaneously after a period of weeks or months, although relapses may occur. A latent period follows which can only be serologically determined; latency has a lifelong duration for about 90% of patients. In the remaining 10%, a tissue-destructive late stage may occur after 5 to 10 years or longer, featuring irreversible lesions of bone, cartilage, soft tissue and skin (Engelkens et al., 1999; Parish, 2000; Antal et al., 2002).

The incidence and clinical expression of yaws shows seasonal effects; during the dry seasons the incidence is lower, the lesions are less florid and become more restricted to the moist skin folds. Because many factors influence the transmission and the natural course of the disease, a patchy distribution in affected populations is a characteristic feature of their epidemiology (Antal et al., 2002).

The subspecies of *T. pallidum* are antigenically highly related. Infection with a particular treponemal subspecies will result in lifelong immunity from re-infection with the homologous organism; however, protection against other subspecies is partial or non-existent. This lack of cross-immunity provides evidence of antigenic differences between the subspecies of *T. pallidum*, but no specific antigens have as yet been identified. Immunoblot studies have revealed no significant antigenic differences between

T. pallidum subsp. *pallidum* (which causes venereal syphilis) and *T. pallidum* subsp. *pertenue*, or between *T. pallidum* subsp. *pallidum* and *T. carateum* (Antal et al., 2002).

Seroconversion takes place after infection with the subspecies of *T. pallidum*, and persists for years or even life (Antal et al., 2002). Attempts to find subspecies-specific monoclonal antibodies have been unsuccessful; treponemal serology cannot differentiate yaws from syphilis, despite extensive attempts to do so (Noordhoek et al., 1990). This cross-reactivity of antibodies is problematic as it precludes a serologic differential diagnosis for both diseases (Engelkens et al., 1999). A recent paper by Marangoni et al. (2005) showed that serum antibodies against the human oral treponeme *T. denticola* do not cross-react with *T. pallidum*.

Penicillin remains the drug of choice for treatment, as these treponematoses have remained exquisitely sensitive to the drug.

2.1.3 Genetic diversity of BDD-associated *Treponema* spp.

Morphological variability of BDD-associated treponemes has been identified using light microscopy (Döpfer et al., 1997; Read et al., 1992). Walker et al. (1995) identified two morphotypes by electron microscopy; these originated from a single treponemal isolate cultured from a BDD lesion. Further EM work was performed by Grund et al. (1995). However, understanding of the corresponding genetic diversity has only relatively recently been developed.

Choi et al. (1997) performed comparative 16S rRNA sequence analysis of treponeme DNA extracted from eight BDD biopsies taken in Germany, describing five phylotypes: DDKL-3, DDKL-4, DDKL-12, DDKL-13 and DDKL-20 (see Figure 2.1). The first three phylotypes were closely related to the human treponemes *T. denticola*, *T. phagedenis* and *T. vincentii* respectively[1]. DDKL-3 was the most frequently identified treponeme. Subsequently, Moter et al. (1998) investigated the spatial distribution of BDD-associated treponemes in lesions; they found that the *T. denticola*-related treponemes were mainly found superficially in the lesions, whilst *T. phagedenis* and *T. vincentii*-related treponemes penetrated much more deeply. The same research group later published details of another, unrelated treponeme species which they named *T. brennaborense* (Schrank et al., 1999), although no aetiological significance could be attached to this finding.

In a UK-based study, BDD lesion material from two cows was tested by PCR; analysis of the 16S rRNA sequence showed them to be closely related to *T. denticola* Rijpkema et al. (1997); Collighan and Woodward (1997). Subsequently, 16S rRNA sequence analysis was performed of a treponeme isolated from a case of an emerging ovine form of the disease (Contagious Ovine Digital Dermatitis, CODD): this isolate

[1] *T. denticola*, *T. vincentii* and the closely-related *T. medium* are human oral pathogens that have been aetiologically associated with periodontitis; *T. phagedenis* is a non-pathogenic commensal of the human genital flora.

was found to be very similar to *T. vincentii*. It became apparent that similar genetic diversity existed for CODD-associated treponemes when Edwards et al. (2003a) analyzed another CODD isolate, which was found to be closely related to *T. denticola*; an isolate cultured from BDD (which originated from the area covered by this study) was closely related to *T. medium*.

In the US, Stamm et al. (2002) performed 16S-23S rDNA intergenic spacer region sequence analysis of six BDD-associated *Treponema* isolates. Whilst this is not directly comparable to 16S rRNA sequence analysis, they identified three phylotypes: a *T. medium* / *T. vincentii* phylotype, a *T. phagedenis* phylotype and a third, less closely related phylotype. Trott et al. (2003) isolated four treponemes from BDD lesions in Iowa and compared these with the two California strains previously characterized by Walker et al. (1995). These were found to be 98% identical to *T. phagedenis*, but only 92% identical to DDKL-3, the *T. denticola*-like treponeme.

2.1.4 Where is the reservoir? Distribution of BDD-associated *Treponema* spp. in the farm environment

Nothing is yet known of the distribution of the lesion-associated *Treponema* spp. in the farm environment (other than in BDD lesions). Assuming that the causative organisms are absent on BDD-negative farms, an initial introduction of infection by an external source is required. However, it is not known whether such events are repeated – and if so, with what frequency. There are indications that this is not a prerequisite for the indefinite persistence of BDD (e.g. farms that have become closed subsequent to the introduction of infection, but that have not eliminated BDD), although seasonal variability in incidence is common. On the other hand, anecdotal information suggests that farms that continue to purchase stock periodically suffer outbreaks of acute clinical BDD.

Haydon et al. (2002) define a reservoir as "one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population." In the case of BDD, it seems that dairy cattle are the only host species or 'target population' that are infected. CODD-associated treponemes have been shown to be closely related to BDD (Harwood et al., 1997; Collighan et al., 2000; Wassink et al., 2003; Dhawi et al., 2005; Moore et al., 2005). However, although shared grazing is common in the UK, cross-species transmission has not yet been demonstrated and it is not known if sheep could constitute a reservoir. Although the treponemal pathogens causing BDD are closely related to human treponemes, there are no indications of any zoonotic potential.

In a situation where there is one target population and no other source populations, either the environment constitutes a reservoir, or the target population is itself the source population, or both. It is possible that transmission of infection is achieved

by dissipation of infectious material from lesions into the environment, thus infecting other animals (i.e. by indirect contact). As BDD-associated treponemes have been found to be fastidiously anaerobic, the indefinite persistence of a free-living population in the environment is unlikely. If the reservoir is not primarily environmental, it follows that it must be an animal-related source. As described in 2.1.2, treponemes make up part of the symbiotic ruminal flora; however, these are morphologically and phylogenetically not closely related to BDD-associated *Treponema* spp. It is possible that BDD-associated *Treponema* spp. inhabit the large intestine, as reported by Shibahara et al. (2002). Another possibility is that these bacteria are part of the commensal digital skin flora, only causing infection when initiating conditions as described above are met. Thirdly, considering their close relatedness with human oral treponemes, a buccal origin is possible. Finally, the close relatedness with *T. phagedenis* makes a urogenital origin a viable possibility.

In the absence of such basic knowledge, the origin and transmission dynamics of the infection cannot easily be clarified.

2.2 Materials and methods

2.2.1 Microbiological investigation of BDD-associated *Treponema* spp.

All microbiological work was carried out at the Connective Tissue Research group of the Faculty of Veterinary Science at the University of Liverpool by Dr. Nick Evans, Ms. Jenni Brown, Dr. Ibrahim Demirkan and the author. The details of the microbiological work will not be presented in detail, as this is not the focus of this chapter; the methods followed the protocols that were fully described by Demirkan et al. (2001) and Demirkan et al. (2006).

BDD lesion tissue biopsies

BDD lesion tissue material was obtained from nine farms in Cheshire, Merseyside, Shropshire and Gloucestershire, UK. A Home Office project license (No. PPL 40/2574) was obtained for this work, and the author acquired a personal license (No. PIL 40/7431). Cows with clinical BDD lesions were identified and restrained in a crush; the affected foot was lifted and fixed. After cleaning the foot by hosing off slurry, the lesion surface was disinfected and dried with a paper towel. Superficial anaesthesia was achieved by applying Lidocaine spray. A biopsy punch with 3mm diameter was used to remove a cylindrical biopsy of 8 to 10mm depth from the central, active part of the lesion.

The biopsy was washed in a sterile PBS solution to remove superficial faecal contamination and placed into 2ml oral treponeme enrichment broth (OTEB, pH 7.2) (Anaerobe Systems, USA) supplemented with rifampicin (1mg/ℓ) and enrofloxacin (1mg/ℓ)

to increase the selectiveness.

Treponemal isolation and culture

The biopsies were transferred to the laboratory, where each biopsy was cut into smaller sections and placed into a fresh OTEB solution, after which they were incubated for 24 hours at 35°C in an anaerobic cabinet. They were then streaked onto anaerobic agars and incubated for up to 3 weeks at 35°C in anaerobic conditions. *Treponema* spp. were selectively subcultured as required to obtain a total of 23 isolates. Isolates were frozen to -70°C for storage.

Gene sequencing and analysis

16S rRNA DNA was extracted from the 23 treponeme cultures as described by Demirkan et al. (2001) and Demirkan et al. (2006). Briefly, appropriate primers were used to amplify nearly the full length of the 16S rRNA genes ($\sim$ 1500 bp). The PCR product was separated by electrophoresis; the amplicons were extracted, purified and subjected to nucleotide sequencing. Sequence editing was performed using DNAstar^{TM}; spirochaete 16S rRNA gene sequences were obtained from GenBank^{TM}. Alignment was performed using CLUSTAL W (Thompson et al., 1994). A phylogenetic tree was generated using genetic distance-based neighbour-joining algorithms (bootstrap values based on 1000 iterations), using MEGA2 software (Kumar et al., 2001).

Flagellin gene These were analysed as detailed by Demirkan et al. (2001) and Demirkan et al. (2006); specific primers were designed for amplification of the flagellin gene sequences of 15 isolates. The amplicons were purified and sequenced. Sequence data of a 567 bp section from the central region of the flagellin gene were used. A phylogenetic tree was generated using genetic distance-based neighbour-joining algorithms (bootstrap values based on 1000 iterations), using MEGA2 software (Kumar et al., 2001).

Enzyme profiling

Enzyme activity patterns were investigated using a commercial, semi-quantitative method (APIZYM®, bioMerieux, http://www.biomerieux.com/). This system consists of a strip consisting of 20 microtubes containing dehydrated substrates.

In accordance with the manufacturer's recommendation, young cultures were used (48-hour OTEB cultures without antimicrobials). After centrifuging and removing the supernatant, the pellet was suspended in phosphate buffered saline (PBS) with pH 7.2 to obtain a bacterial suspension with a turbidity of 5-6 McFarland. Each cupule of the system was inoculated with $60\mu\ell$ of this suspension to reconstitute the media. After incubation in an anaerobic chamber for four hours at 37°C, one drop of the

specific reagents ZYM A and ZYM B were added to each cupule. The intensity of the colour development brought about by enzymatic metabolism was interpreted using the manufacturer's reference chart.

Electron microscopy

Electron microscopy was performed to study the morphology of a representative of each of the three antigenic groups (see 2.3.1), namely T19, T320 and T3552B. The method as described by Demirkan et al. (2006) was followed.

2.2.2 Investigation of serology

To investigate the possible existence of serogroups, we utilized and further developed a well-described indirect ELISA (Demirkan, Walker, Murray, Blowey and Carter, 1999). The treponemal antigen used for the initial development of this test was a bovine strain; at the time, this was the only suitable antigen available. However, upon repeated subculturing, this antigen was discovered to be an impure mixture of two isolates. Phylogenetic analysis of the 23 new isolates obtained showed clustering into three different subdivisions (see 2.3.1). We wished to evaluate whether these isolates elicited distinct serologic responses, and to assess the evidence of existence of serotypes / -groups. A two-step process was employed.

In the first step, 20 of the 23 available treponemal isolates were selected (the remaining isolates were not used as there were some doubts as to their purity). These were harvested, centrifuged and sonicated. For each antigen, a non-activated, 96-well microtitre ELISA plate (Dynatech) was coated with 5μg/mL of each of the test antigens. A panel of 44 test sera was selected from the cross-sectional study serum bank. These sera were not randomly chosen, but were selected on the basis of the results of testing with the original ELISA; samples from different farms, management groups and clinical status were included. We deliberately included 'abnormal' or unexpected results, i.e. sera from animals with no BDD lesions but high titre (i.e. 'false positives') and animals with BDD lesions but low titre (i.e. 'false negatives'). The objective was to include the widest range of possible outcomes, and to investigate whether the false positive and negative samples selectively reacted (or failed to react) with any of the antigens. In summary, we were more interested in the consistency of response of the antigen-specific ELISAs, as well as identifying any deviations in reactivity from the original ELISA. The relationships between the humoral response and incidence of BDD lesions were more thoroughly explored in the observational studies (Chapters 4 and 5).

The sera were diluted to 1/100, and 50μL were pipetted into ELISA plate wells in duplicate. All plates included positive and negative control sera, and two wells were left blank. After incubation, the plates were washed and dilutions of mouse anti-bovine IgG1, IgG2 and IgM (Serotec) were added to each well. Plates were incubated at 37°C

for 1 hour, and at 4°C for 1 hour. Following washing, bound antibodies were detected using an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). The antibody-conjugated reaction was visualized with p-nitrophenyl phosphate (Sigma) diluted in glycine buffer. The absorbency (OD 405nm) was measured with a multi-well ELISA plate reader (Multiskan, Titertek), and adjusted for differences between plates (quantity of substrate, incubation time) by expressing it as a percentage positive (PP), which is defined as

$$\text{PP} = 100 * \left(\frac{\overline{\text{OD}}_{\text{sample}}}{\overline{\text{OD}}_{\text{positive control}}} \right)$$

In the second step, a subset of five antigens including representatives from each of the three subdivisions was selected from the original 20 on the basis of serological reactivity. These antigens were also blended into a 'cocktail' preparation, and one plate was prepared for this cocktail: the objective was to study whether it would identify a sample as positive regardless of the antigenic infective strain, and hence be useful as a test for routine screening. Two plates were prepared for each antigen and the cocktail. A further 44 serum samples were selected to double the panel to 88; as before, selection was not random but was based on criteria to achieve representation of a broad range of outcomes. We wished to investigate whether the reactivity of the antigens, as determined in the first step, was consistent when we increased the sample size.

As the 43 samples tested in the first step were repeat tested in the second step, the repeatability of the test could be evaluated. A straightforward method to visually assess this was by plotting scatter plots. More formal tests included calculation of the Pearson correlation coefficient, which measures the strength of association between the first and second test outcomes; this was calculated for each antigen and all antigens combined. A limitation of the Pearson coefficient is that it does not directly compare the values obtained; therefore, it is not a measure of agreement (Dohoo et al., 2003). To assess the reliability of the test, the intraclass clustering coefficient (ICC) was also computed. The ICC is the ratio of the variance between the 'true' measurements of the first and second test to the total test variance, which is the sum of the between-test variance and the within-test variance (or measurement error) (Kirkwood and Sterne, 2003).

2.3 Results

2.3.1 Microbiological investigation of BDD-associated *Treponema* spp.

Gene sequencing and analysis

16S rRNA On the basis of 16S rRNA gene sequence similarity, the treponemal isolates were grouped into three clear subdivisions. Within these subdivisions, isolates were closely related, showing similarities of 98% or more. Between the subdivisions,

sequence similarity was 89 to 92%. The isolates were arbitrarily designated as groups 1, 2 and 3 (see the similarity matrix in Table 2.1).

	16S rRNA similarity matrix (%)																						
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1–T184																							
2–T18A1	99																						
3–T19	98	99																					
4–T35	98	99	99																				
5–T54	98	99	99	99																			
6–T56	98	99	99	100	99																		
7–T52A	91	90	90	90	91	90																	
8–T119A	91	91	90	90	90	90	99																
9–T136	90	90	90	90	91	91	99	99															
10–T167	90	90	90	90	90	90	99	99	99														
11–T257	90	90	90	90	91	91	99	99	99	99													
12–T272	90	90	90	90	90	90	99	99	99	99	99												
13–T296A	90	90	90	90	90	90	99	99	99	99	99	99											
14–T320A	91	90	90	90	91	90	99	99	99	99	99	99	99										
15–T354B	90	91	90	90	90	90	99	99	99	99	99	99	99	99									
16–T380A	91	91	90	90	90	90	99	99	99	99	99	99	99	99	99								
17–TW35	90	90	90	90	90	90	99	99	99	99	99	99	99	99	99	99							
18–TG169A	91	90	90	90	90	90	99	99	99	99	99	99	99	99	99	99	99						
19–TG187	90	90	90	90	90	90	99	99	99	99	99	99	99	99	99	99	99	99					
20–T354A	90	90	90	90	90	90	92	92	92	92	92	92	92	92	92	92	92	92	92				
21–T354C	89	90	90	90	89	89	91	91	92	91	91	91	91	92	91	92	91	92	91	99			
22–T3552B	89	89	89	89	89	89	91	91	91	91	91	91	91	91	91	92	91	91	91	99	99		
23–TG819C	90	90	90	90	90	90	91	92	92	91	92	91	91	92	92	92	92	92	91	99	99	99	

Table 2.1: Similarity matrix (%) of 16S rRNA sequences of 23 treponemal isolates cultured from BDD lesions. Isolates 1 – 6 have been designated as antigenic group 1, isolates 7 – 19 have been designated as antigenic group 2 and isolates 20 – 23 have been designated as group 3. Within-group homology is 98 – 100%; between-group similarity ranges from 89 – 92%.

Flagellin gene The flagellin gene was less conserved than the 16S rRNA gene. However, the same subdivisions were identified. Sequence similarity ranged from 91 to 100% within the groups, and from 72 to 82% between the groups (see the similarity matrix in Table 2.2).

	Flagellin DNA similarity matrix (%)														
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1–T18A1															
2–T19	100														
3–T54	100	100													
4–T56	100	100	99												
5–T52A	80	80	80	80											
6–T119A	80	80	79	80	98										
7–T136	81	80	80	81	98	98									
8–T167	80	80	80	80	97	98	98								
9–T257	79	79	79	79	94	93	94	94							
10–T272	80	80	79	80	99	98	99	98	94						
11–T320A	81	81	80	81	98	98	99	97	94	97					
12–TG169A	78	78	78	78	95	94	95	96	92	95	94				
13–TG187	78	77	77	78	94	94	94	95	91	94	94	95			
14–T354A	75	73	75	75	75	79	76	76	76	76	76	80	75		
15–T3552B	73	73	73	72	76	80	80	80	78	76	80	82	76	97	

Table 2.2: Similarity matrix (%) of flagellin gene sequences of 15 treponemal isolates cultured from BDD lesions. Isolates 1 – 4 have been designated as antigenic group 1, isolates 5 – 13 have been designated as antigenic group 2 and isolates 14 – 15 have been designated as group 3. Within-group homology is 91 – 100%; between-group similarity ranges from 72 – 82%.

Phylogenetic analysis Phylogenetic analysis of the 16S rRNA sequence reflected clustering of the isolates into three species-level subdivisions. The dendrogram in Figure 2.1 shows the relatedness of various spirochaetes, including the human periodontal treponemes, the treponemes causing syphilis and yaws, and the five phylotypes described by Choi et al. (1997). *Brachyspira hyodysenteriae* is included in the dendrogram as an outgroup. The group 2 isolates are closely related to *T. phagedenis*; group 1 isolates are closely related to *T. medium*; and group 3 isolates are related to *T. denticola* and *T. putidum*. No representatives of the two phylotypes DDKL12 and DDKL20, as reported by Choi et al. (1997), were identified; nor any isolates similar to *T. brennaborense* (Schrank et al., 1999).

The phylogenetic tree of the flagellin gene sequence (Figure 2.2) shows a nearly identical pattern. The group 2 antigens show a greater similarity with *T. phagedenis* compared to the 16S rRNA analysis, while the group 1 antigens are less similar to *T. medium*. The phylotypes described by Choi et al. (1997) could not be included, as flagellin gene analysis was not performed.

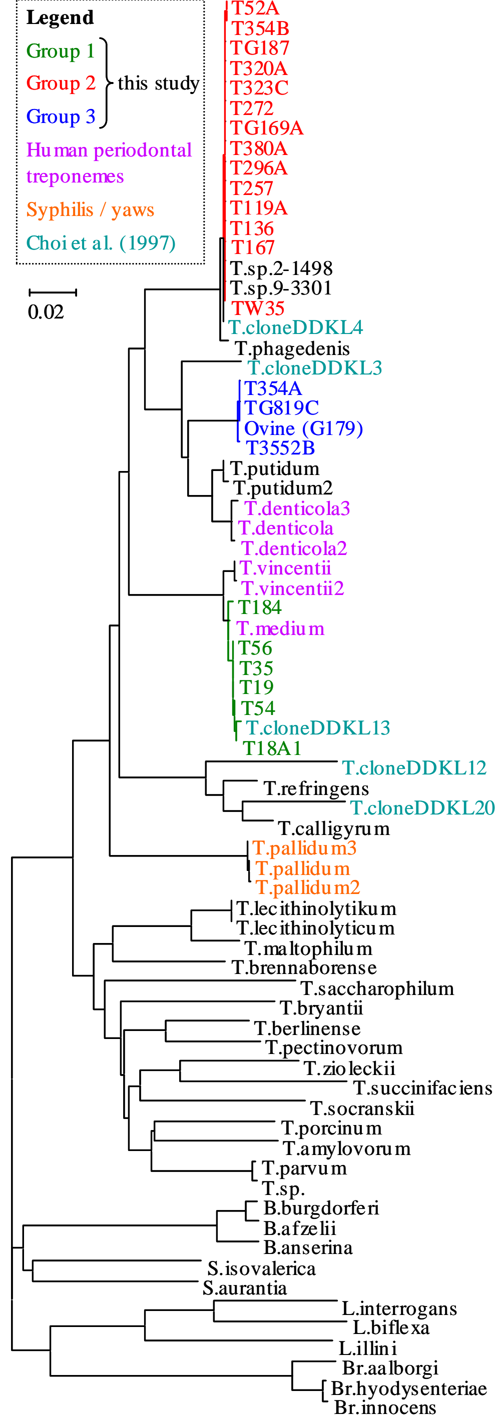


Figure 2.1: 16S rRNA dendrogram of spirochaetal spp. Alignment was performed using CLUSTAL W; the dendrogram was drawn using MEGA2. The dendrogram was derived by genetic distance-based neighbour-joining algorithms (bootstrap values based on 1000 iterations). The marker bar represents a 2% difference in nucleotide sequences. The BDD-associated treponemes derived in this study have been designated as groups 1, 2 and 3. Human treponemes have been included for reference, as well as the five BDD-associated phylotypes described by Choi et al. (1997)

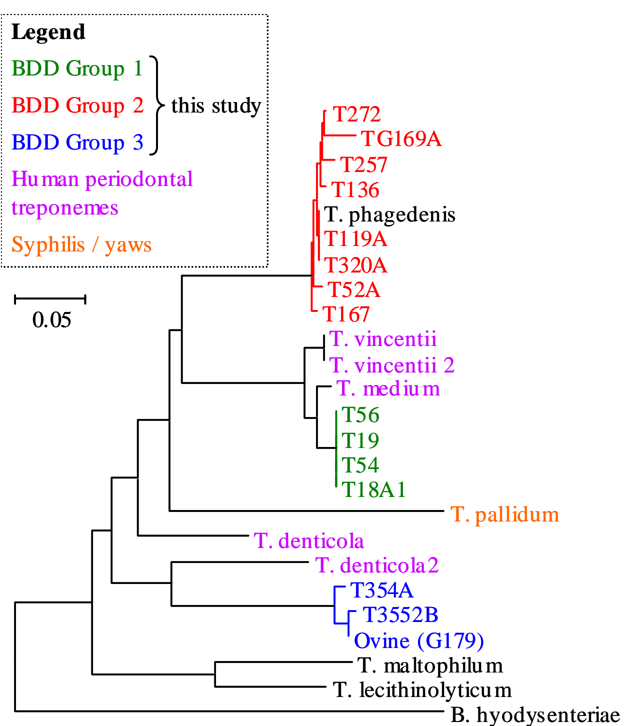


Figure 2.2: Flagellin gene dendrogram of spirochaetal spp. Alignment was performed using CLUSTAL W; the dendrogram was drawn using MEGA2. The dendrogram was derived by genetic distance-based neighbour-joining algorithms (bootstrap values based on 1000 iterations). The marker bar represents a 5% difference in nucleotide sequences. The BDD-associated treponemes derived in this study have been designated as groups 1, 2 and 3. Human treponemes have been included for reference

Enzyme profiles The results of the enzyme analysis are presented in Table 2.3. The isolates have been sub-grouped in the same order as above, and the closely-related human treponemes have been included for comparison. Distinct patterns emerge: within each group, isolates consistently express a number of enzymes. The profiles differ between the groups; C_8 esterase lipase is the only enzyme expressed by all groups, and C_4 esterase is expressed by groups 1 and 3. The closely-related human treponemes differ from the BDD-associated treponemes by at least one enzyme.

Electron microscopy The morphology of the group 1 isolate T19, as determined by transmission electron microscopy, was very similar to *T. medium*, with only the number of flagella differing (see Figure 2.5 at the end of this chapter). The group 2 isolate T320 was substantially larger than *T. phagedenis*, to which it is genetically closely related (Figure 2.6). The group 3 isolate T3552B also differed from *T. denticola*, showing a different flagellation pattern (Figure 2.7).

Isolate	Group	Enzyme activity																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
T. medium		+	+	+	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−
T184	1	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
T18A1	1	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
T19	1	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
T54	1	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
T56	1	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
T. phagedenis		−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	−
T52A	2	+	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	+	−	−
T119A	2	+	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	+	−	−
T136	2	+	−	+	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	+
T167	2	+	−	+	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	−
T257	2	+	+	+	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	+
T272	2	+	+	+	−	−	−	−	−	−	+	+	−	+	+	−	−	+	−	−
T296A	2	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−
T320A	2	+	−	+	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	+
T354B	2	+	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	+	−	+
T380A	2	+	−	+	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	+
T323C	2	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−
TW35	2	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	−	+	−	−
TG169A	2	+	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−	+	−	+
TG187	2	+	−	+	−	−	−	−	−	−	+	−	−	+	+	−	−	+	−	−
T. denticola		−	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
T354A	3	+	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
T3552B	3	+	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
TG819C	3	−	+	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−
G179	3	−	+	+	−	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−

Table 2.3: Enzyme reactivity patterns of 22 treponemal isolates cultured from BDD lesions and 1 isolate cultured from a CODD lesion (G179). The closely related *T. medium*, *T. phagedenis* and *T. denticola* have been included for comparison. Enzymes tested: 1, alkaline phosphatase; 2, C_4 esterase; 3, C_8 esterase lipase; 4, C_{14} lipase; 5, leucine arylamidase; 6, valine arylamidase; 7, cystine arylamidase; 8, trypsin; 9, chymotrypsin; 10, acid phosphatase; 11, naphtholphosphohydrolase; 12, α-galactosidase; 13, β-galactosidase; 14, β-glucuronidase; 15, α-glucosidase; 16, β-glucosidase; 17, N-acetyl-β-glucosaminidase; 18, α-mannosidase; 19, α-fucosidase.

Origin of isolates As the 23 isolates were collected from nine farms in different geographic locations, the origin of these samples could provide clues about the distribution of BDD-associated treponemes. Table 2.4 lists relevant details. All group 1 isolates were cultured from a single farm in Merseyside; a group 3 isolate was cultured from a cow co-infected with a group 1 isolate. On the other farms of origin, group 2 isolates predominated, with several group 3 isolates less commonly identified. In one other case, a single cow was found to be co-infected with a group 2 and a group 3 isolate.

Isolate	Isolation date	Location			Antigenic group
		County	Farm	Cow	
T184	06/10/2003	Merseyside	1	1	1
T19	07/10/2003	Merseyside	1	2	1
T54	07/10/2003	Merseyside	1	3	1
T56	07/10/2003	Merseyside	1	4	1
T18A	07/10/2003	Merseyside	1	5	1
T18B	07/10/2003	Merseyside	1	5	3
T320	24/02/2004	Merseyside	2	1	2
T380	24/02/2004	Merseyside	2	2	2
T272	24/02/2004	Merseyside	2	3	2
T3552B	24/02/2004	Merseyside	2	4	3
T323C	20/07/2004	Merseyside	2	5	2
TW35	17/03/2004	Merseyside	3	1	2
T167	08/12/2003	Cheshire	1	1	2
T257	12/01/2004	Cheshire	2	1	2
T296A	12/01/2004	Cheshire	2	2	2
T354B	12/01/2004	Cheshire	2	3	2
T354A	12/01/2004	Cheshire	2	3	3
T136	04/02/2004	Shropshire	1	1	2
T119A	04/02/2004	Shropshire	1	2	2
T52A	04/02/2004	Shropshire	1	3	2
TG819C	07/05/2004	Gloucestershire	1	1	3
TG187	12/05/2004	Gloucestershire	2	1	2
TG169A	13/05/2004	Gloucestershire	3	1	2

Table 2.4: Locations of origin, dates of isolation and antigenic grouping of BDD-associated *Treponema* spp. isolates

2.3.2 Investigation of serology

Evidence of serotypes

For each serum sample, the response (PP) against each of the 20 treponemal antigens was investigated. The antigens were subdivided into three groups as before. The serologic responses elicited by the isolates of each subdivision were plotted in trellis plots; an example is given in Figure 2.3. In this plot, four samples each from animals with no BDD lesions, acute lesions, chronic lesions and regressing lesions were randomly selected from the test sample of 44 sera. Both false positives and false negatives were apparent; as stated in 2.2.2, these samples were not randomly chosen, but were selected on the basis of such discrepancies after testing with the original ELISA (results not given).

For each group, the serologic responses tended to be of comparable magnitude,

which is reflected by clustering of the antigens; however, there was a degree of cross-reactivity between the groups. The antigens of group 2 appeared to raise the strongest antibody response. As only two group 3 antigens were included, the degree of clustering is difficult to determine.

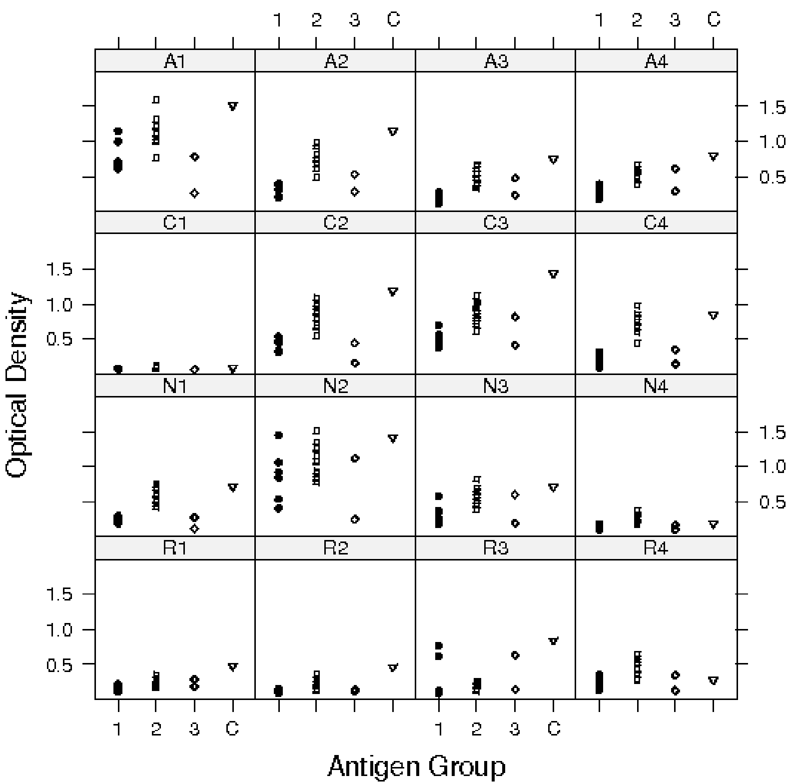


Figure 2.3: Reactivity of serum antibodies from 16 test samples with different clinical status (N: negative; A: acute lesion; C: chronic lesion; R: regressing lesion). The 20 antigens tested have been grouped into their phylogenetic subdivision (1, 2 and 3); the cocktail (C) has been included for illustration. Both false positives and false negatives are apparent (see the text for a fuller discussion)

When the analysis was repeated in the second step (i.e. double the number of samples tested against five antigens), the results were highly comparable; the same clustering pattern was seen. No trellis plots of this second step is presented; as only five antigens were included, the clustering of the antigens is not as visible. The serologic response to the cocktail ELISA tended to be as high as the highest individual antigen, i.e. it performed well as a 'catch-all'.

Repeatability of the ELISA

Scatter plots were made for each of the five antigens (see Appendix A), as well as for all samples combined (Figure 2.4). Both the raw optical density (OD) as well as

the percentage positive (PP), the adjusted value enabling comparison of results across plates, were plotted; the PP showed better agreement.

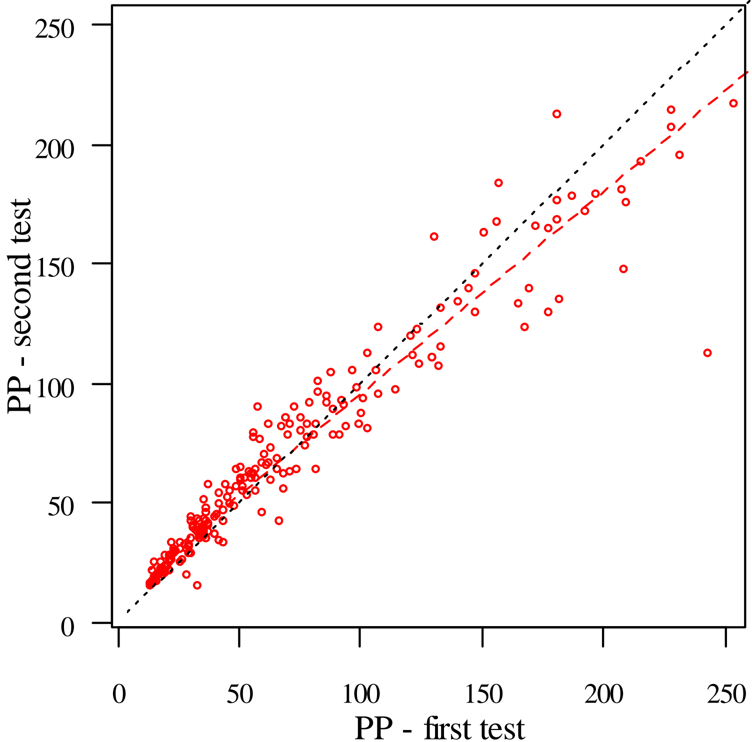


Figure 2.4: Scatter plot of the indirect BDD treponeme ELISA of 43 samples that were tested against five separate antigens (i.e. n=215) on two occasions: the percentage positive (PP) result of the first test was plotted against the second test; the red dashed line was fitted to the results, and the black dotted line represents perfect agreement

The Pearson correlation coefficients equate to the degree of clustering about the fitted line in the scatter plots (Dohoo et al., 2003); they are given in Table 2.5. The intraclass clustering coefficients (ICCs) have also been listed.

For the OD values, the fitted line deviated from the line of equality (see Appendix A); this was a consequence of differences in incubation times between the first and second test. As expected, this was corrected by using the PP. The scatter plots also indicated that there was greater disagreement for higher OD / PP values; this appears to be inherent to the assay, i.e. the ELISA can less precisely quantify the levels of antibodies in highly-reacting samples. The Pearson correlation coefficients are identical for the OD values and PP values, but the ICCs are substantially higher for the PP values.

Antigen	Measurement scale	Pearson correlation	95% CI	Intraclass clustering coefficient (ICC)
T184	OD	0.95	0.91 – 0.97	0.90
	PP	0.95	0.91 – 0.97	0.95
T354A	OD	0.96	0.93 – 0.98	0.70
	PP	0.96	0.93 – 0.98	0.94
T3552B	OD	0.94	0.89 – 0.97	0.88
	PP	0.94	0.89 – 0.97	0.92
T56	OD	0.97	0.95 – 0.99	0.97
	PP	0.97	0.95 – 0.99	0.95
TG187	OD	0.99	0.98 – 0.99	0.85
	PP	0.99	0.98 – 0.99	0.97
ALL	OD	0.92	0.90 – 0.94	0.87
	PP	0.97	0.96 – 0.98	0.96

Table 2.5: Repeatability of the BDD treponeme ELISA. Each of five antigens were twice tested with 43 samples; the results for all antigens were also combined. Both the raw optical density (OD) and the percentage positive (PP) were analyzed. The Pearson correlation coefficient with corresponding 95% confidence intervals measures the strength of association between the measures; the ICC is a measure of the reliability of the test

2.4 Discussion

Our findings indicate that the populations of BDD-associated *Treponema* spp. are more diverse and widespread in the dairy farm environment than hitherto appreciated. This may explain differences in clinical presentation of the disease between farms or regions. It also concurs with the empirical finding that once a farm has acquired infection, elimination is unsuccessful.

Evidence from microscopic studies, immunohistochemistry (including *in-situ* hybridization) and microbiological investigation have left little doubt of the primary importance of various treponemes for the aetiology of BDD. Light microscopy and electron microscopy have previously shown variability in the morphotypes of treponemes (Read et al., 1992; Walker et al., 1995; Grund et al., 1995; Döpfer et al., 1997). Genetic studies of these treponemes, which have made increasing progress since 1997 due to advances in microbiological techniques, have identified a correspondingly large degree of antigenic variability. A consistent finding has been a clustering into three distinct phylogroups, which were identified as being closely related to the human treponemes *T. denticola*, *T. phagedenis* and *T. vincentii / medium* (Choi et al., 1997; Colligham

and Woodward, 1997; Collighan et al., 2000; Stamm et al., 2002; Trott et al., 2003; Edwards et al., 2003b). Several other phylotypes have been less commonly identified (Choi et al., 1997; Schrank et al., 1999).

Many questions remain. As isolation and culture of the bacteria has not been possible until relatively recently, it is unclear whether the bacteria have been present in the farm environment for a longer period; there is some evidence for this (Egerton and Parsonson, 1966). No reservoir of the bacteria has yet been identified. Likewise, there is no explanation for the close relatedness between the BDD-associated treponemes and human treponemes: it has not been possible to make any inferences regarding putative associations, or, in terms of bacterial evolution, a common ancestry. While microbiological description and characterization of a number of individual BDD-associated treponemes has been performed, we do not yet have any insights into the variability of the populations of these bacteria. Assuming they play a primary role in the aetiology of BDD, it is unclear whether representatives of the three antigenic groups all play a part in the pathogenesis of the disease, and if so, whether any of these groups is more important than others. The virulence determinants remain a matter of speculation. Initially, interest was focused on the BDD-associated, *T. denticola*-like treponemes; these bacteria are demonstrated pathogens in human oral periodontitis and possess a number of virulence factors (e.g. proteases) which made an aetiological role biologically plausible (Collighan and Woodward, 1997; Edwards et al., 2003a). However, a study investigating the *in situ* distribution indicated that these bacteria tended to remain in the more superficial parts of the lesion (Moter et al., 1998); and in the US, they were less frequently identified in lesion tissue (Stamm et al., 2002; Trott et al., 2003).

The objectives of our study were twofold: to further refine molecular detection techniques and serology, and to perform sampling of farms on which we were simultaneously conducting observational epidemiological studies. In comparison to the prior studies cited we have obtained more isolates from a larger number of farms. Combined analysis of the 16S rRNA sequence, the flagellin gene sequence, enzyme activities and serology enables us to make inferences about the molecular epidemiology of BDD-associated treponemes in our study area, where our interest is focused primarily on patterns in the populations of bacteria, rather than on characteristics of individual strains.

Microbial-level The findings of our study were entirely consistent with the literature: of the 23 isolates cultured by us, three very distinct groups were identified using 16S rRNA sequence analysis. We arbitrarily characterized these as groups 1, 2 and 3; they should perhaps be more correctly described as *T. medium*-like, *T. phagedenis*-like and *T. denticola*-like, respectively. Sequence similarity for the representatives of these groups was greater than 98%, whereas similarity between the groups was no more than

92%. We did not find any representatives of the remaining two phylotypes described by Choi et al. (1997), nor any isolates similar to *T. brennaborense* (Schrank et al., 1999).

Comparison of the flagellin gene sequences has not been performed by other researchers. As expected, this gene was less conserved; however, an identical grouping pattern emerged. There were two slight differences: the group 2 representatives appeared more closely related to *T. phagedenis*, while the group 1 representatives appeared somewhat less related to *T. medium* than on the basis of the 16S rRNA sequence.

The enzyme profiles showed that representatives of one group consistently expressed a number of enzymes; the pattern differed between the groups. Interestingly, the group profiles also differed from that of the most closely related human treponemes. The enzyme profile described for *T. brennaborense* (Schrank et al., 1999) is distinct from our profiles. The results of the two morphotypic groups described by Walker et al. (1995) would appear to be a mixture of a *T. phagedenis*-like strain and a *T. denticola*-like strain (referred to in the paper as 1-9185MED), and a mixture of a *T. phagedenis*-like strain and a *T. medium*-like strain (referred to in the paper as all non-1-9185MED isolates). Edwards et al. (2003a) performed enzyme assays on two *T. medium*-like isolates and a *T. denticola*-like isolate, and found the latter to produce trypsin and chymotrypsin, while both of the former did not; this is consistent with our results.

Electron microscopy performed by us on three representatives of the groups confirmed that they represent different morphotypes.

It has hitherto been unclear whether the three species-level clusters of BDD-associated *Treponema* spp. are separate species, or in fact strains of the human treponemes they are closely related to. Adopting the criterium of 98% similarity as a species cut-off, our phylogenetic analysis indicates that the *T. phagedenis*-like and *T. denticola*-like clusters can be designated as separate species; the *T. medium*-like cluster is borderline (98.4% similarity). In addition, our results have shown that the isolates cultured by us differ morphologically and in enzyme expression from the human treponeme species.

Cow-level It appears that simultaneous infections with treponemes from different antigenic groups are possible: on two occasions, we cultured isolates from two antigenic groups from a single lesion biopsy. In both cases, a group 3 (i.e. *T. denticola*-like) isolate was involved. The FISH study of Moter et al. (1998) showed these bacteria to be present in higher numbers in the superficial parts of the lesion. Our research did not investigate the spatial distribution of representatives of the three antigenic groups in the lesion.

We have demonstrated (by high Pearson correlation coefficients and ICCs) that the ELISA is repeatable and hence reliable, for the five individual antigens as well as the 'cocktail'. The variability of the test is small; it is precise, where precision relates to the consistency of the test (Dohoo et al., 2003; Thrusfield, 1997). Use of PPs is preferable

over raw ODs, as this compensates for differences in incubation time and standardizes the results across test plates. Also, the ICCs were substantially better for the PP values.

Our results of antigen-specific serology provided evidence of the existence of multiple serogroups / -types – there was marked similarity of ELISA results for representatives of each group. The group 2 isolates solicited the highest response, regardless of clinical status; this corresponds with the group that were identified by Moter et al. (1998) to penetrate most deeply in the lesion, and it is possible that they therefore bring about the strongest immunologic response.

While we showed clustering of ELISA results per antigenic group (Figure 2.3), it was apparent that there was substantial cross-reactivity. This is in sharp contrast with Walker et al. (1997), who performed adsorption studies using immune sera from calves immunized with two morphotypes, 1-9185MED and 2-1498; they found that the reaction of the ELISA was predominantly directed against the specific spirochaete group being evaluated. However, in view of subsequent findings which appear to indicate that neither of these groups constituted pure cultures (as mentioned above), it is difficult to interpret these results.

Several explanations are possible for our findings:

a) infection with one specific treponemal antigen elicits antibodies, which cross-react with antigens from the other groups (i.e. true cross-reactivity);

b) mixed infection with treponemal antigens from multiple groups elicits a mixture of antibodies, which specifically react with antigens from a single group;

c) mixed infection with treponemal antigens from multiple groups elicits a mixture of antibodies, which cross-react with antigens from other groups.

This could not be clarified, as we lacked information on the treponemes that may have infected the animals from which the test sera were taken.

In the absence of such information, interpretation of the serology is not straightforward. We are currently unable to distinguish between the serogroups. Serological diversity is well documented for *Brachyspira hyodysenteriae*, to which the treponemes are related (see Figure 2.1). More research is required to clarify whether comparable diversity exists for BDD-associated treponemal serology.

Also, we currently cannot explain the occurrence of false negative and false positive outcomes; a more detailed understanding of the humoral response is required. In initial studies (Walker et al., 1997; Demirkan, Walker, Murray, Blowey and Carter, 1999), the serology was strongly associated with clinical BDD. However, these, and more recent studies such as that of Trott et al. (2003), were experimental case-control studies rather than observational in design, and smaller numbers of animals were studied. It is therefore possible that these authors did not identify these inconsistent serologic results.

Another possibility is that in the initial period, study animals had only been relatively recently exposed to the infection, and hence showed a pattern consistent with primary infection; in the intervening years, the increasing morbidity and spread of the disease has caused animals to be repeatedly challenged by the causal *Treponema* spp., as a result of which the serology is not as clear-cut. Repeated tests of animals, such as performed during the longitudinal study (see Chapter 5), might provide clarification.

The 'cocktail' ELISA functioned well as a 'catch-all' test for the investigation of serology, and hence was adopted for use in routine testing for subsequent observational studies. By including antigenic representatives from the three treponemal species-level clusters, the assumption was therefore made that all have pathological importance. This assumption was based on the 'best available information', i.e. the fact that antigenic representatives from all three groups were cultured from BDD lesion tissue. There is currently no way to test this assumption, as we have not yet developed an understanding of the microbiological determinants of pathogenicity of the associated *Treponema* spp. The ELISA cannot discriminate between eventual differences in farm-level expression of antigenic strains. Should further research provide evidence that (a) specific strain(s) has specific significance, it may well be necessary to modify the ELISA, and re-evaluate the results obtained with the 'cocktail' ELISA.

Farm-level In our study, representatives of the group 1 (*T. medium*-like) treponemes were identified on only one farm (Table 2.4). On the other farms, group 2 (*T. phagedenis*-like) treponemes predominated; group 3 (*T. denticola*-like) treponemes were found less frequently. It is possible that the isolation and culture procedures selectively favoured *T. phagedenis*-like treponemes; however, we have no indications to believe this. We cultured multiple isolates from four of the ten farms; and identified more than one treponemal species on three of these. While these results represent a small sample, they suggest that farms are frequently infected by more than one treponemal species.

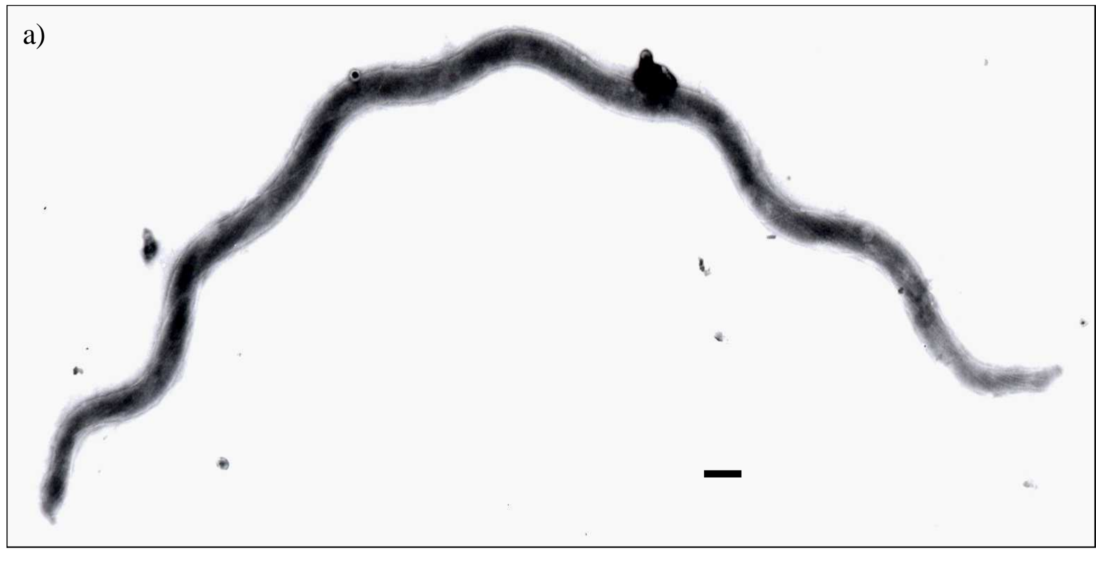
On all three farms from which different species were identified, a group 3 (*T. denticola*-like) treponeme was the secondary isolate; in two cases, they were cultured from the same lesion biopsy, i.e. appeared to constitute a mixed infection. In combination with the finding of Moter et al. (1998) that these bacteria were found more superficially in the lesion as well as our finding that they do not elicit as strong a humoral response, it is possible that these bacteria are of secondary significance for the aetiology and pathogenesis of BDD. Alternatively, they could play a role in the initiation of the infection, after which other treponemes become predominant. We cannot yet relate the isolates to specific clinical findings or farm-level morbidity of BDD. Trott et al. (2003) postulate about the relative importance of the treponemal groups as 'primary' and 'secondary' invaders, but as we currently lack information on predominating treponemal morphotypes which sequentially shift during the infection, we are unable to make any

inferences.

Choi et al. (1997) most frequently identified treponemes of the *T. denticola*-like phylotype. In contrast, the US-based studies (Walker et al., 1995; Stamm et al., 2002; Trott et al., 2003) did not commonly find these. The earlier work performed in the UK (Collighan and Woodward, 1997; Collighan et al., 2000) identified *T. denticola*-like treponemes; however, more recent results (Edwards et al., 2003a; Demirkan et al., 2006) also identified the *T. phagedenis*-like species.

To date, we have not been able to identify any reservoirs of infection. As persistence in the environment is unlikely due to the fastidious anaerobic nature of the treponemes, an animal-related source is more likely. Exploratory work performed by us has failed to locate treponemes related to those associated with BDD in any part of the gastrointestinal tract. The skin or urinary tract are other locations we have sampled, but have consistently failed to identify treponemes in. BDD lesions are currently the only known reservoirs of the treponemes; our current hypothesis therefore is that transmission occurs by dissemination of treponemes into the underfoot environment and 'foot to foot' infection. The frequent concurrent incidence of lesions on both feet supports this.

We are currently further developing tools that will enable us to extend the findings presented here. Observational studies are clarifying the within-herd distribution and transmission of infection (see Chapters 4, 5 and 6). Phylotype-specific PCRs are being developed, that will identify cow-related and environmental sources of BDD-associated treponemes. Eventually, this will allow us to determine the determinants of pathogenicity and identify reservoirs of infection. It is hoped that the aggregate of this information will provide clues about the bacterial evolution, possible explanations for emergence of the disease and assist the design of effective intervention strategies.



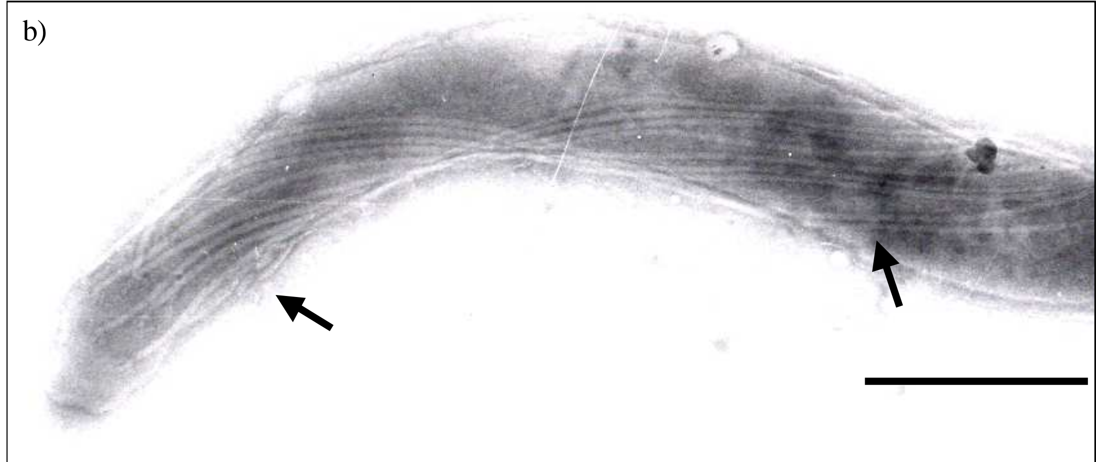
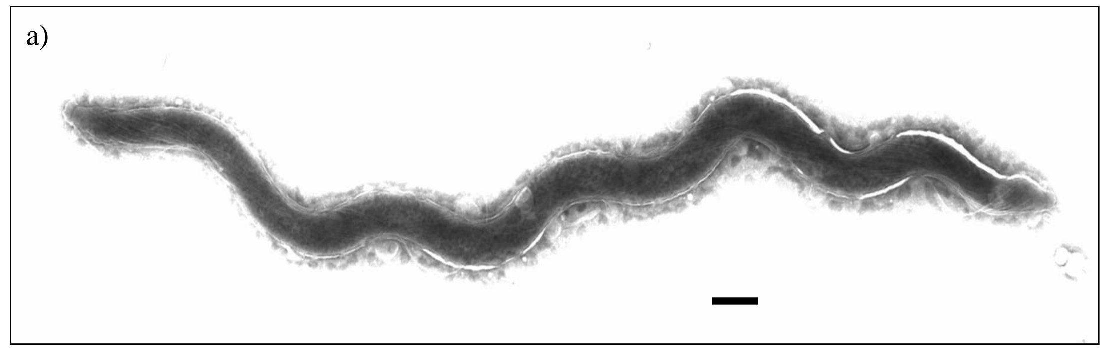


Figure 2.5: a) Electron micrograph of negatively stained cells of T19 (*T. medium*-like) isolate, b) showing 9 flagellar attached subterminally (indicated by arrows). Bars, 0.5μm



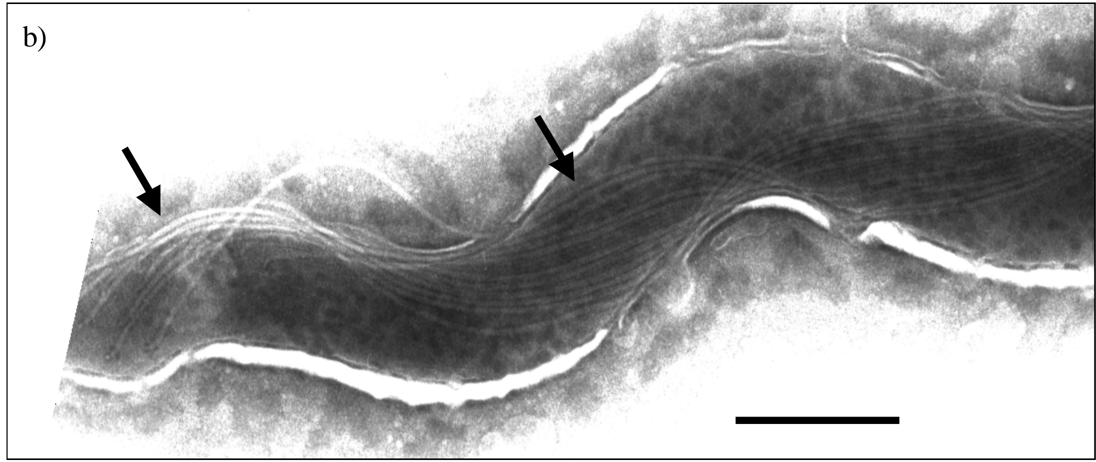


Figure 2.6: a) Electron micrograph of negatively stained cells of T320A (*T. phagedenis*-like) isolate, b) showing 11 flagellar attached subterminally (indicated by arrows). Bars, 0.5μm

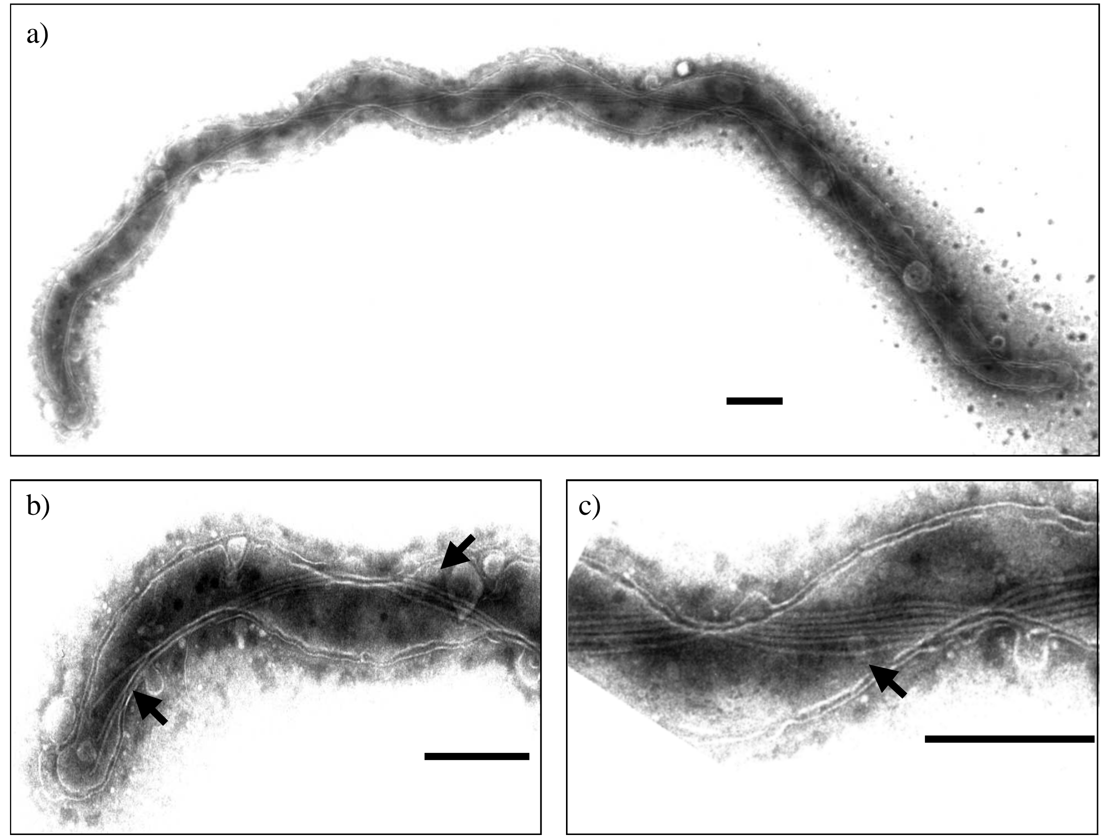


Figure 2.7: a) Electron micrograph of negatively stained cells of T3552B (*T. denticola*-like) isolate, b) to demonstrate the 3:6:3 flagellar pattern the end of the cell is shown with 3 flagellar attached subterminally, c) 6 flagellar are then shown across the centre of the cell. Flagellar are indicated by arrows. Bars, 0.5μm

Chapter 3

Revising the case definition of Bovine Digital Dermatitis: validation and assessment of diagnostic protocols

3.1 Introduction

The complexity of BDD and the methodological problems associated with its study (as outlined in the previous chapters) have impeded our understanding of its causality, which is still largely hypothetical. Similarly, it has hindered development of a comprehensive case definition. The presentation and progression of lesions have been clinically well described (Chapter 1), and diagnosis is based on visual inspection. However, although there have been attempts at describing and characterizing lesions systematically (Döpfer and Willemen, 1998), no uniformly accepted standard exists of classifying lesions, for instance on a scale of stage or severity. Lesions may be prominent and easily visible; however, in the early or late stages of lesion development, they are frequently small and easily missed. The location of the lesions means they are frequently masked from sight, particularly when environmental hygiene is poor; it is likely that the prevalence of the condition on dairy farms is therefore underestimated. Many dairy farmers tend to overlook BDD as a condition affecting welfare and production, and the best incentive for farmers to initiate effective control measures – tangible economic impacts – remain difficult to quantify.

Diagnostic assessment is the cornerstone of the analysis, interpretation and modelling of data generated by observational epidemiological studies. In this chapter, we investigate two contrasting diagnostic approaches: the first is based on clinical inspection for BDD lesions, and the second relies on serology of BDD-associated *Treponema* spp. We wish to differentiate between these, which necessitates specifying unambiguous definitions pertaining to BDD states.

We consider that an individual's *disease* status is defined by presence or absence of clinical BDD lesions (i.e. 'dis-ease' in the most literal sense); this can be directly observed by inspection. Therefore, we consider clinical inspection of the lifted foot to be the 'Gold Standard' for determining disease status.

The serologic identification of animals that are not diseased but have a high antibody titre indicates that absence of lesions may not necessarily imply absence of infection. It is reasonable to postulate that an individual's 'true' *infection* status is unobserved; we do not currently have the tools or knowledge to determine this status, which we therefore consider to be a latent variable. Consequently, no 'Gold Standard' test exists for determining infection status. We investigate the usefulness of serology for diagnosis, combined with clinical observation data, by fitting a model applying Bayesian techniques. The outputs of this model contribute to an improved case definition, and enable inferences to be made on the infection levels on the management group and farm levels (Chapter 4).

The differentiation between disease (which is verifiable, with a gold standard test) and infection (which is a latent variable and cannot be determined by a gold standard) is consistently applied throughout this chapter.

Performing clinical inspection of the foot is currently the only recognised diagnostic procedure for the disease. As lifting the feet is a time- and labour – intensive procedure, visual inspection of the feet of the standing animal is frequently performed. The only formal attempt at validation of this as a diagnostic test has been performed by Rodriguez-Lainz et al. (1998), who performed screening in the milk parlour of a dairy (described as the 'bright light, water jet' test) and compared the results to a more thorough clinical inspection of feet in a chute (n=117). The screening method had a sensitivity of 0.72, and a specificity of 0.99; the authors concluded that although of limited accuracy, visual screening was useful as a cost-efficient method of estimating BDD prevalence on the herd level. However, for the observational studies carried out within this project, we wished to have a higher degree of confidence in our observations, without needing to manually lift each foot. To achieve this, we made use of a rigid industrial borescope modified according to Blowey (Appendix C). This scope facilitates visualization of the 'typical' lesion area on the plantar digital skin in the standing animal, and is hence an improvement to the aforementioned 'bright light, water jet' test. We anticipated that it would have higher sensitivity (where lifting of the feet was taken as the reference 'Gold Standard'), yet be quicker and less demanding to perform than lifting the feet. As we employed this scope in both of our observational studies, it was desirable to quantify its diagnostic accuracy.

No diagnostics based on molecular detection techniques (e.g. serology, PCR) have yet been developed for routine application, firstly because the aetiology has not yet been confirmed, secondly because microbiological research of the assumed causative agents

(*Treponema* spp. bacteria) has been problematic, and thirdly because our understanding of the immunology is limited. An indirect ELISA has been tested using *Treponema* spp. antigen (Demirkan, Walker, Murray, Blowey and Carter, 1999; Dhawi et al., 2005; Laven et al., 2000; Murray et al., 2002; Walker et al., 1997). Investigation showed that antibody titres of lesion positive animals were significantly higher than lesion negative animals; the humoral response had a short duration, and was correlated with the presence of BDD lesions. Interestingly, there was also evidence that the antibody titre rises before clinical disease is apparent (Carter, unpublished data), which suggests that an animal may be exposed to infection before developing disease. This indicates that a subclinical or prodromal state may exist. This ELISA has been further developed (Chapter 2), but its discriminatory ability has not been investigated in population studies.

Bayesian approaches have been increasingly applied for serological modelling in recent years, particularly for diagnostic test validation in the absence of a 'Gold Standard' test (Branscum et al., 2004, 2005; Johnson et al., 2001; Joseph et al., 1995). Bayesian methodology requires the modelling of uncertainty with probability, and therefore implicitly allows for uncertainty about parameter estimates to be incorporated. Therefore, the unknown accuracies and prevalences are modelled by specifying prior probability distributions for all parameters of interest, i.e. sensitivities and specificities of the test and disease prevalences of the sample populations. Such models can easily accommodate unobserved variables such as an individual's true disease status, which is relevant to our problem. If available, scientific information about the prevalences of the sampled populations, derived from the literature or 'expert opinion', can be incorporated. This enables the prior probability distributions to be specified more precisely, resulting in a more accurate and robust model. In Bayesian analysis involving proportions, these prior distributions are often specified using of beta distributions, due to flexibility and ease of computation (Enøe et al., 2000).

Typically, validation of a diagnostic test based on serology involves specifying an appropriate cut-off point which has been optimized for the purposes of the test's application. As there is often considerable overlap in the serological distributions of the disease positive and disease negative sub-populations, the decision where to place this cut-off influences the sensitivity and specificity of the test; a relationship that is commonly investigated by making use of ROC (receiver operating characteristic) curves (Greiner et al., 2000). A relatively high threshold will improve the specificity at the cost of the sensitivity, and vice-versa. By dichotomizing the test, an individual test result is classified as either 'positive' or 'negative'; the degree by which this result falls above or below the threshold is not considered. This information, which is inherent in the continuous scale of the assay, is therefore discarded. In addition, there is a 'grey zone' of serologic outcomes which are near the cut-off; these are accordingly classified

as 'positive' or 'negative' although this may not correspond to the true disease status. Recent Bayesian approaches have developed methods which do not dichotomize, but rather assign a conditional probability of infection to an individual on the basis of the serologic test result (Choi et al., 2006). We apply this method.

3.2 Materials and methods

3.2.1 Validation of the borescope

The dataset used here was derived from a longitudinal study carried out on four commercial dairy farms in North West England (fully described in Chapter 5) between February 2004 and December 2005. These farms participated in a preceding cross-sectional study (see 3.2.2) and included two high clinical BDD prevalence farms, one medium prevalence farm and one low prevalence farm.

The methodology was as follows. Over the entire duration of the study, inspection with the borescope of the hind feet was performed on all animals at each sampling visit (standing in A.I. stalls on three farms, and in a crush on the fourth), after hosing down the feet to remove slurry. The front feet were visually inspected using a torch only due to the practical difficulty of using the scope; these results will not be considered here. The hind feet of on average every sixth animal were routinely lifted in a crush after inspection, irrespective of whether this indicated presence of lesions or not, corresponding to a total of 371 animals. Observed lesions were characterized on the basis of three criteria:

- *Size*: the greatest diameter of the lesion, in centimetres.
- *Presentation*: acute (erosive to beginning keratinization), chronic (granulomatous to proliferative), and regressing.
- *Location*: midway between heels, above inner or outer bulb, or other locations.

While clinical inspection of the lifted foot is currently considered to be the 'Gold Standard' test, we did not define it as such. We assessed the level of agreement between the observations using the borescope (predicted value) and the corresponding lesion status when picking up the feet (actual value) by calculating Cohen's kappa statistic, which is used as a means of determining the level of agreement of tests with categorical outcomes (Dohoo et al., 2003). A value of 1 indicates perfect agreement, and values > 0.75 are considered excellent agreement (Kirkwood and Sterne, 2003). Kappa was calculated for the binary outcomes of both tests. For tests measured on an ordinal scale where the categories are ordered (in this case, lesion presentation or size), it may be assumed that adjacent categories are less dissimilar than further removed ones. In such cases, a weighting factor may be incorporated to allow for partial agreement.

A weighted kappa was calculated for both lesion presentation and lesion size, using Fleiss-Cohen weights (Dohoo et al., 2003).

A simple graphic representation of the strength of agreement in the contingency tables was made by plotting agreement charts, which can be intuitively interpreted (Bangdiwala, 1987; Friendly, 2003).

3.2.2 Dataset used for the Bayesian model

The dataset used was derived from an in-depth population-based cross-sectional study carried out on eight commercial dairy farms in North West England and North Wales (fully described in Chapter 4, section 4.2.1).

Serum samples were taken from all animals on the study farms on the dates of sampling ($n = 2215$). They were analyzed with the 'cocktail' ELISA (see Chapter 2), as per the standardized protocol. Samples were tested in duplicate; duplicates that showed poor agreement were retested (for a description of criteria, see 4.2.4). The mean of the optical densities of the duplicates was expressed as a percentage of the plate reference positive sample, i.e. as a percentage positive (PP). Exploratory data analysis was carried out to investigate the serological distributions.

The clinical BDD status was determined by visual inspection, using the borescope, of a subset of animals from all management groups ($n = 609$). Not all feet were inspected due to time constraints. The inspection is described in 4.2.3. Observed lesions were characterized by the same criteria as described above.

Data on the foot hygiene score (FHS) and age of the animals were also recorded. For the final dataset, records with missing ELISA values (due to loss or test failure: $n = 11$) and missing age data ($n = 40$) were removed; a total of 2165 records remained.

3.2.3 Construction of the Bayesian model

Background

Our goal is to estimate the conditional probability of BDD infection of an individual in the absence of a 'Gold Standard' test. We applied two screening tests: serology and clinical inspection. A serologic test outcome was obtained for every animal; clinical BDD inspection was performed on a subset of animals. Applying latent class analysis, we assume both diagnostic tests to be imperfect indicators of the unobserved infection status, which we designate as a latent variable; and furthermore, that observed associations between the diagnostic tests are attributed to the unobserved heterogeneity induced by this latent variable. We therefore assume that the responses to the two tests are conditionally independent (Yang and Becker, 1997). As clinical manifestation of BDD and development of the humoral immune response are based on different pathophysiological mechanisms, this assumption is reasonable.

Model specification

Let I denote (unobserved) infection, and $\overline{I}$ denote no infection. Presence of apparent disease is defined by the presence of at least one lesion and noted as L, absence thereof as $\overline{L}$. We hypothesize that an animal is exposed to *Treponema* spp. and becomes infected before disease develops, and remains infected for (at least) as long as the animal shows apparent disease.

The serologic response is given by S (this is measured as the percentage positive, PP). The model enables an individual's probability of being truly infected to be made given the serologic outcome, i.e. $P(I \mid S)$, incorporating the data on L.

The shapes of serologic population distributions are presented in 3.3.2 below; a log transformation provides the closest approximation to the normal distribution. The log serologic mean is taken as μ and precision (which is defined as the inverse of the variance, i.e. $1/\sigma$) as τ, i.e.

$$S_i \sim \text{Normal}(\mu_i, \tau_i)$$

From Figure 3.3, it is apparent that the distributions differ for the infected and uninfected sub-populations. This is incorporated in the model as follows:

$$\mu_i = ((1 - I_i) * \mu_1) + (I_i * \mu_2)$$

$$\tau_i = ((1 - I_i) * \tau_1) + (I_i * \tau_2)$$

Hence, when $I_i = 0, S_0 \sim \text{Normal}(\mu_1, \tau_1)$ and if $I_i = 1, S_1 \sim \text{Normal}(\mu_2, \tau_2)$. From 3.3.2 below, we know that this is strictly not the case; but the log transformation provides the best approximation of normality.

Lesion status is binary, hence Bernoulli distributed with probability q_i of an animal having BDD lesions:

$$L_i \sim \text{Bernoulli}(q_i)$$

Presence or absence of lesions is modelled by logistic regression with two covariates, applying the standard logit link function:

$$\log\left(\frac{q_i}{1 - q_i}\right) = \beta_1 + (\beta_2 * xc_i) + (\beta_3 * zc_i)$$

Age and foot hygiene score (FHS) were selected as the explanatory variables, as initial statistical models showed these to be strongly associated with BDD. These variables were standardized; xc is the standardized and centred covariate of FHS, and zc is the standardized and centred covariate of age. In order to specify the coefficients β_1, β_2 and β_3, informed prior distributions were defined (see below).

We now need to specify the true infection status I in the model. As this is a dichotomous variable, it is Bernoulli distributed with probability p_i of an animal being infected, i.e.

$$I_i \sim \text{Bernoulli}(p_i)$$

We wish to express p_i as a function of q_i, as this can be quantified by specifying informed prior distributions. In probability terms, we express $P(I)$ as a function of $P(L)$, as follows:

$$P(I) = [P(L) * P(I \mid L)] + [P(\overline{L}) * P(I \mid \overline{L})]$$

As I is the 'true' status and L is the 'apparent' status, $P(I \mid L)$ is the predictive value positive (PVP) (i.e. corresponding to a / (a + b) in the 2x2 table below) and $P(\overline{I} \mid \overline{L})$ is the predictive value negative (PVN) (i.e. corresponding to d / (c + d) in the 2x2 table below).

		Infection		
		Positive	Negative	Total
BDD lesion	Positive	a	b	(a+b)
	Negative	c	d	(c+d)
	Total	(a+c)	(b+d)	(a+b+c+d)

Hence:

$$P(I) = [P(L) * \text{PVP}] + [(1 - P(L)) * (1 - \text{PVN})]$$

Sensitivity, Se ($P(L \mid I)$, or a / (a + c)) and specificity, Sp ($P(\overline{L} \mid \overline{I})$, or d / (b + d)) are innate test properties. PVP and PVN are diagnostic test characteristics which are dependent on the Se, the Sp and the study population prevalence, Prev. As the prevalence differs between populations, the PVP and PVN are not constant (Thrusfield, 1997). Incorporating PVP and PVN in our model would thus limit its applicability to our specific study population – this is undesirable. Therefore it is preferable to express PVP and PVN in terms of Se, Sp and Prev. This can be achieved by rearranging, as a result of which we have

$$\text{PVP} = \left(\frac{\text{Prev} * \text{Se}}{(\text{Prev} * \text{Se}) + [(1 - \text{Prev}) * (1 - \text{Sp})]} \right)$$

and likewise,

$$\text{PVN} = \left(\frac{(1 - \text{Prev}) * \text{Sp}}{[(1 - \text{Prev}) * \text{Sp}] + [\text{Prev} * (1 - \text{Se})]} \right)$$

Or in probability terms,

$$\text{PVP} = \left(\frac{\text{Prev} * P(L \mid I)}{(\text{Prev} * P(L \mid I)) + [(1 - \text{Prev}) * (1 - P(\overline{L} \mid \overline{I}))]} \right)$$

and likewise,

$$\text{PVN} = \left(\frac{(1 - \text{Prev}) * P(\overline{L} \mid \overline{I})}{[(1 - \text{Prev}) * P(\overline{L} \mid \overline{I})] + [\text{Prev} * (1 - P(L \mid I))]} \right)$$

We then substitute PVP and PVN with these formulas, thus bringing Se, Sp and Prev into the model.

As our hypothesis is that infected animals may not (yet) show lesions, the probability of observing false negatives (i.e. $P(\overline{L} \mid I)$) will be appreciable, therefore Se will be relatively low. On the other hand, it is improbable that animals with lesions are uninfected (few false positives, i.e. negligible $P(L \mid \overline{I})$), therefore Sp will be high. We apply a non-informative prior for Prev in order to extend applicability of this model to any population. We consider Se, Sp and Prev to be beta-distributed, since beta distributions provide a family of conjugate prior distributions for binomial distributions.

Although Rodriguez-Lainz et al. (1998) determined a Se of 0.72 and a Sp of 0.99 for their 'bright light, water jet test', and we expect our borescope to be more sensitive while maintaining good specificity, we specified our prior distributions for Se and Sp more conservatively. This is primarily because Rodriguez-Lainz et al. (1998) considered clinical inspection to be the 'Gold Standard'; as discussed above, we do not consider this to be the case. Our 'best estimate' for Se was 0.6 with a 95th percentile of 0.4, and for Sp, 0.9 with a 95th percentile of 0.8. The prior distribution for Se was kept relatively broad as there is more uncertainty around this parameter. Using these estimates, we obtained Se $\sim$ Beta$(10.9, 7.6)$ and Sp $\sim$ Beta$(42.6, 5.6)$. A distribution of Prev $\sim$ Beta$(1.9, 1.9)$ was taken for the prevalence.

Deriving informed prior distributions These were derived by utilizing information independent of the data. To perform this exercise, it is necessary to standardize the variables by centring. Starting with FHS, which we denote x:

$$xc_i = \frac{(x_i - \overline{x})}{\text{sd}(x)}$$

where $\text{sd}(x)$ is the standard deviation of x.

Consider the simple regression equation

$$\text{logit}(q_i) = b_1 + (b_2 * x_i)$$

Standardizing x_i as above and substituting with the standardized FHS xc_i,

$$\text{logit}(q_i) = b_1 + \left(b_2 * \frac{(x_i - \overline{x})}{\text{sd}(x)} \right)$$

Now let $\widetilde{Q}_1$ be the q_i for an animal with an 'average' value of FHS, where this is given by x_1, i.e. $\widetilde{Q}_1 = P\{L \mid (x_1 = \bar{x})\}$. We utilize experience on dairy farms in the study area to provide a 'best estimate' of $\widetilde{Q}_1$. As $x_1 = \overline{x}$, $\text{logit}(\widetilde{Q}_1) = b_1$. As we have prior information for $\widetilde{Q}_1$, we can now solve for b_1.

Now let $\widetilde{Q}_2$ be the q_i for an animal with a specific FHS that is 'above average', where this score is given by x_2, i.e. $\widetilde{Q}_2 = P\{L \mid (x_2 > \bar{x})\}$. As with $\widetilde{Q}_1$, we provide estimates and derive a distribution for $\widetilde{Q}_2$. As above,

$$\text{logit}(\widetilde{Q}_2) = b_1 + \left(b_2 * \frac{(x_2 - \bar{x})}{\text{sd}(x)}\right)$$

As $\text{logit}(\widetilde{Q}_1) = b_1$, we can substitute, rearrange and solve for b_2:

$$b_2 = [\text{logit}(\widetilde{Q}_2) - \text{logit}(\widetilde{Q}_1)] * \left(\frac{\text{sd}(x)}{x_2 - \bar{x}}\right)$$

This process is repeated when extending the model with the second covariate, age, denoted as z:

$$\text{logit}(q_i) = b_1 + (b_2 * xc_i) + (b_3 * zc_i)$$

or

$$\text{logit}(q_i) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right) + \left(b_3 * \frac{(z_i - \bar{z})}{\text{sd}(z)}\right)$$

where $\widetilde{Q}_3 = P\{L \mid (z_3 = \bar{z})\}$ and $\widetilde{Q}_4 = P\{L \mid (z_4 > \bar{z})\}$. So

$$\text{logit}(\widetilde{Q}_3) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right) + \left(b_3 * \frac{(\bar{z} - \bar{z})}{\text{sd}(z)}\right)$$

i.e.

$$\text{logit}(\widetilde{Q}_3) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right)$$

and

$$\text{logit}(\widetilde{Q}_4) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right) + \left(b_3 * \frac{(z_4 - \bar{z})}{\text{sd}(z)}\right)$$

therefore

$$\text{logit}(\widetilde{Q}_4) = \text{logit}(\widetilde{Q}_3) + \left(b_3 * \frac{(z_4 - \bar{z})}{\text{sd}(z)}\right)$$

so rearranging and solving for b_3 we get

$$b_3 = [\text{logit}(\widetilde{Q}_4) - \text{logit}(\widetilde{Q}_3)] * \left(\frac{\text{sd}(z)}{z_4 - \bar{z}}\right)$$

The model was implemented in OpenBUGS software, and the beta distributions were determined using BetaBuster. The prior distributions for average and 'dirty' FHS ($\widetilde{Q}_1$ and $\widetilde{Q}_2$) and average age and older cows ($\widetilde{Q}_3$ and $\widetilde{Q}_4$) were based on 'best estimate' information; they will not be reproduced here, but are given in Appendix D, along with the full model code.

3.3 Results

3.3.1 Validation of the borescope

Contingency tables containing data about actual (i.e. characterization of lesion presentation and size from the lifted foot) versus predicted (i.e. corresponding observations using the borescope) classifications are shown in Tables 3.1, 3.2 and 3.3. Matrices for the binary outcome of absence or presence of lesion and for the ordinal outcomes of lesion presentation and size are presented. As the lesion location was almost always midway between the heel bulbs, directly proximal to the interdigital cleft, location was discounted from further analysis.

		Borescope		
		Negative	Positive	Total
Inspection	Negative	270	16	286
	Positive	7	78	85
	Total	277	94	371

Table 3.1: 2x2 table for comparing observations of BDD lesions by borescope and clinical inspection of the lifted foot

For the binary outcome (Table 3.1), McNemar's χ^2 test was carried out to assess whether test bias exists, i.e. whether the proportions positive of these paired tests are significantly different (Dohoo et al., 2003). The test value was 3.53, with a binomial P-value of 0.10. The kappa value was 0.83.

The kappa values for the ordinal outcomes (Tables 3.2 and 3.3) are of a similar order of size as the binary outcome, and show that agreement between the actual clinical status and the observation made by the borescope are good to excellent. Applying Fleiss-Cohen weights has no effect for lesion presentation, but does improve the kappa of lesion size.

		Borescope				
		Negative	Acute	Chronic	Regressing	Total
Inspection	Negative	270	0	2	14	286
	Acute	1	16	3	0	20
	Chronic	1	0	22	1	24
	Regressing	5	0	7	29	41
	Total	277	16	34	44	371

Table 3.2: Test agreement of BDD lesion presentation as characterized by borescope and clinical inspection of the lifted foot (unweighted kappa: 0.77; weighted kappa: 0.77)

		Borescope					
		Negative	< 2 cm	2 to 3 cm	3 to 4cm	> 4cm	Total
	Negative	270	15	1	0	0	286
	≤ 2 cm	6	44	3	0	0	53
Inspection	>2 to 3 cm	0	9	12	1	0	22
	>3 to 4 cm	0	0	4	3	0	7
	> 4 cm	0	0	0	1	1	2
	Total	276	68	20	5	1	370

Table 3.3: Test agreement of BDD lesion diameter as characterized by borescope and clinical inspection of the lifted foot (unweighted kappa: 0.73; weighted kappa: 0.80)

Agreement charts (Figure 3.1) provide a simple graphic representation of the strength of agreement in a contingency table (Bangdiwala, 1987). Black squares showing observed agreement are positioned within larger rectangles which show the maximum possible agreement. Partial agreement (analogous to the weighted kappa) can be incorporated by grey shaded rectangles. The sizes of the boxes are proportional to the corresponding number of observations.

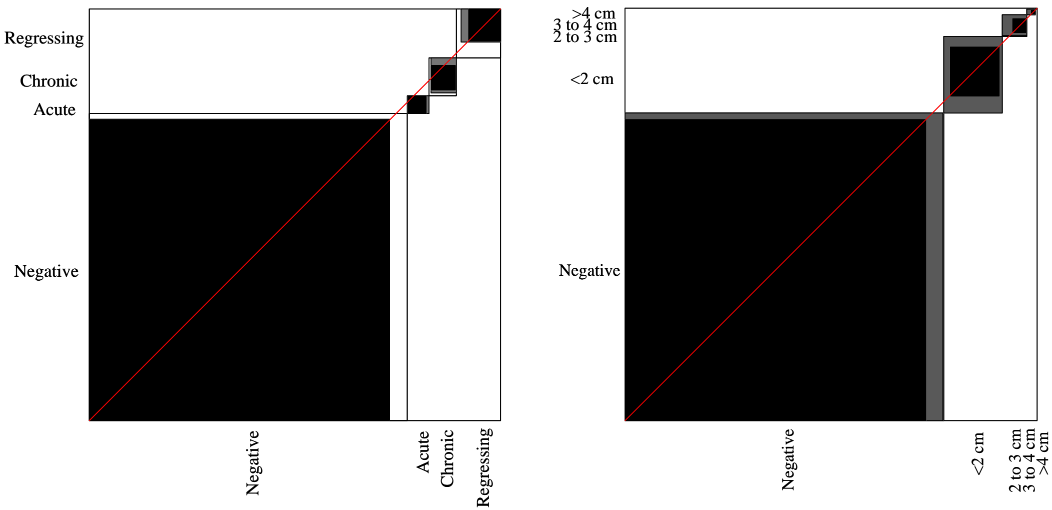


Figure 3.1: Agreement plots of lesion presentation (left) and lesion size (right). Borescope observations on the horizontal axis; lifted foot observations on the vertical axis. The white squares show maximum possible agreement; grey squares show partial agreement (analogous to weighting); and black squares show observed agreement. Systematic over- or underestimation by one test would result in deviation from the diagonal

3.3.2 Serologic frequency distributions

Figure 3.2 shows untransformed and log transformed serologic relative frequency distributions of the whole study population. The untransformed distribution has a suggestion of bimodality; we hypothesize that this is due to the mixture of uninfected and infected sub-populations, where the uninfected sub-population is strongly right-skewed and the positive sub-population appears to be more normally distributed. The distributions of the eight individual farms were comparable, although low prevalence farms were more strongly right-skewed and high prevalence farms were more normally distributed. Distributions of the individual farms are given in Appendix F. Due to the bimodal nature of the distribution, it could not be normalized through an appropriate transformation. However, the log transformation approximates a bivariate normal distribution, which would be represented as a two-dimensional normal distribution in which the negative and positive sub-populations are normally distributed and independent of each other. For this reason, the log transformed values were used, and a normal distribution was assumed in the model (see Appendix D).

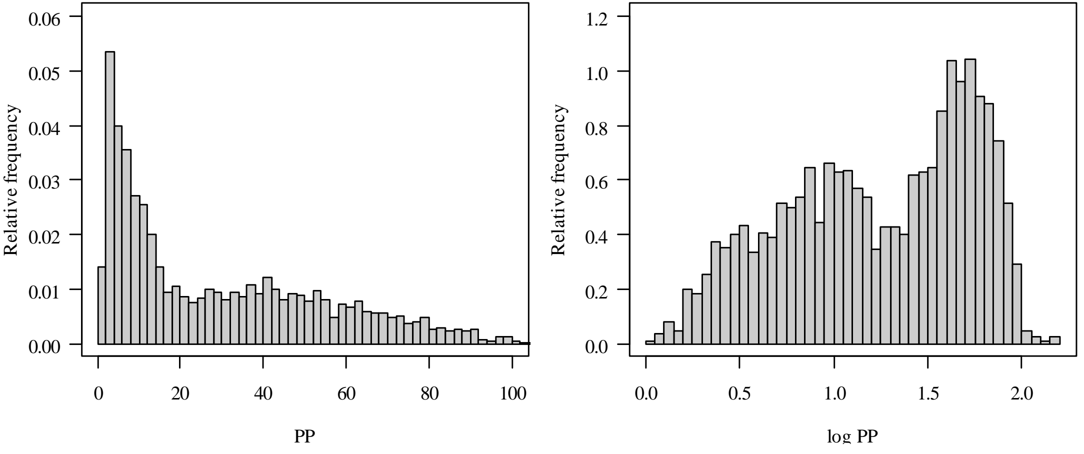


Figure 3.2: Relative frequency histograms of the ELISA of the entire study population ($n = 2215$), showing untransformed (left) and log transformed (right) distributions

Figure 3.3 shows the serologic relative frequency distributions of clinical positive (diseased) and clinical negative (non-diseased) animals. The antibody titre was significantly higher for the diseased group, but there was a large degree of overlap between the sub-populations. The distributions of the diseased and non-diseased groups was as we had expected, i.e. normal and right-skewed, respectively.

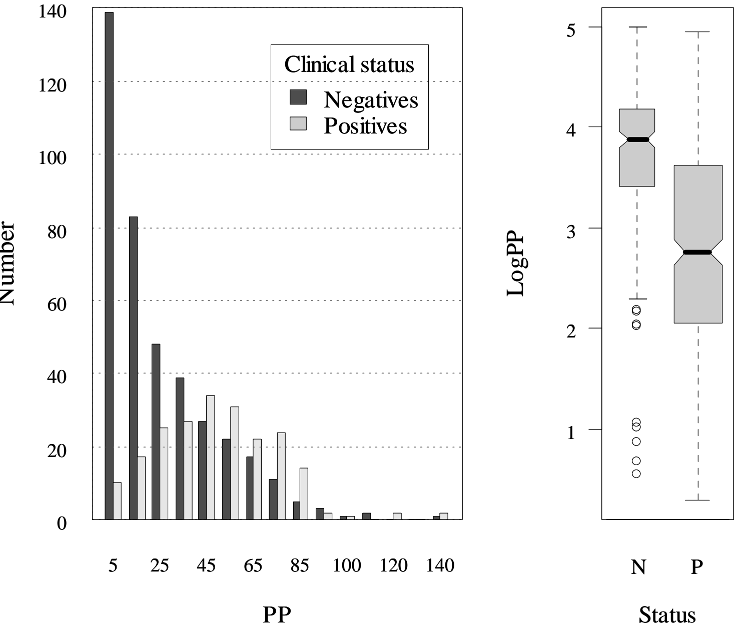


Figure 3.3: Relative frequency histogram and notched boxplots of the ELISA of animals clinically inspected for BDD (n=609), classified into clinical negatives (non-diseased) and clinical positives (diseased). The length of the notch along the box is a graphical representation of a confidence interval about the median of a sample: statistically, if the notches on side-by-side boxplots do not overlap then the medians will be significantly different. The width of the box is proportional to the the square root of the number of observations

3.3.3 Bayesian analysis of serology

The model converged well and gave sensible output. The final model output was taken from a run of 20000 iterations, of which the first 250 were discarded as the burn-in phase. Graphical output relevant to model performance (trace plots, autocorrelation plots and quantile plots of model variables) is given in Appendix D.

The posterior densities of the sensitivity, specificity, positive and negative predictive values, and the prevalence are given in Figure 3.4. The model outputs are summarized in Table 3.4.

The mean probability of infection of an individual with average foot hygiene score (about 39%) was higher than the prior estimate of 30%. A similar pattern was found for an individual with high foot hygiene score (posterior of 53%, where the prior estimate was 45%). For average age, the posterior model estimate of approximately 26% was lower than the prior estimate of 35%; but for older cows, the posterior of 42% was higher than the prior estimate of 30%.

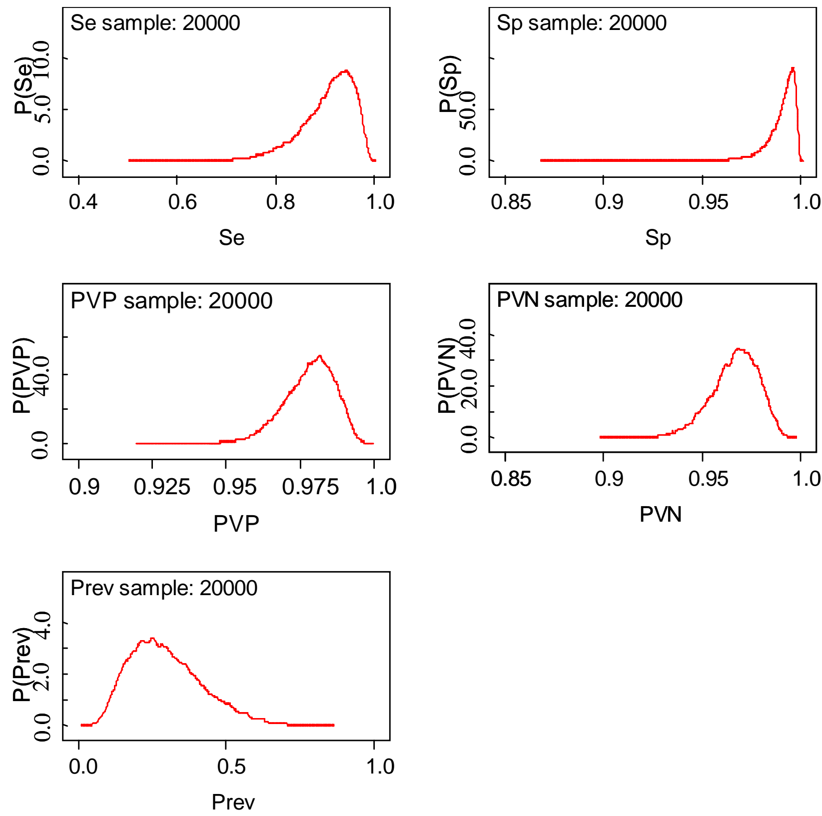


Figure 3.4: Kernel density plots of model estimates of sensitivity, specificity, predictive value positive, predictive value negative and prevalence

	Mean	Standard deviation	MC error	2.5 %ile	Median	97.5 %ile
Prevalence	0.30	0.12	0.0029	0.11	0.29	0.58
Sensitivity	0.90	0.06	0.0012	0.77	0.92	0.98
Specificity	0.99	0.01	0.0002	0.97	0.99	0.99
PVP	0.98	0.01	0.0001	0.96	0.98	0.99
PVN	0.97	0.01	0.0001	0.94	0.97	0.99
Average FHS	0.39	0.02	0.0003	0.36	0.39	0.42
High FHS	0.53	0.02	0.0003	0.49	0.53	0.57
Average age	0.27	0.06	0.0035	0.15	0.26	0.40
High age	0.42	0.09	0.0049	0.24	0.42	0.60

Table 3.4: Summary statistics as estimated by the Bayesian model

Figure 3.5 shows the estimated probabilities of infection given the serologic outcome for lesion negative animals (n=376), lesion positive animals (n=208) and all animals, regardless of whether they were inspected or not (n=2160). The lines were fitted with a smoothing spline. For a representative sample, this curve can be used predictively to

assess the conditional probability of infection given the serologic outcome. The curves almost overlap; this implies that additional information on the lesion status is not a requirement for making an inference of an animal's conditional probability of infection. For a PP of approximately 20%, there is a probability of about 20% that the animal is infected; for a PP of approximately 30%, this probability of infection has risen to about 80%. The PP interval between 20% and 30% is the 'grey' zone which is difficult to interpret.

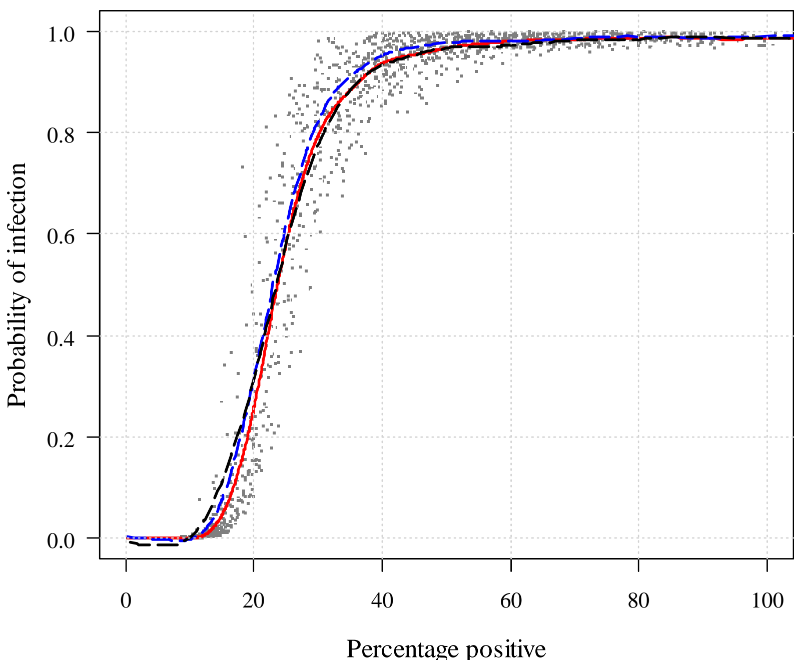


Figure 3.5: Probability of infection plot as a function of serology, given the data. The blue dashed line represents the predictive probability of infection (PPI) of lesion negative animals (i.e. $P(I|S, L = 0,\ \text{data})$; n=376); the dashed black line represents the PPI of lesion positive animals ($P(I|S, L = 1,\ \text{data})$; n=208). The red line represents the PPI of all animals, regardless of lesion status ($P(I|S,\ \text{data})$; n=2160). All lines were fitted using a smoothing spline; the grey points represent the conditional probability of infection given its serologic test result for every individual animal

It is also interesting to correlate the lesion presentation, as determined by inspection, with the probability of infection, as estimated by the model. A graphic of this relationship is presented in Figure 3.6. The model classified animals with acute and chronic lesions with a high conditional probability of infection or CPI (small interquartile range). However, negative animals, or those with regressing lesions, showed a wide interquartile range of CPIs, depending on the serologic result.

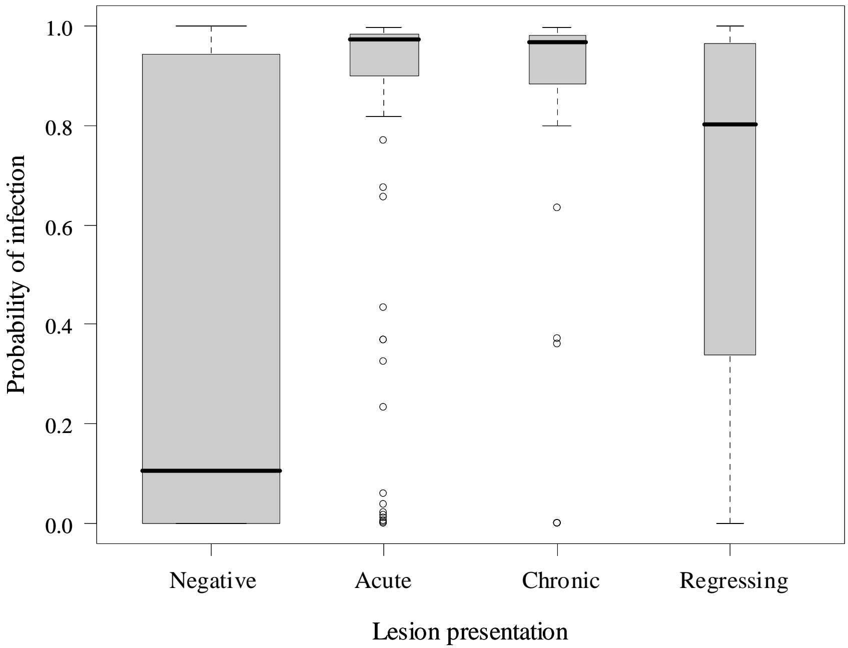


Figure 3.6: All animals inspected for BDD lesions (n=609) were classified as negative or having acute, chronic or regressing lesions. The conditional probability of infection (CPI) was obtained for each animal from the Bayesian model, and boxplots were plotted

3.4 Discussion

3.4.1 Utilization of the borescope for visual inspection of lesions

The borescope was initially employed as a labour- and time – saving device. Although useful in this experimental setting, this instrument is not one that will have a widespread practical application. It was necessary to perform the validation exercise because the foot inspection data which were used in the Bayesian model and in other statistical models of the cross-sectional and longitudinal study datasets (see Chapters 4 and 5) were obtained using this method.

The values of the kappa statistic indicate that there is excellent agreement between observations made with the borescope and of the lifted foot; kappa is of the same order of magnitude when binary or ordinal outcomes are considered, or when the size of the lesion is considered. For the binary outcome, McNemar's χ^2 test is non-significant (which indicates that the two proportions positive are not different). This is a consequence of the relatively high number of false positives with the borescope: these were instances where the borescope showed what appeared to be lesions, which upon inspection of the lifted foot were classified as regressed or 'old' lesions.

For the ordinal outcomes, weighting has no effect for lesion presentation, but does improve the kappa of lesion size. This indicates that presentation is less likely to be

classified into adjacent categories i.e. is more consistently classified, while size is more variable – this is a possible effect of the slight distortion caused by magnification of the image.

Within the agreement charts, the observed agreement (black squares) corresponds almost entirely with the maximum possible agreement (white squares); which is a reflection of the kappa statistic. For lesion presentation, weighting makes very little difference and hence grey shading (which signifies partial agreement) is nearly absent; for lesion size, the effect of weighting is much larger, which results in substantial partial agreement. As the squares lie on or very near the diagonal line, the two tests (borescope and inspection) consistently tend to classify observations into the same categories.

It is relevant to address possible sources of bias which could affect the outcome of the validation. Random sampling was performed, which should eliminate selection bias; this was carried out on all study farms. As all observations were made by a single observer, no observer bias between borescope and clinical observations would be expected; although the cumulative experience over the study duration of using the scope may have influenced the outcomes. The observations for the validation were made during the longitudinal study: repeated observations of the cows implies that detection bias is certainly a possibility. However, the number of cows entered in the study and the variable and fast-changing presentation of the lesions were such that detection bias is unlikely to have been a major problem.

3.4.2 Bayesian analysis of serology

Foot inspection still necessitates restraining cows in a crush or A.I. stalls, cleaning their feet and performing the inspection. Even if an instrument such as the borescope reduces the amount of effort required to perform an inspection, taking a blood sample for serology is even more straightforward and less labour intensive. In addition, should a preclinical or latent state exist which is missed by clinical inspection, serology may provide us with more sensitive and specific information.

We have previously investigated and developed an improved ELISA (Chapter 2). Using the observations of the foot inspections as well as the serologic outcome of the ELISA, we have here developed a model which enables us to make an inference about the conditional probability of infection on the basis of the serologic result alone. We have chosen not to dichotomize the diagnostic test as this would lead to loss of information; hence we make an inference of the probability of infection given the serologic result (rather than classifying an animals as seropositive or seronegative using a predetermined cut-off). This is an appropriate approach: the high degree of overlap in the serological distributions of clinical positive and clinical negative animals would mean that determining cut-offs would inevitably lead to low sensitivity or specificity.

The model's posterior estimates for sensitivity and specificity were higher and nar-

rower than our prior estimates. The prior distribution for the sensitivity was rather conservative; however, the model estimated the sensitivity at about 90%. As expected, the specificity was very high.

Our prior estimates for foot hygiene score were approximately 10% lower than the posterior model estimates. On the basis of EDA (see Chapter 4), we had anticipated advancing age to exert a protective effect; this was contradicted by the model results.

Referring to the PPI plot in Figure 3.5, there is an interval of serologic outcomes (roughly from PP 20 to 30) within which it is difficult to make inferences about the conditional probability of infection (CPI); if we were to dichotomize, the cut-off would likely be placed in this range. However, the PPI plot has quite a steep gradient, which is desirable to limit this indeterminate interval.

Using this model, a predictive probability of infection can be determined, given a serologic test outcome (i.e. $P(I|S)$). However, this does not provide us with information on the predictive probability that the animal has clinical BDD lesions (i.e. $P(L|S)$), which is a requirement for diagnostic application of the test. Without a better understanding of the humoral response and its relation to the development of clinical lesions over time, the diagnostic usefulness of the ELISA is limited.

Given our current understanding of the biology of the disease (multifactorial, polymicrobial, with titres possibly rising before clinical lesions are evident), incorporation of true infection status as a latent variable was appropriate. Furthermore, in this scenario a probabilistic, Bayesian approach is attractive.

Informed prior distributions for the model were based on both the literature and on the author's experience on dairy farms in the study region. The inherent risk of using such a 'best guess' approach is of mis-specifying these parameters. To investigate the effects of the specified priors on the model output, a range of distributions was investigated for the different parameters; the model remained stable and robust, continued to converge, and produced very comparable output.

Assuming that the study population is representative of the target population, the model has been correctly specified and the priors have been defined using reliable information, the conditional probability of infection curve developed here can be applied to ELISA results of samples from other dairy cows in the UK, i.e. it can be used predictively. If available information of another population to be studied were to differ markedly from the assumptions used to derive the priors here, these could be easily modified.

Interpretation of the boxplots of probability of infection versus lesion presentation (Figure 3.6) highlights some interesting points. Lesion negatives have a low median probability of infection, but have a large interquartile range; in other words, the model identifies a number of clinically negative animals as having a high probability of infection (on the basis of a high serologic result). Animals with acute and chronic lesions

have high probabilities of infection, but with some negative outliers; as we assume that the presence of lesions confirms disease, the outliers either represent animals that did not seroconvert, or are an artefact of the ELISA. Regressing lesions have a significantly lower probability of infection than acute and chronic lesions, and the range is larger. This implies that there is more variability, which is to expected as the distinction between regressing lesions and 'old' lesions or lesion scars is a subjective one. This has implications for the mathematical model discussed in Chapter 6.

The greater variability of CPIs for the negative and regressing lesions groups presumably reflects animals transferring from uninfected to infected states (or vice-versa). Whereas the model appears to identify infected animals (low variability of CPIs for animals with acute and chronic lesions), the model is less able to identify the infectious status of clinically negative animals, or animals with regressing lesions.

3.4.3 Case definition of BDD

Case definition was previously based on clinical inspection alone. We have developed serology for diagnostic application (Chapter 2); we have applied and investigated this here. Furthermore, we have included both clinical inspection and serology in a Bayesian model, incorporating 'true' infection status as a latent variable. Thus we can now make comparisons between clinical inspection, serology and latent infection status:

- *Relationship between serology and clinical disease.* Previous experimental evidence indicated that titres rose before manifestation of BDD lesions, and decayed quickly after regression; this finding gained plausibility when preliminary inspection of the cross-sectional study dataset identified animals without lesions with high antibody titres, and vice-versa. This was confirmed during exploratory data analysis, which showed considerable overlap between the serological distributions of clinically negative and positive animals (Figure 3.3). It was clear that the humoral response was complex, and that interpretation was not straightforward. However, there were sufficient indications to suggest that an understanding of serology might reveal useful epidemiological insights. Serologic analysis of the longitudinal study dataset revealed more insights; these are discussed in Chapter 6.

- *Relationship between clinical disease and infection status.* From the Bayesian model output in Figure 3.6, the probabilities of infection of animals with acute and chronic lesions are very high, which is to be expected. However, the probabilities of infection of animals with no or regressing lesions are very variable. According to the model, clinical lesion status is not the best predictor of the probability of infection.

- *Relationship between serology and infection status.* The steepness of curve in Figure **??** and the clustering of points along the line indicate that serology is more effective than inspection for clinical disease for differentiating between animals with high or low probability of infection. However, interpretation of PP results in the 20 to 30 range is difficult without extra information on history and lesion status.

BDD is a dynamic disease both clinically and serologically: the findings change rapidly over time, and not necessarily in conjunction with each other. For outcomes that are clear-cut (i.e. no lesion with low titre or lesion with high titre), interpretation is straightforward; but in other cases, this becomes problematic in the absence of additional relevant information. For a cross-sectional study, where the infection status is determined at a single point in time, such information is unavailable.

The serological work developed in Chapter 2 and the Bayesian model presented here indicate that basing the case definition on clinical inspection alone is inadequate for a fundamental understanding of the epidemiology of BDD. On the other hand, the high degree of overlap between the serological distributions of clinical positive and clinical negative sub-populations makes interpretation of serology on its own problematic. However, it is clear that serology is an informative tool for diagnostic assessment when used in conjunction with inspection. The complexity of the disease (specifically, our limited understanding of infection dynamics and immunity) is such that we cannot yet apply a definitive or universal case definition. Under our current understanding of BDD, modelling the infection status as a latent variable is a pertinent option, particularly when performing this within a Bayesian framework.

Chapter 4

Investigation of farm-level distribution and risk factors for BDD in UK dairy herds

4.1 Introduction

The causal mechanisms of BDD are not clearly understood, but the disease is evidently multifactorial and numerous risk factors have been identified. The putative causal web presented in Chapter 1.3 incorporates plausible risk factors as derived and interpreted from the literature, and provides a frame of reference for further investigation. It can inform the design of epidemiological studies by determining possible risk factors and variables of interest to be studied, as well as identifying possible confounders and sources of interaction. This is a useful conceptual exercise and can assist the design and formulation of appropriate statistical models (Dohoo et al., 2003).

Such an approach has not been taken for previous epidemiological observational studies which have investigated risk factors for BDD. This may be because these studies addressed broader objectives (e.g. concurrently investigating various infectious conditions causing lameness, such as Frankena et al. (1991) or Somers et al. (2005)), and / or were primarily interested in management, husbandry and production factors (the same authors, and Rodriguez-Lainz et al., (1996); Rodriguez-Lainz et al. (1998, 1999)). Further limitations of these studies were that they only considered the disease in the lactating cow groups and excluded the young stock, and hence did not study the distribution of the disease on the farm level. In addition, they relied on visual inspection for clinical lesions only. Relevant findings on risk factors from these studies are summarised in Chapter 1.2.4.

The limited understanding of the fundamental disease dynamics is an impediment to the interpretation of findings presented in such studies. While significant risk factors have been identified, and we have empirical knowledge of effective control strategies, we have very little understanding of the underlying mechanisms which explain their action.

The emphasis of the observational studies described in this thesis is to improve our understanding of the determinants, distribution and transmission of infection. Through the microbiological work (described in Chapter 2), we have attained better insights into the pathogenic *Treponema* spp. bacteria that are considered the 'necessary cause'; we have utilized this information to develop serology for diagnostic screening (Chapter 3), which has in turn improved our case definition.

In this section, the results of a cross-sectional study will be presented. The farm-level prevalence and distribution of BDD will be described using clinical inspection data as well as serological data. Exploratory data analysis (EDA) is a useful tool for visualizing such data. Statistical models are presented to investigate risk factors as presented in the causal diagram formulated in Chapter 1.3. The Bayesian model for making inferences of an individual's conditional probability of 'true' infection, as described in Chapter 3.2.3, will be extended to assess probabilities of infection on the management group and herd levels.

4.2 Materials and methods

4.2.1 Study farm selection and sampling populations

A detailed cross-sectional study of eight farms was carried out in North West England and North Wales (see Appendix B) towards the end of the housing season (March to May 2003 and February and March 2004), when the prevalence of BDD was expected to be high.

Information from regional veterinary practices was utilized to identify farms of different prevalence level (four high, two medium, one low and one negative). All farms were commercial dairy operations using cubicle type housing, excepting the negative control farm, which used a straw yard system (this farm was selected as it was the only one which could be verified with a high degree of confidence to be clinically negative). Therefore, although selection of participating farms was not random, it was assumed that the study population was representative of, or had similar characteristics to, the larger population.

4.2.2 Data collection

During an initial visit to each farm, a structured questionnaire was performed by interview (see Appendix E.1.1). Data relevant to BDD were collected for inclusion as group- and farm-level explanatory variables in the statistical analysis; they included husbandry factors (e.g. cleanliness and hygiene of housing) and management procedures (e.g. buying in stock, movement of animals between groups, footbathing procedures, foot care, BDD farm history and control).

Data on the dates of birth were obtained for all animals included in the study. More

detailed cow records including parity, last calving date and individual milk production were acquired where possible; however, two farms did not have automated record keeping systems, and these data could not be retrieved.

After administering the questionnaire, an inspection of the farm premises was performed (see Appendix E.1.2). The housing of all management groups was assessed according to a standardized checklist and factors pertinent to hygiene and comfort were scored.

4.2.3 Sampling protocol

Blood samples were obtained by coccygeal venepuncture into 7 ml vacutainer tubes (Becton Dickinson, UK). All cattle on the study farms at the time of sampling were bled; a total of 2217 animals were included in the study (see Table 4.1).

BDD prevalence level	Farm ID	No. in group					
		unweaned calves	weaned calves	heifers	lactating cows	dry cows	total
High	01	10	39	8†	174	14	245
	04	23	22	110	185	29	369
	06	0†	0†	16	215	75	306
	08	17	23	43	86	16	185
Medium	02	26	59	34	136	19	274
	05	8	17	66	174	17	282
Low	07	0†	0†	5	127	35	167
Free	03	34	60	74	162	59	389
Overall	All	118	220	356	1259	264	2217

Table 4.1: Population details of cross-sectional study farms †stock reared on separate premises and could not be sampled

Body hygiene scoring of all animals was performed, according to a protocol adapted from the literature (Chaplin et al., 2000; Eicher et al., 2001; Fregonesi and Leaver, 2001; Hughes, 2001; Jordan et al., 1999; Lowe et al., 2001; Schreiner and Ruegg, 2002). Four areas (flank, hind leg, udder and tail area) were assessed on left and right side and scored on a four-point scale (1: very clean / minimal dirt; 2: mostly clean with some fresh soiling; 3: mostly soiled with superficial dirt; 4: very soiled with caked-on dirt). Foot hygiene was recorded for all animals on a similar four-point scale.

A visual inspection of the feet was carried out of the feet of a subset of animals from all management groups (n=609). Not all feet were inspected due to time constraints. Inspection was performed on the standing animal, after hosing down the feet to remove slurry (if necessary). Hind feet were inspected using the borescope, following

the protocol described in Chapter 3 and Appendix C. The front feet were inspected by torch, as use of the borescope was not practicable. For the cows, on average every third individual was inspected, corresponding to a predetermined place or order in the restraint facility (A.I. stalls, race, crush or parlour)(n=504). For the young stock, selection was performed by simple random sampling (calves, n=30 and heifers, n=75). All observations were performed by the author.

A clinical description of BDD lesions is provided in Chapter 1.2.2. A lesion scoring system was adapted from those devised by other researchers (Dopfer, 1994; Laven and Hunt, 2002; Shearer and Hernandez, 2000). Briefly, observed lesions were characterized on the basis of three criteria:

- *Size*: the greatest diameter of the lesion, in centimetres.
- *Presentation*: acute (erosive to beginning keratinization), chronic (granulomatous to proliferative), and regressing.
- *Location*: midway between heels, above inner our outer bulb, or other locations.

A sampling record form is reproduced in Appendix E.1.3.

4.2.4 Laboratory analysis

The blood samples were left to settle overnight at 4°C. Aliquots of serum were obtained by centrifuging at 3000 rpm for 6 minutes and pipetting into storage tubes (Eppendorf). The method of the indirect ELISA was followed as described in Chapter 2; the 'cocktail' of antigens was used to coat the plates, as prior investigation showed this to function consistently as a 'catch-all'.

All serum samples were tested in duplicate; samples which showed poor replicate agreement were retested. This was assessed not just on the absolute difference between the OD values, as the difference between highly-reacting samples tended to be naturally larger than lower reacting samples (see Chapter 2.3.2), and this was hence not a good criterium. Neither was replicate agreement assessed by the relative difference (expressed as a percentage) between the OD values, as a small absolute difference in the lower range would translate to a large percentage difference. The methodology used was as follows: the higher OD of the duplicate samples was used to calculate the PP (by dividing it with the plate reference positive OD and multiplying by 100). These were then stratified into three levels: upper (positive) range (PP>50), middle (undecided) range (PP 40-50) and lower (negative) range (PP<40). A relative difference between the duplicate OD values was then specified for each level as a cut-off for retesting (7% for the upper range, 9% for the middle range and 12% difference for the lower range). As the middle range was considered to be undecided and hence critical, the criteria for retesting were more conservatively defined.

After exclusion of broken, lost or unidentifiable sample tubes, 2209 samples were tested by ELISA. 55 samples were retested due to poor duplicate agreement. A further 5 samples were excluded from analysis due to test failure – i.e. after repeated analysis, the duplicates still showed poor agreement. The final test result was expressed as the PP of the mean of the duplicates.

4.2.5 Statistical analysis

Data handling and validation

The final dataset consisted of 2204 animal records; a unique ID was assigned to each record. Table 4.2 lists the variables collected in each record. Of the animal level variables, body hygiene score, foot hygiene score, age and serology were available for all animals. Lesion inspection data were available for 609 animals; the outcome was recorded as a binary variable (i.e. absence or presence of BDD lesion), as well as an ordinal variable (i.e. no lesion, acute, chronic or regressing lesion). Of the 1541 cows in the dataset that had calved at least once, data on parity and lactation stage could only be retrieved for 609.

Level	Variable	Type
Animal	Lesion inspection performed?	binary
	– if so, lesion status	binary
	– if so, lesion presentation	ordinal
	Serology	continuous
	Foot hygiene score	categorical
	Body hygiene score	categorical
	Age	continuous or categorical
	Parity‡	categorical
	Days in milk / lactation stage‡	continuous / categorical
Management	Management group	categorical
	Environmental hygiene	categorical
	Environmental comfort	categorical
	BDD treatment	categorical
	Footbaths	categorical
	Foot care	categorical
Farm	Farm code	nominal

Table 4.2: Overview of variables registered per animal record ‡ lactating cows only (n=609)

Animal-based composite indices were calculated for foot hygiene score and body hygiene score by summation of the individual scores. For foot hygiene scores, the range of values was 4-16; for body hygiene score, this was 8-32. An ordinal scale of 1-4

was calculated for both hygiene scores by division by four and eight respectively, and rounding to the nearest digit.

The indices for environmental hygiene and environmental comfort were more complex to obtain; they were derived from information from the questionnaire and housing inspection. Per management group on every study farm, scores were assigned to each of the housing factors listed in Table 4.3. A compound score for the housing hygiene and housing comfort was then derived for each group (and hence every animal). A similar procedure was followed to derive compound scores for footcare protocols and BDD control procedures (Table 4.4).

These indices were recategorized as follows:

- *Housing comfort* 1: worse than average; 2: better than average
- *Housing hygiene* 1: very poor; 2: poor; 3: good; 4: very good
- *Footbathing* 0: none; 1: worse than average; 2: better than average
- *BDD control* 0: none; 1: worse than average; 2: better than average

Inevitably, the creation of indices is a subjective procedure. Also, such indices are a simplification of the existing conditions, and preclude evaluation of the individual constituent factors. On the other hand, the number of farms and management groups was probably insufficient to derive more detailed indices.

Exploratory data analysis

Clinical BDD lesion prevalence was tabulated for the different farm, age and management groups, for both binary (absence / presence of lesions) and ordinal (absence / presentation of lesions) outcomes. Barplots were used to visualize the results after stratification of the study population into high, medium and low prevalence farms.

The serological data were applied to investigate frequency distributions on the farm level, and were likewise stratified into farm prevalence level and age and management groups. For the subset of clinically inspected animals, frequency distributions of clinical positives were compared to clinical negatives. Boxplots were plotted to summarize patterns in the data.

Finally, parity and lactation stage effects were investigated. First, tests were carried out to verify that the subset of cows (i.e. animals that had calved at least once) for which these data were available (n=609) was representative of the larger group of cows (n=1541). Subsequently, serological boxplots of parity and lactation stage were generated.

	Variable	Categories
Housing comfort	Housing system	Cubicles; group pens; cubicles; straw yards
	Bedding material	None; straw; sawdust; sand
	Frequency of removing bedding	Number of times per week
	Floor condition	Poor; adequate; good
	Stocking density	High; appropriate for area; low
Group pens: hygiene	Faecal consistency	Liquid; loose; average; stiff; dry / lumpy
	Frequency of use of additives	Number of times per week
	Frequency of removing bedding	Number of times per week
	Frequency of steam cleaning	Never; after each group; less frequently
	Frequency of bedding down	Number of times per week
	Hygiene score: lying area	Worse than average; average; better than average
	Hygiene score: feeding area	Worse than average; average; better than average
Cubicles: hygiene	Faecal consistency	Liquid; loose; average; stiff; dry / lumpy
	Frequency of use of additives	Number of times per week
	Frequency of removing bedding	Number of times per week
	Frequency of steam cleaning	Number of times per year
	Frequency of bedding down	Number of times per week
	Frequency of slurry removal	Number of times per day
	Method of slurry removal	Automatic scrapers; tractor scraper; hand; combinations
	Pools of standing slurry after scraping?	Yes / no
	Do cows have to pass through these?	Yes / no
	Hygiene score: cubicle beds	Worse than average; average; better than average
	Hygiene score: floor surface	Worse than average; average; better than average
	Hygiene score: passageways	Worse than average; average; better than average

Table 4.3: Observed variables used to derive environmental comfort and hygiene scores

	Variable	Categories
Footcare	Footbaths used?	No; single; tandem
	Frequency of use	Never; weekly; fortnightly; monthly; less frequently
	Number of uses per solution	Number
	Footbath procedures	N.a.; no pre-washing; pre-washing
	Footbathing: which animals?	None; in-calf heifers and cows; cows only
	Foot trimming: which animals?	None; all cows; lame cows
	Which feet?	All feet; all overgrown feet; back feet only
	Frequency of trimming	At fixed interval; at drying off; overgrown feet only; lame only
	Formal training?	No; yes
BDD control	Which management groups?	None; cows only; cows and heifers
	When treated?	Never; early stage; easily visible; large lesion
	Individual treatments?	No; yes
	Formulations used	None; topical antibiotic; injected; other; combinations
	Supporting treatments?	No; cleaning; trimming; bandaging; combinations
	Preventive measures	None; closed herd; trimming; breeding; combinations
	Control measures	None; footbaths; hygiene; biosecurity; combinations

Table 4.4: Observed variables used to derive scores for footbathing protocols and BDD control procedures

4.2.6 Statistical modelling

Background and approach

The development of a causal diagram was a preliminary exercise for model building (see Chapter 1.3), as it identified putative causal relationships between the outcome and explanatory variables, and enabled the biological plausibility of the final model to be assessed.

The model building consisted of various stages. As an initial step to assess correlation between explanatory variables (hence confounding), correlation analysis was performed – this was performed for all individual-level and management-group-level variables. Pairwise correlations were assessed using Spearman's method, which estimates a rank-based measure of association (the rho statistic) which ranges from -1 to 1, with 0 indicating no association. A two-sided alternative hypothesis was chosen, i.e. association could be either positive or negative. The test computes the probability of this value being zero.

Regression models were then constructed to examine risk factors for the outcome of interest. Two different outcomes were considered, namely clinical identification of BDD lesions (binary) and serology (continuous); as these were on different scales, separate models were constructed.

Animals within the dataset are stratified by farm and management group, i.e. the dataset is hierarchical with three levels (animal, management group and farm). As animals share a common environment with other animals in the same group, they tend to be more similar. This clustering effect implies that the assumption of independence, which is fundamental to regression modelling, is violated (Dohoo et al., 2003); there are now additional components of variability due to these unobservable effects. This must be accounted for in the model to avoid spurious outcomes (specifically, underestimation of the standard errors). Various models were fitted and compared to identify the most appropriate structure.

A simple solution with fixed-effects models is to include the level at which clustering is assumed (e.g. farm) as a fixed effect; the model effectively estimates a separate parameter for every herd, and thus separates between-herd variation from the residual variation (Dohoo et al., 2003). This is a feasible option for this dataset as the number of farms is small; if this were large, the number of parameters estimated by the model would be unwieldy.

A more common technique is by extending the fixed effects model to include random effects terms. Fixed effects have a discrete set of possible levels which are repeatable across management groups and / or farms, and random effects are non-repeatable / unobservable (Song and Lee, 2006; Bates, 2005; Faraway, 2005). The random effects account for the variability about the fixed effects. Mixed effects models were formulated: with farm as a random effect, with management group as a random effect (as the

number of farms was relatively small), and with management group nested within farm as random effects (which more explicitly defined a multilevel structure).

Univariable analyses were conducted, sequentially analyzing each of the explanatory variables listed in Table 4.2 above. The continuous variables age and days in milk were treated as quadratic polynomials (as EDA indicated an absence of a linear relationship), as well as categorical variables. The univariable relationships between the explanatory variables and the outcome variable were compared for the different models. Model performance was assessed and compared using appropriate measures, and a choice of the most appropriate random effects structure was made.

Subsequently, all explanatory variables with p-values of <0.25 were considered for inclusion in multivariable models. Rather than performing automated selection procedures, the multivariable models were iteratively constructed; this was possible as the number of explanatory variables was not large. Variables of interest were manually selected (where choice of selection was guided both by results of the univariable analysis and the causal diagram, i.e. biological plausibility), and different permutations of these variables were assessed. The choice of the final model was made by comparison of the relevant information measures.

BDD lesions: generalized linear models

The dataset was derived from the subset of animals of which the hind feet were inspected (n=609). Data on age could not be obtained for 21 animal records, which were removed; hence 590 animals records were included in the dataset.

If Y_i is the (binary) clinical BDD lesion status of animal i, the probability of a positive outcome p_i is Bernoulli distributed, i.e.

$$Y_i \sim \text{Bernoulli}(p_i)$$

The observations are assumed to be conditionally independent. Because the outcome variable is binary, the models constructed were of the generalised linear model (GLM) family, i.e. using a 'link' function, specifically, the logit transformation:

$$\text{logit}(p_i) = \ln\left[\frac{p_i}{(1-p_i)}\right] = \beta_0 + \Sigma\beta_{iv}X_{iv}$$

for a set of v explanatory variables. After backtransformation, the value of p_i is bounded between 0 and 1 due to this logit transformation; this hence enables this probability to be modelled as a linear function of a set of explanatory variables.

Rearranging, the probability of the outcome can be computed:

$$p_i = \frac{\mathrm{e}^{(\beta_0+\Sigma\beta_{iv}X_{iv})}}{1+\mathrm{e}^{(\beta_0+\Sigma\beta_{iv}X_{iv})}}$$

The scale on which the explanatory variables are modelled is therefore different than that of the outcome. The usual error term is not included in the equation for

this reason: the distribution of the errors is on the original scale and hence cannot be incorporated in the transformed regression equation (Dohoo et al., 2003).

Generalized linear mixed models (GLMMs) are a direct extension of GLMs, to accommodate random effects in the model – which are appropriate for a hierarchical or multilevel structured dataset, e.g. if Y_{ijk} is the (binary) clinical BDD lesion status of animal i within management group j on farm k. A random effects term is added to the regression equation (on the transformed scale), as follows:

$$\text{logit}(p_{ijk}) = \ln\left[\frac{p_{ijk}}{(1-p_{ijk})}\right] = \beta_0 + \Sigma\beta_{ijkv}X_{ijkv} + U_k$$

where U_k is the random effect for farm k. The random effects are assumed to be normally distributed with mean zero, i.e.

$$U_k \sim \text{Norm}(0, \sigma^2)$$

Analysis of GLMMs is not straightforward. The parameters β, and the parameters describing the distribution of the random effects, can be estimated by maximum likelihood (ML) analysis based on the marginal distribution of the observations Y_{ijk}. For linear mixed effects models, the distributions of both Y_{ijk} and U_k are assumed to be normal, and this technique is therefore feasible. However, in GLMMs this requires many high-dimensional non-analytic integrations of the random effects (as a consequence of the logit link function), which is in most cases very computationally intensive, even if they are normally distributed (Song and Lee, 2006; Edmond et al., 2006; Schall, 1991; Venables and Ripley, 2002). Alternative, approximate methods of inference have been developed to avoid the computationally-intensive integration. Common procedures include marginal and penalised quasi-likelihood (PQL) and, more recently, maximum likelihood approaches based on Markov chain Monte Carlo (MCMC) simulation (Breslow and Clayton, 1993; Dohoo et al., 2003; Song and Lee, 2006; Edmond et al., 2006; Venables and Ripley, 2002). Advantages of the PQL algorithms are that they are relatively robust and less computationally intensive; however, they tend to bias the estimates towards zero (i.e. underestimate them), especially for clustered binary data and when the data are sparse.

As estimation is not exact, comparison of GLMMs is a contentious subject. As the PQL algorithm does not perform ML fitting, it does not provide an exact value of the log-likelihood, which is used to calculate the 'standard' information measures for comparing models, the AIC and BIC (this issue is presumably resolved when using an ML algorithm). The approximate log-likelihood can be used to perform likelihood ratio tests (LRTs). The Wald test is another method for comparing models; although this test may yield similar results to the LRT, it is not preferable for reasons outlined by Kirkwood and Sterne (2003).

Serology: linear models

For the continuous outcome of the ELISA serological test, a linear mixed effects model was developed and fitted. As a serological result was available for every animal, the full dataset (n=2204) could now be used. As age was considered as an explanatory variable in the models, records missing data on date of birth of the animal were removed, resulting in 2165 records.

As the dataset used was not the same as that for the GLMMs, correlation analysis was repeated, using the same technique. This dataset had the same hierarchical structure (i.e. animal, management group and farm levels). Model fitting, estimation and comparison were more straightforward.

In analogy with the above, for the continuous outcome of serology Z_i of animal i, the probability of a positive outcome q_i is normally distributed, i.e.

$$Z_i \sim \text{Norm}(q_i)$$

The outcomes are assumed to be conditionally independent. In this case, no link function is required for the regression of the linear predictor on the outcome, hence a simple linear regression model applies:

$$Z_i = \beta_0 + \Sigma\beta_{iv}X_{iv} + \varepsilon_i$$

for the same set of v explanatory variables. Note that the error term, which was dropped for generalised linear models, must be included.

For a hierarchical or multilevel structured dataset, e.g. where Z_{ijk} is the serological status of animal i within management group j on farm k, a random effects term is added to the regression equation:

$$Z_{ijk} = \beta_0 + \Sigma\beta_{ijkv}X_{ijkv} + \varepsilon_i + U_k$$

where U_k is the random effect for farm k. This is referred to as a linear mixed model, or a mixed effects model. A similar random effect could be inserted for the second level in the model, i.e. management group:

$$Z_{ijk} = \beta_0 + \Sigma\beta_{ijkv}X_{ijkv} + \varepsilon_i + U_k + U_j$$

where U_j is the random effect for management group j. The random effects and error terms are assumed to be normally distributed with mean zero, i.e.

$$\varepsilon_i \sim \text{Norm}(0, \sigma^2) \quad \text{and} \quad U_k \sim \text{Norm}(0, \sigma_k^2) \quad \text{and} \quad U_j \sim \text{Norm}(0, \sigma_j^2)$$

We therefore estimate variances between individuals, between management groups and between farms (if the last two are specified). The total variance in the data can be decomposed to the sum of the component variances.

The top level of the hierarchy (i.e. farm) would normally be modelled as a random effect, i.e. the variability between farms is not directly observable. As the number of farms in the dataset is relatively small, and representativity is not guaranteed as selection of farms was not random, it is defensible to model farm as a fixed effect (Dohoo et al., 2003).

Since multiple linear regression is based on the assumption of a normal distribution, analysis is based on likelihood approaches. Estimation can be performed by 'full' maximum likelihood (ML) or restricted maximum likelihood (REML); both are approximations which are asymptotically unbiased; in our models, we used ML. The estimated log likelihood was then used to calculate likelihood ratio tests (LRTs) for model comparison.

4.2.7 Investigation of group and herd level of infection

The Bayesian model developed in Chapter 3 was used to investigate the management group and herd levels of infection. As the model output describes the probability of infection as a latent variable, the term 'prevalence' should not be used: this is defined as the proportion of cases within the population at risk at a point in time. The Bayesian model does not identify BDD cases; rather, it assigns a conditional probability of infection (CPI) for each individual (whether clinical lesions are apparent or not).

From these individual CPIs, we wished to make inferences about the infection levels of groups of individuals. Graphical data analysis was performed to investigate this. The frequency distributions of the CPIs were examined per management group. For these management groups, the mean was taken of the constituent individuals to denote the level of infection; 95% confidence intervals were derived. Consequently, barplots were made for the farms stratified by prevalence level and the whole study population. Medians of the CPIs and the corresponding quartiles were plotted in boxplots.

4.3 Results

4.3.1 Exploratory data analysis

Clinical data

Few lesions were observed in the in-calf heifer groups and no lesions were seen in younger stock. Figure 4.1 shows the clinical prevalence by age and management group, stratified by farm prevalence level. As the age of first calving (2 to 3 years) showed some variability between farms, this age category was subdivided into lactating and non-lactating cows. Prevalence per farm (for the lactating and dry cow groups only) is presented in Table 4.5.

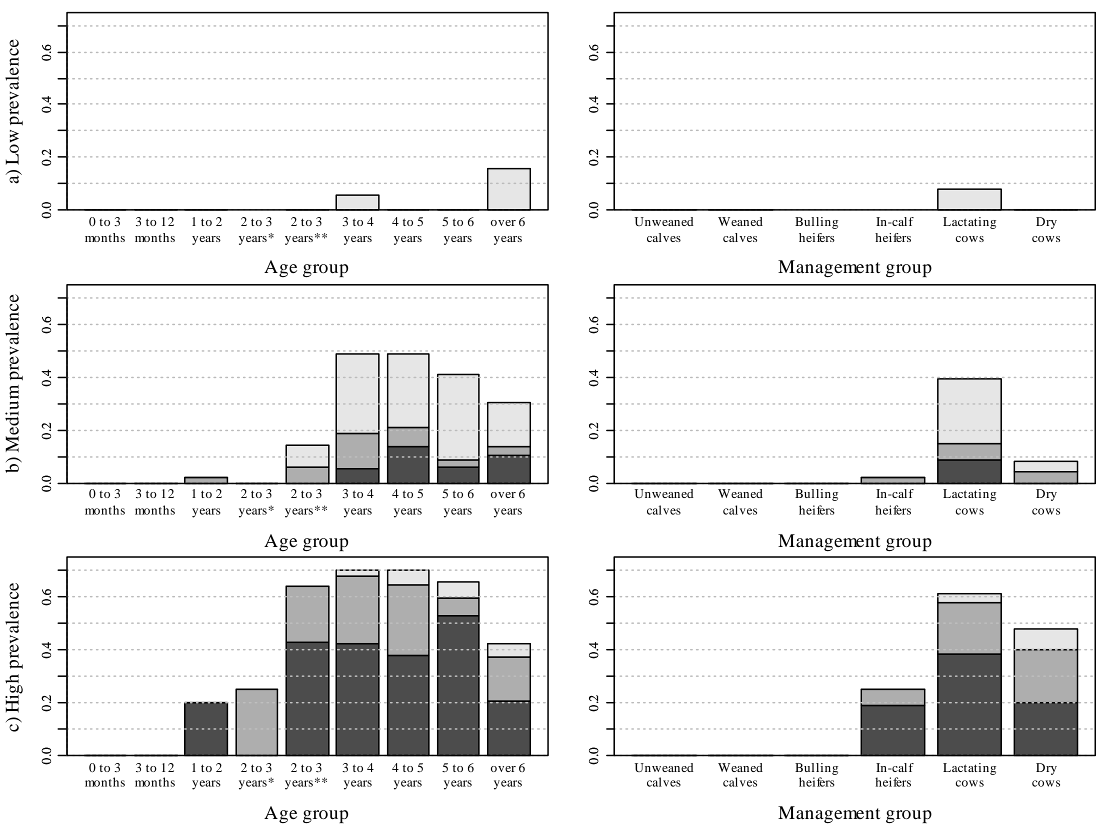


Figure 4.1: Clinical prevalence of BDD lesions by age group and management group of low prevalence farms, medium prevalence farms and high prevalence farms. Acute, chronic and regressing lesions are shaded dark grey, grey and light grey respectively. (Note: * non-lactating, ** lactating)

Serological data

Histograms of the serological distributions showed evidence of a bimodal response, which indicated the existence of infected and uninfected sub-populations. The negative sub-population was strongly right skewed, whereas the positive sub-populations appeared to be more normally distributed. This pattern was more or less consistent for the individual farms. The distributions on the negative and low clinical prevalence farms showed more skewing and less bimodality, whereas the distributions on the high prevalence farms showed strong evidence of bimodality, for one farm even approaching a normal distribution (histograms and Q-Q plots of all farms have been included in Appendix F). The negative control farm (i.e. clinically free of BDD) showed a similar distribution, with approximately a dozen cows showing high titres. These samples were retested to exclude spurious results.

Distributions were also investigated for the clinically inspected animals (n=609). This further substantiated evidence of sub-populations: clinical negatives showed a strongly right-skewed distribution, while the positives' distribution was more normal

BDD prevalence level	Farm code	Clinical lesion prevalence (%)			
		Acute	Chronic	Regressing	Overall
High	01	34.6	9.2	16.2	60.0
	04	0.0	20.0	15.0	35.0
	06	0.0	26.9	30.8	57.7
	08	16.7	41.7	0.0	58.3
Medium	02	8.6	1.4	23.7	33.8
	05	3.0	12.1	9.1	24.2
Low	07	0.0	0.0	12.9	12.9
Free	03	0.0	0.0	0.0	0.0
Overall	All	15.7	8.6	16.7	41.0

Table 4.5: Clinical BDD lesion prevalence (lactating and dry cow groups only) of the study farms, stratified by BDD prevalence level. The young stock has been excluded due to the low prevalence in these groups

(Figure 4.2). The notched boxplots indicate that the median logPP is significantly higher for clinically positive animals. A two-sample t-test confirmed that clinically positive animals have a highly significantly ($p \ll 0.001$) higher titre than clinical negatives.

Further stratification of lesion positives into acute, chronic and regressing lesions showed that animals with acute and chronic lesions had significantly higher titres than clinically unaffected animals and animals with regressing lesions, but the difference between animals with acute and chronic lesions was not significant (Figure 4.3).

Finally, the study population was stratified into age and management groups (Figure 4.4). Boxplots of the serology showed a gradual rise in titres up to calving (which takes place between 2 to 3 years of age), and then rose rapidly. Titres remained high as age increased, i.e. for increasing parities. The titres of the dry cows were not significantly different than the lactating cows.

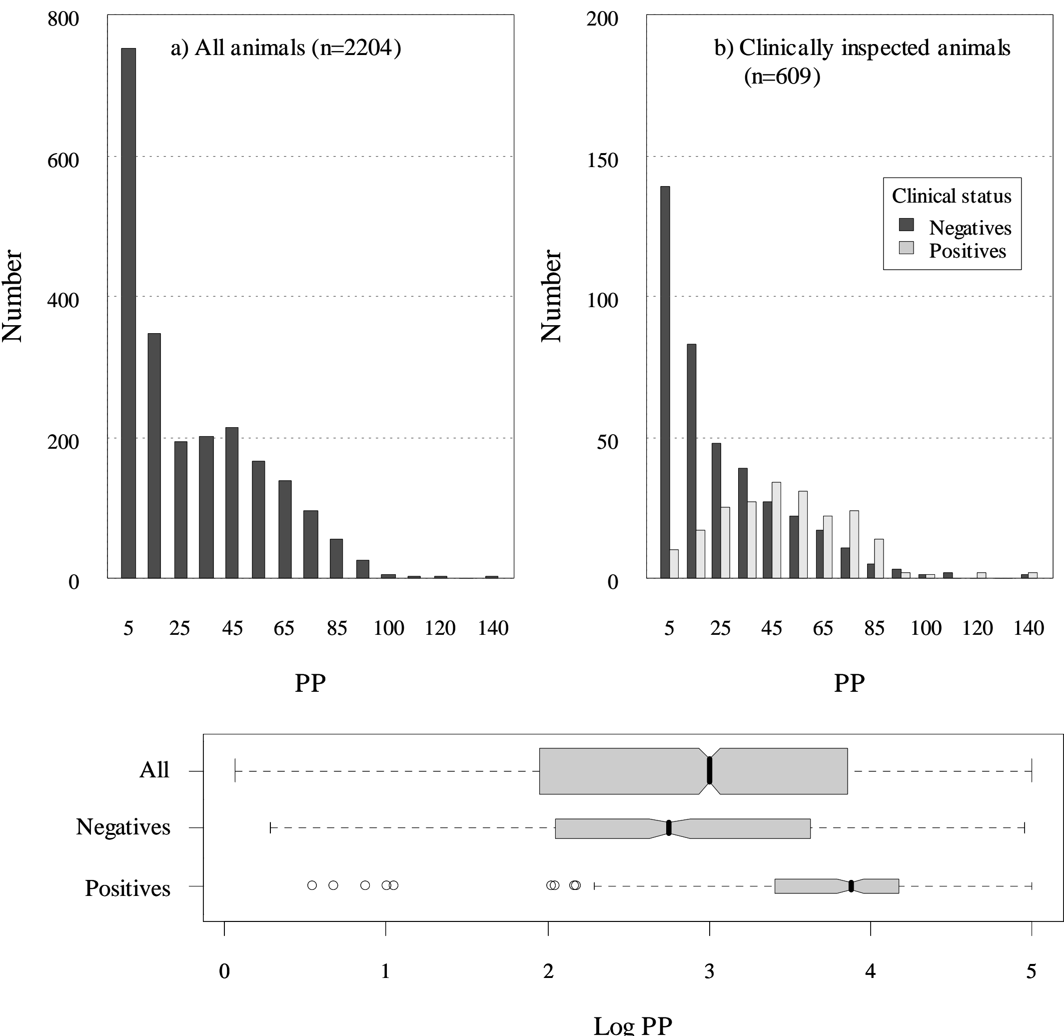


Figure 4.2: Histograms and notched boxplots of the ELISA of a) the entire study population, and b) animals inspected for BDD, classified as clinical negatives and clinical positives. Note that the scale of the y-axes is not equivalent. The width of the plotted box is proportional to the the square root of the number of observations. The length of the notch along the box is a graphical representation of a confidence interval about the median of a sample: statistically, if the notches on side-by-side boxplots do not overlap then the medians will be significantly different

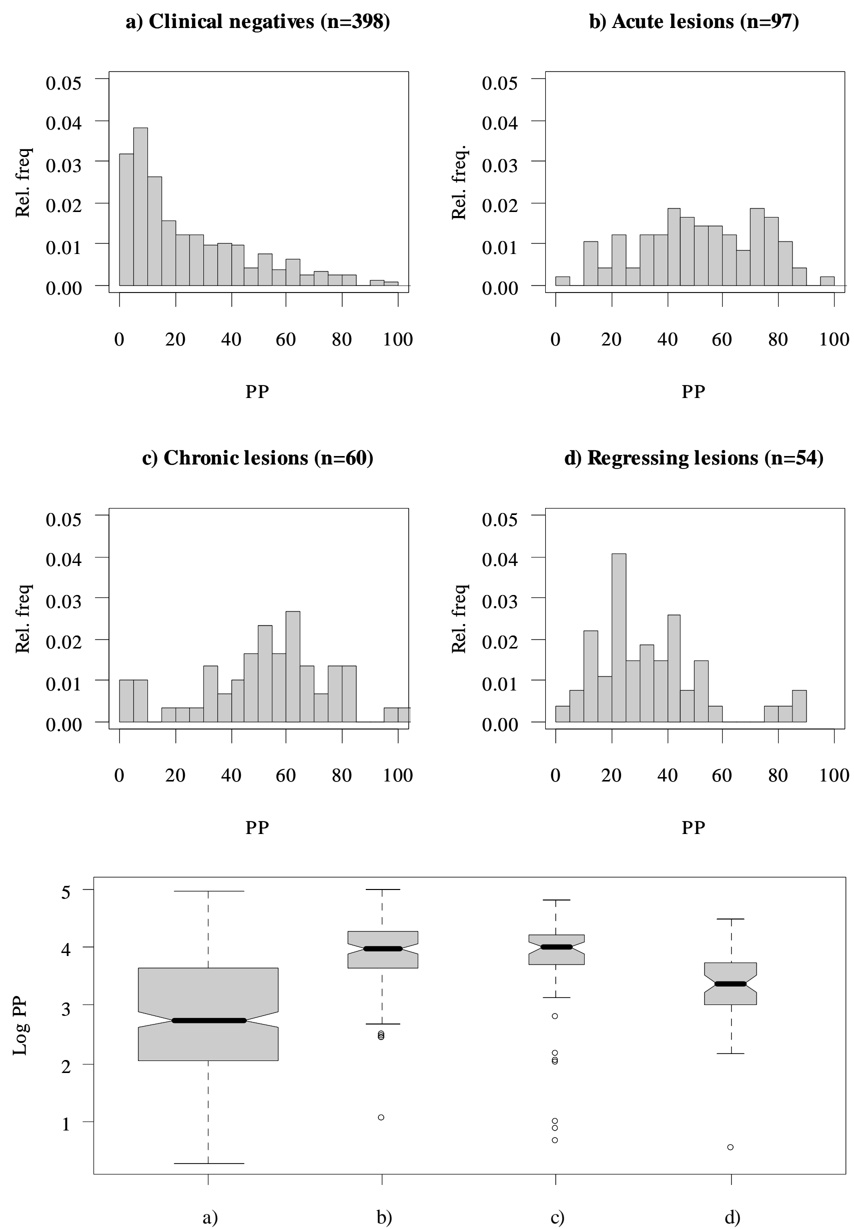


Figure 4.3: Relative frequency histograms and notched boxplots of the ELISA of animals clinically inspected for BDD (n=609), classified by BDD lesion presentation. The length of the notch along the box is a graphical representation of a confidence interval about the median of a sample: statistically, if the notches on side-by-side boxplots do not overlap then the medians will be significantly different. The width of the box is proportional to the the square root of the number of observations

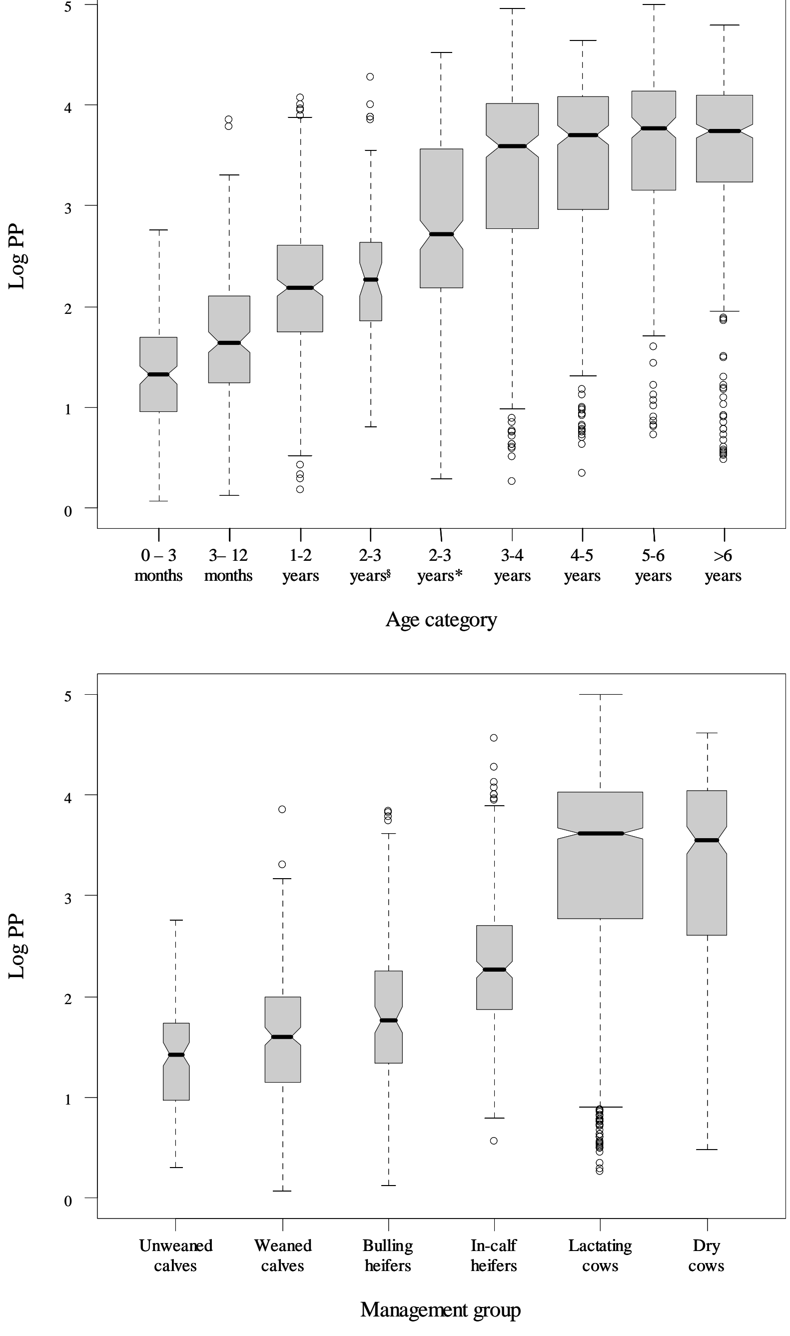


Figure 4.4: Notched boxplots of ELISA titres per age category (§not lactating; *lactating) and management group of eight study farms (n=2204). The length of the notch along the box is a graphical representation of a confidence interval about the median of a sample: statistically, if the notches on side-by-side boxplots do not overlap then the medians will be significantly different. The width of the box is proportional to the the square root of the number of observations

Lactation data

Of the total of 1541 cows included in the study, data were obtained on the parity of 761 cows (from six farms); the last calving date (hence lactation stage) and previous lactation production were known of 609 of these (from five farms). The serological distribution of the sub-groups for which lactation number and last calving date were known were visually compared to the distribution of all cows included in the study; this showed that they were comparable (see Appendix F.3). Subsequently, boxplots were used to investigate parity and lactation stage (Figure 4.5). As already indicated by Figure 4.4, lactation number was not significantly associated with antibody titre. Similarly, it appeared that there were no significant associations with lactation stage.

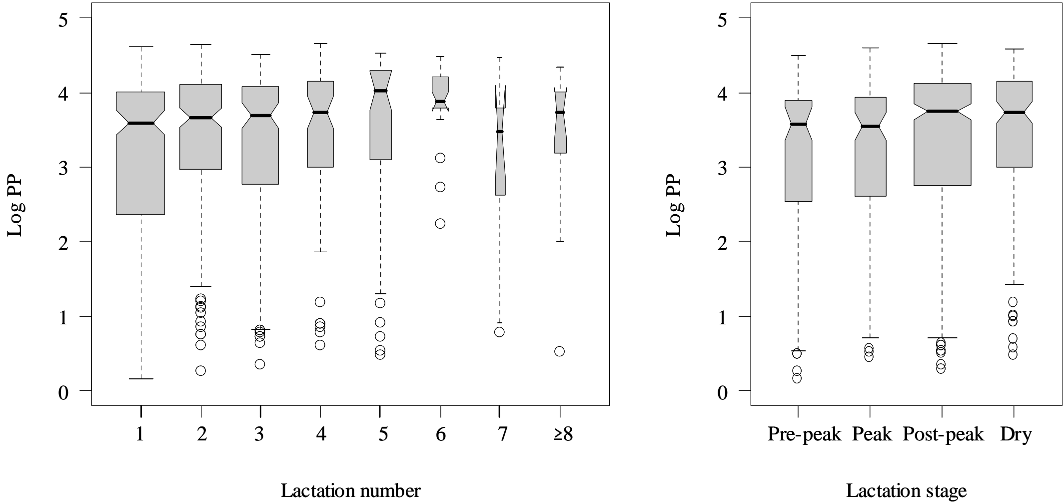


Figure 4.5: Notched boxplots showing the relationships between lactation number and lactation stage, and serology (n=609). The length of the notch along the box is a graphical representation of a confidence interval about the median of a sample: statistically, if the notches on side-by-side boxplots do not overlap then the medians will be significantly different. The width of the box is proportional to the the square root of the number of observations

4.3.2 Statistical models

BDD lesions: generalized linear models

Correlation analysis The results of the pairwise correlation tests using Spearman's method for individual-level and management group level variables are given in Table 4.6. This did not show strong evidence of collinearity between all individual-level variables, but reasonably strong correlation between some of the management group level variables.

Variable 1	Variable 2	Spearman's rho	p-value
Individual animal level			
Body hygiene score	Foot hygiene score	0.31	< 0.001
Body hygiene score	Age (categorical)	0.13	< 0.01
Body hygiene score	Management group	0.17	< 0.001
Foot hygiene score	Age (categorical)	0.37	< 0.001
Foot hygiene score	Management group	0.26	< 0.001
Age (categorical)	Management group	0.53	< 0.001
Management group level			
Housing hygiene	Housing comfort	0.24	< 0.001
Housing hygiene	BDD treatment	0.04	0.35
Housing hygiene	Footbaths	-0.12	< 0.01
Housing comfort	BDD treatment	-0.71	< 0.001
Housing comfort	Footbaths	-0.61	< 0.001
BDD treatment	Footbaths	0.73	< 0.001

Table 4.6: Correlation analysis among explanatory variables of the inspected animals data subset (n=609) using Spearman's method: the rho statistic ranges from -1 to 1, with 0 indicating no association; the p-value represents the probability of this value being zero

Univariable analysis Tables with the full output of all explanatory variables for the four models are given in Appendix G.1. All explanatory variables remained available for inclusion in multivariable models ($p< 0.25$), except the production variables parity and lactation stage (in all models) and foot hygiene score (model specifying management group nested within farm as the random effects term).

Univariable associations of the explanatory variables with clinical BDD were very similar for the GLM including farm as fixed effect, and the GLMM specifying farm as random effect ($GLMM^1$). The trends in the odds ratios were broadly comparable between models. For foot hygiene score (FHS) and body hygiene score (BHS), the odds ratios rose with increasing score, except in the GLMM with management group as random effect ($GLMM^2$). For the categorised age variable, the odds ratios were highest for the 3 to 4 years category, and subsequently declined; this is consistent with the EDA (see Figure 4.1). This corresponded to parity, for which the odds ratio was highest for second parity cows. The odds ratios for the lactating cows were more than double those of the dry cows. No patterns emerged from analysis of the lactation stage variable.

When management group was nested within farm as a random effect ($GLMM^3$), FHS became non-significant. It appeared that the significant relationship identified was a result of confounding: dirty feet were highly significantly associated with BDD; however, cows tend to have dirty feet whereas young stock have clean feet. When cor-

recting for this by stratifying the analysis within management group, this association proved to be spurious, i.e. within the cow groups, cows with dirtier feet are not significantly more likely to have BDD. This was confirmed by including management group as a second fixed effect (after farm ID) in the GLM: FHS again became non-significant. This indicates that $GLMM^3$ best fits the data.

For the three GLMMs, the information measures AIC (Akaike's Information Criteria), BIC (Bayesian Information Criteria), log likelihood and deviance are useful parameters for assessing model performance. For the GLM, only the AIC and residual deviance were given by the software. These are summarised in Table 4.7.

Variable	GLM		GLMM[1]				GLMM[2]				GLMM[3]			
	AIC	deviance	AIC	BIC	logLik	deviance	AIC	BIC	logLik	deviance	AIC	BIC	logLik	deviance
Foot hygiene score	686.98	664.98	704.05	726.12	-347.02	694.05	730.49	752.56	-360.24	720.49	676.13	698.20	-333.06	666.13
Body hygiene score	674.72	652.72	692.33	714.41	-341.16	682.33	726.09	748.17	-358.05	716.09	670.01	692.09	-330.01	660.01
Age (quadratic)	625.09	605.09	21995.05	22012.57	-10993.50	21987.05	703.68	721.20	-347.84	695.68	21954.45	21971.97	-10973.20	21946.45
Age (categorised)	625.28	599.28	644.02	674.68	-315.01	630.02	705.78	736.44	-345.89	691.78	641.25	671.91	-313.62	627.25
Housing hygiene	661.27	639.27	683.69	705.77	-336.85	673.69	–	–	–	–	–	–	–	–
Housing comfort	698.25	680.25	715.03	728.27	-354.51	709.03	–	–	–	–	–	–	–	–
Footbaths	654.50	634.50	666.95	684.61	-329.47	658.95	–	–	–	–	–	–	–	–
BDD treatment	646.43	626.43	662.81	680.47	-327.41	654.81	–	–	–	–	–	–	–	–
Parity	166.48	148.48	177.82	193.17	-83.91	167.82	194.40	209.74	-92.20	184.40	182.04	197.38	-86.02	172.04
Lactation stage (quadratic)	140.57	124.57	150.88	162.39	-71.44	142.88	162.99	174.49	-77.49	154.99	150.88	162.39	-71.44	142.88
Lactation stage (categorised)	184.93	156.93	199.84	226.11	-91.92	183.84	229.74	256.00	-106.87	213.74	207.09	233.35	-95.54	191.09

Table 4.7: Comparison of summary measures for the univariable generalized linear models and generalized linear mixed models. GLMM[1]: farm as random effect; GLMM[2]: management group as random effect; GLMM[3]: management group nested within farm as random effect. Group-level outputs are not available for GLMM[2] and GLMM[3], as group is specified as a random effect. Note: the models of the production data (parity and lactation stage) make use of a subset of the data; as the number of observations is not equal to the larger dataset, the information measures are not comparable

Random effects structure While comparison of information measures is important, consideration should also be given to biological and statistical principles. The GLM was not considered further as the data were assumed to be highly clustered, and a random-effects model was more appropriate given this data structure. Another disadvantage of the GLM was that by fitting farm as a fixed effect, the model estimates applied only to the study herds and not to the larger population, as was the case when specifying it as a random effect. While it was defensible to model farm ID as a fixed effect within the random effects models (because the number of farms is relatively small), it was therefore more suitable to include it as a random effect. Also, in this case the model was more parsimonious, as it did not include the farm parameters in the fixed effects. Since we are not primarily interested in comparing the study farms, these are referred to as 'nuisance' parameters.

The most appropriate method for management group depends to an extent on assumptions about the uniformity of these groups across the farms. Assuming that management practices of the groups on the study farms are more or less consistent, then the management group variable has a discrete set of possible levels which are repeatable; in order to characterize the change in response between these different levels, it could be modelled as a fixed effect. On the other hand, if the assumption is made that management practices are not comparable across farms, this variable would be non-repeatable, and to assess variation in the response, it would be modelled as a random effect. By modelling it as a fixed effect, we estimate a separate parameter for each management group of the study farms; by modelling it as a random effect, we estimate it as a single parameter which, assuming the study population is representative, applies to the larger population. A drawback of modelling it as a random effect is is that other group-level fixed effects cannot be included in the model, as they are already accounted for by the management-group random effect. As we considered the study farms to be representative, and the management procedures to be typical of dairy farms in the region, either option was valid. The EDA showed marked differences in BDD prevalence within management groups on the study farms, so it might be safer to model it as a random effect. Conversely, GLMM^{2} did not account for any differences between farms, which probably did exist; therefore this model was thought to be less suitable.

The most appropriate model structure was therefore either GLMM^{1} or GLMM^{3}. Further explanatory variables were fitted to these models, and the candidate models evaluated using likelihood ratio statistics.

Model comparison For GLMM^{3}, the results (AIC, BIC and log likelihood) were obtained with age (categorised) or BHS as the first explanatory variable. No further variables could be added to without the model losing significance. BHS gave better results; also, age and management group are moderately correlated (with a rho of 0.53

– see Table 4.6).

With GLMM[1], different combinations of explanatory variables were assessed, using first management group as the first variable, and subsequently age as the first variable. No more than a second variable could be added without the model losing significance. In both cases, the best results were obtained using BHS as the second explanatory variable.

The candidate models could not be directly compared because they incorporated different random effects structures. However, they could be compared to their respective null models, which enabled the LRT to be calculated (Table 4.8).

Model	Variables	AIC	BIC	logLik	deviance	LRT	P-value	df
Null models								
GLMM[1]	–	720.32	729.15	-358.16	716.32	–	–	–
GLMM[3]	–	673.05	681.88	-334.52	669.05	–	–	–
Candidates for the final full model								
GLMM[1]	Management group Body hygiene score	655.85	686.75	-320.92	641.85	74.47	< 0.0001	2
GLMM[1]	Body hygiene score Age (categorical)	643.03	686.83	-311.51	623.03	93.30	< 0.0001	2
GLMM[3]	Body hygiene score	623.55	644.65	-306.78	613.55	55.48	< 0.0001	1

Table 4.8: Comparison of null models and final candidate generalized linear mixed models (GLMMs) with clinical BDD as binary outcome variable. GLMM[1]: farm as random effect; GLMM[3]: management group nested within farm as random effect. The deviance compares the likelihood of the model with the saturated model

The overall likelihood ratio test (LRT) statistic is calculated as $2 * (\ln L_M - \ln L_0)$, where L_M is the log likelihood of the candidate model and L_0 is the log likelihood of the null model; as this has an approximate χ^2 distribution, the P-value represents the proportion of the relevant sampling distribution that falls to the right of the χ^2, with as many degrees of freedom (df) as there are covariates in the model.

The GLMM[1] candidate models can be compared with a reduced model (i.e. first variable only). For the GLMM[1] with management group and BHS, this 'improvement χ^2' was 7.22 with a Chi-squared distribution with 2df (i.e. p=0.03). For the GLMM[1] with age and BHS, the 'improvement χ^2' was 7.00 with a Chi-squared distribution with 2df (i.e. p=0.03). In both cases, fitting the second covariate significantly improved the model.

The output from these three candidate models is presented in Table 4.9. The LRT was lower for the GLMM[1] models, i.e. they were more improved compared to their null models. However, GLMM[3] has better AIC and BIC; it is more explicitly multilevel,

accounts for the data better, is more parsimonious and has lower deviance. Therefore, this was probably the best model.

Variable		Coefficient	SE	OR	95% CI	*P*-value
Candidate 1: GLMM[1]						
Management group	YS	Referent				
	LC	2.44	0.51	11.44	4.25 – 30.81	< 0.01
	DC	1.54	0.59	4.67	1.48 – 14.72	< 0.01
Body hygiene score	1	Referent				
	2	1.30	0.60	3.68	1.13 – 12.05	0.03
	3	1.46	0.61	4.30	1.31 – 14.13	0.02
	4	1.53	0.65	4.61	1.30 – 16.34	0.02
Candidate 2: GLMM[1]						
Age (categorical)	0-2y	Referent				
	>2-3y	1.93	0.78	6.87	1.49 – 31.75	0.01
	>3-4y	2.96	0.78	19.30	4.21 – 88.54	< 0.01
	>4-5y	2.83	0.78	16.90	3.67 – 77.93	< 0.01
	>5-6y	2.70	0.79	14.90	3.19 – 69.57	< 0.01
	> 6y	2.07	0.77	7.90	1.74 – 35.89	< 0.01
Body hygiene score	1	Referent				
	2	0.93	0.61	2.53	0.76 – 8.43	0.13
	3	1.24	0.61	3.47	1.05 – 11.50	0.04
	4	1.42	0.65	4.14	1.17 – 14.68	0.03
Candidate 3: GLMM[3]						
Body hygiene score	1	Referent				
	2	1.43	0.70	4.16	1.06 – 16.35	0.04
	3	1.63	0.69	5.09	1.30 – 19.84	0.02
	4	1.58	0.73	4.88	1.17 – 20.35	0.03

Table 4.9: Outputs of three candidate generalised linear mixed models (GLMMs). GLMM[1]: farm as random effect; GLMM[3]: management group nested within farm as random effect. YS: young stock; LC: lactating cows; DC: dry cows

Serology: linear models

Correlation analysis The results of the pairwise correlation tests using Spearman's method for individual-level and management group level variables are given in Table 4.10. The individual-level variables were more highly correlated than was the case for the subset of inspected animals, but consistently so; in no case is the coefficient rho higher than 0.9, in which case collinearity would become a problem (Dohoo et al., 2003). Of the management group level variables, only BDD treatment and footbath protocol were as strongly correlated; the other variables were less correlated for the larger dataset.

Variable 1	Variable 2	Spearman's rho	p-value
Individual animal level			
Body hygiene score	Foot hygiene score	0.42	< 0.001
Body hygiene score	Age (categorical)	0.31	< 0.001
Body hygiene score	Management group	0.29	< 0.001
Foot hygiene score	Age (categorical)	0.53	< 0.001
Foot hygiene score	Management group	0.45	< 0.001
Age (categorical)	Management group	0.75	< 0.001
Management group level			
Housing hygiene	Housing comfort	0.19	< 0.001
Housing hygiene	BDD treatment	0.20	0.35
Housing hygiene	Footbaths	-0.05	0.01
Housing comfort	BDD treatment	-0.23	< 0.001
Housing comfort	Footbaths	-0.25	< 0.001
BDD treatment	Footbaths	0.72	< 0.001

Table 4.10: Correlation analysis among explanatory variables of the entire dataset (n=2204) using Spearman's method: the rho statistic ranges from -1 to 1, with 0 indicating no association; the p-value represents the probability of this value being zero

Univariable analysis Tables with the full output of all explanatory variables for the four models are given in Appendix G.2.

All explanatory variables remained available for inclusion in multivariable models ($p < 0.25$), except housing comfort, which now became non-significant; the overall p-value for parity and lactation stage (in all models) was now less than 0.1. For the model specifying management group nested within farm as the random effects (LME^{3}), the overall foot hygiene score was significant($p < 0.001$), but category 2 had a p-value of 0.8.

As with the generalised models, univariable associations of the explanatory variables were very similar for the linear regression model (LM) and the linear mixed-effects model specifying farm as random effect (LME^{1}). The trends in the coefficients were broadly comparable between models.

For foot hygiene score (FHS) and body hygiene score (BHS), the coefficients rose with increasing score. For the categorised age variable, the coefficients rose rapidly up to the 3 – 4 years category (i.e. generally the second lactation), and subsequently rose slowly with increasing age. This is a marked contrast with the clinical BDD models (which showed highest odds ratios in the 3 – 4 year category and a subsequent decline), but is consistent with the EDA (see Figure 4.4). This corresponded to parity, for which the coefficients rise sharply in the second parity and thereafter keep increasing slightly. In contrast to the clinical BDD models, the coefficients for the lactating cows were comparable to those of the dry cows. While the lactation stage variable now remained

significant, the trend was weak; the coefficients rose slightly with increasing days in milk.

Only the higher categories (3 and 4) of FHS were significantly associated with serology when management group was nested within within farm as a random effect (LME^3). As for the GLMMs, the significant relationship identified in the other models was a result of confounding; the young stock tended to have lower FHS (1 and 2) than the cows (which generally scored 3 or 4). When the same strategy for analysing this was applied, i.e. by including management group as a second fixed effect (after farm ID) in the LM, FHS score 2 once again became non-significant.

For the three LMEs, the AIC, BIC and log likelihood are given (as estimation of the models was based on maximum likelihood). These are given in Table 4.11.

Random effects structure The discussion on determining the most appropriate random effects structure given in 4.3.2 above applies equally. The most appropriate model structure was either LME^1 or LME^3. Further explanatory variables were fitted to these models, and the candidate models evaluated using likelihood ratio statistics.

Model comparison With LME^1, different combinations of explanatory variables were assessed, using first management group as the first variable, and subsequently age as the first variable. No more than a second variable could be added without the model losing significance. The best bivariate combinations were management group and BHS, and management group and age. Of the bivariate models that were fitted for LME^3, the best results were obtained incorporating age (categorised) and BHS as explanatory variables.

The candidate models could not be directly compared because they incorporated different random effects structures. However, they could be compared to their respective null models, which enabled the LRT to be calculated (see Table 4.12). The candidate models were also compared to the reduced models to obtain the 'improvement χ^2' (Table 4.13). In all cases, fitting the second covariate significantly improved the model.

The output from these three candidate models is presented in Table 4.14. As LME^3 more explicitly specifies a multilevel structure, it was probably the most suitable model.

Variable	LME[1]			LME[2]			LME[3]		
	AIC	BIC	logLik	AIC	BIC	logLik	AIC	BIC	logLik
Foot hygiene score	19186.17	19220.23	-9587.09	19407.45	19441.51	-9697.73	18916.21	18955.95	-9541.11
Body hygiene score	19406.69	19440.76	-9697.35	19476.67	19510.74	-9732.34	18949.90	18989.63	-9467.95
Age (quadratic)	18930.80	18959.18	-9460.40	19333.51	19361.90	-9661.76	18815.94	18850.00	-9401.97
Age (categorised3)	18872.88	18918.29	-9428.44	19318.49	19363.91	-9651.25	18774.39	18825.48	-9378.20
Age (categorised2)	18860.36	18911.46	-9421.18	19312.65	19363.75	-9647.33	18767.49	18824.26	-9373.75
Age (categorised)	18861.99	18918.76	-9421.00	19313.62	19370.39	-9646.81	18768.99	18831.44	-9373.50
Housing hygiene	19428.38	19462.44	-9708.19	19393.14	19427.20	-9690.57	18946.15	18985.99	-9466.08
Housing comfort	19571.03	19593.73	-9781.51	19367.73	19390.44	-9679.87	18950.71	18979.09	-9470.35
Footbaths	19119.37	19147.75	-9554.68	19381.38	19409.77	-9685.69	18922.75	18956.81	-9455.38
BDD treatment	19289.02	19317.40	-9639.51	19423.76	19452.15	-9706.88	18945.17	18979.24	-9466.59
Parity	7577.12	7605.52	-3782.56	7798.35	7826.74	-3893.17	7579.12	7612.25	-3782.56
Lactation stage (quadratic)	7590.50	7614.16	-3790.25	7792.52	7816.18	-3891.26	7592.50	7620.89	-3790.25
Lactation stage (categorised)	7590.90	7628.76	-3787.45	7792.11	7829.96	-3888.05	7592.90	7635.49	-3787.45

Table 4.11: Comparison of univariable linear mixed models. LME[1]: farm as random effect; LME[2]: management group as random effect; LME[3]: management group nested within farm as random effect. Note: the models of the production data (parity and lactation stage) make use of a subset of all data

Model	Variables	AIC	BIC	logLik	LRT	P-value	df
Null models							
LME[1]	–	19570.07	19587.10	-9782.04	–	–	–
LME[3]	–	18951.52	18974.23	-9471.76	–	–	–
Reduced models							
LME[1]	–	19005.88	19034.27	-9497.94	568.19	< 0.0001	1
LME[3]	–	18767.49	18824.26	-9373.75	196.03	< 0.0001	1
Candidates for the final full model							
LME[1]	Management group Body hygiene score	19004.43	19049.85	-9494.22	575.64	< 0.0001	2
LME[1]	Management group Age (categorical)	18843.40	18905.84	-9410.70	742.68	< 0.0001	2
LME[3]	Age (categorical) Body hygiene score	19765.27	18839.07	-9369.63	204.25	< 0.0001	2

Table 4.12: Comparison of null models and final candidate linear mixed effects models (LMEs) with serology as continuous outcome variable. LME[1]: farm as random effect; LME[3]: management group nested within farm as random effect

Model	Variables	L_{full}	L_{full}	LRT	P-value	df
LME[1]	Management group Body hygiene score	575.64	568.19	14.90	0.0001	1
LME[1]	Management group Age (categorical)	742.68	568.19	348.98	< 0.0001	1
LME[3]	Age (categorical) Body hygiene score	204.25	196.03	16.44	< 0.0001	1

Table 4.13: 'Improvement χ^2' of candidate linear mixed effects models (LMEs) with serology as continuous outcome variable. L_{full}: logLik of full model; L_{red}: logLik of reduced model. LME[1]: farm as random effect; LME[3]: management group nested within farm as random effect

Variable		Estimate	SE	95% CI	t-value	*P*-value
Candidate 1: LME[1]						
Management group	YS	Referent				
	LC	23.60	1.16	21.33 – 25.87	20.41	0.01
	DC	22.87	1.53	19.86 – 25.87	14.90	< 0.01
Body hygiene score	1	Referent				
	2	3.24	1.46	0.38 – 6.09	2.22	0.03
	3	3.93	1.62	0.75 – 7.11	2.43	0.02
	4	5.25	2.11	1.13 – 9.38	2.49	0.01
Candidate 2: LME[1]						
Management group	YS	Referent				
	LC	9.49	2.37	4.84 – 14.13	4.01	< 0.01
	DC	6.19	2.63	1.04 – 11.34	2.35	0.02
Age (categorical)	0-1y	Referent				
	>1-2y	5.89	1.58	2.80 – 8.99	3.73	< 0.01
	>2-3y	5.43	2.34	0.84 – 10.02	2.32	0.02
	>3-4y	19.36	2.80	13.86 – 24.85	6.90	< 0.01
	>4-5y	21.41	2.79	15.94 – 26.89	7.67	< 0.01
	>5-6y	23.77	2.89	18.11 – 29.43	8.32	< 0.01
	> 6y	23.79	2.78	18.34 – 29.25	8.55	< 0.01
Candidate 3: LME[3]						
Age (categorical)	0-1y	Referent				
	>1-2y	6.64	2.50	1.74 – 11.53	2.66	< 0.01
	>2-3y	10.33	2.81	4.81 – 15.84	3.67	< 0.01
	>3-4y	25.23	2.91	19.52 – 30.94	8.66	< 0.01
	>4-5y	26.85	2.92	21.13 – 32.57	9.20	< 0.01
	>5-6y	28.75	2.98	22.90 – 34.59	9.64	< 0.01
	>6y	29.15	2.88	23.49 – 34.80	10.10	< 0.01
Body hygiene score	1	Referent				
	2	3.19	1.93	-0.60 – 6.97	1.65	0.10
	3	4.20	2.02	0.23 – 8.16	2.08	0.04
	4	6.68	2.40	1.98 – 11.37	2.79	< 0.01

Table 4.14: Outputs of three candidate linear mixed effects models (LMEs). LME[1]: farm as random effect; LME[3]: management group nested within farm as random effect. YS: young stock; LC: lactating cows; DC: dry cows

4.3.3 Using clinical and serological data to assess group and herd levels of infection

The individual conditional probabilities of infection (CPI) derived from the Bayesian model (see Chapter 3.2.3) were stratified by management group. The multiple histogram in Figure 4.6 shows the resulting frequency distribution. This shows that the model tends to dichotomize quite strongly. Also, nearly all calves and heifers are assigned a low CPI. Roughly half as many cows are likely to be uninfected as infected.

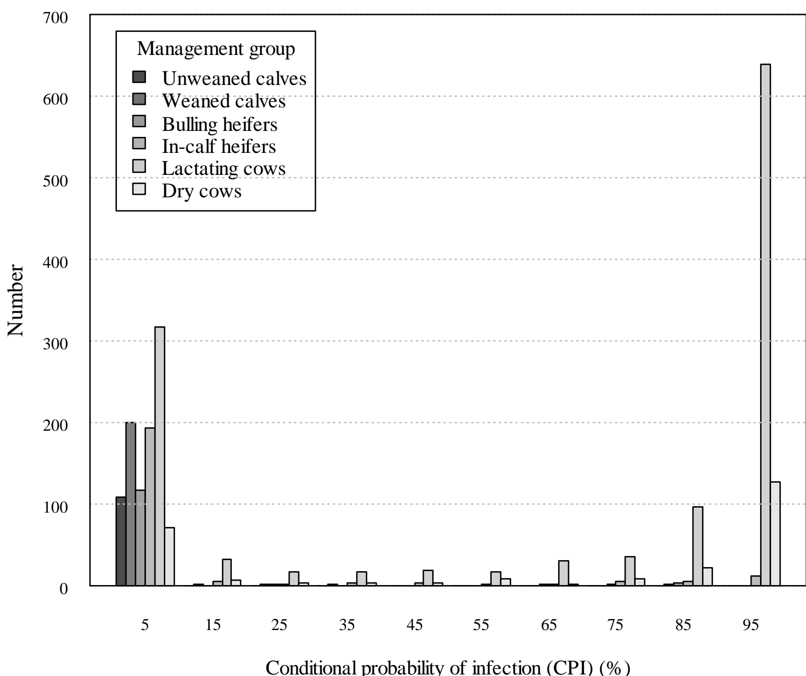


Figure 4.6: Multiple histogram showing the conditional probability of infection (CPI) of animals per management group as determined by the Bayesian model (n=2165); the CPI ranged from 0 to 100% and was subdivided into 10 categories (midpoints shown)

Per management group, the mean CPIs (with corresponding 95% confidence intervals) were calculated. The dataset was further stratified by farm clinical prevalence level, and plotted in a barplot (Figure 4.7). As the data are clearly strongly non-normal (but right skewed for the young stock and bimodal for the cows, as shown in Figure 4.6), notched boxplots were also plotted.

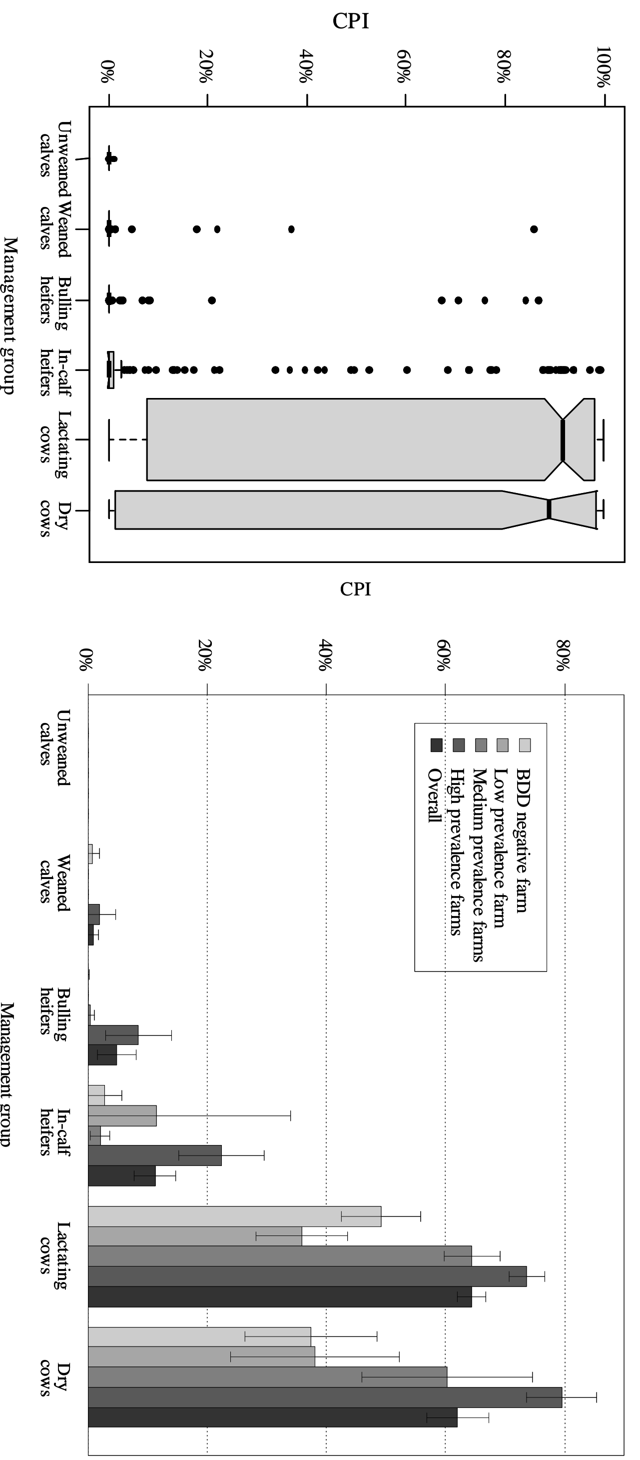


Figure 4.7: Notched boxplots and barplot showing the BDD infection level per management group (all farms, n=2165), as determined by the Bayesian model. The boxplots show the median conditional probability of infection (CPI), 25th and 75th percentiles, and outliers; the barplots show the mean CPI and the corresponding 95% confidence intervals. The differences between the levels in the plots can be explained by the shape of the underlying frequency distributions. Note that the model assigns high mean CPIs to cows from the clinically BDD free farm

4.4 Discussion

Several authors have associated increases in the prevalence of foot and leg disorders (including BDD) with changes in housing systems from tie-stall systems to loose-housing systems in which hygiene is poorer and the opportunity for animal contacts is greater (Capion, 2004; Enevoldsen et al., 1991; Hultgren, 2002). A number of independent investigators have consistently reported increasing morbidity of BDD (Frankena et al., 1991; Laven, 2003; Manske et al., 2002; Somers et al., 2003; Holzhauer et al., 2006). However, the distribution of infection on the farm level (i.e. within and between management groups) and between farms has not been well investigated.

In this population cross-sectional study, we have combined clinical inspection of BDD with serology of BDD-associated treponemes, applying statistical methods to provide a detailed characterization of the farm-level prevalence, distribution and risk factors for BDD. Given this objective, we elected to perform a highly detailed study on a small number of farms, rather than a less detailed study on a larger number of farms. This is at odds with a risk-factor study such that of Holzhauer et al. (2006), where the investigators were more interested in effects across all herds.

We strove to select a representative study population; as the numbers of farms was small, participating farms were selected partly on the basis of information from veterinary practices, to include farms with different BDD prevalences but mostly comparable management practices. Our findings on BDD prevalences are comparable to those reported in other studies (Frankena et al., 1991; Rodriguez-Lainz et al., 1996; Rodriguez-Lainz et al., 1999; Somers et al., 2005; Holzhauer et al., 2006), which indicates that our study population is indeed representative.

4.4.1 Farm-level distribution of BDD

Clinical findings After stratification of the study population into high, medium and low clinical prevalence farms, we found that high prevalence farms not only had higher clinical prevalence, but also had a higher proportion of acute lesions, i.e. new infections. Very few BDD lesions were found in young stock (calves and heifers); those identified were almost all on the high prevalence farms. This implies that the level of exposure is higher on these farms, which probably results in a higher force of infection. Anecdotal information from farmers suggests that when BDD becomes a 'problem', it tends to spread in epidemic waves; at other times, it shows a more endemic pattern, at lower prevalence.

Serology Serology was very useful for further exploring the farm-level dispersion of the infection. The serological frequency distribution of the whole dataset (i.e. all farms) is positively-skewed, with evidence of a bimodal response. Frequency histograms of the farm populations stratified by clinical BDD prevalence level show that the lower

clinical prevalence farms tend to have more strongly skewed distributions and less bimodality (median: 11.14, standard deviation: 17.30) than medium clinical prevalence farms (median: 15.53, standard deviation: 21.73) and high prevalence farms (median: 45.06, standard deviation: 29.49), which tend to approximate normal distributions. The evidence of bimodality indicates that the ELISA distinguishes between positive and negative sub-populations. This becomes more evident when comparing the distributions of the clinical positives to the clinical negatives. The mean titre of clinical positives is significantly higher than clinical negatives; however, there is substantial overlap between these sub-populations. Animals with acute or chronic lesions have significantly higher titres than those with regressing lesions. This indicates that the antibody half-life is short.

Statistical modelling As the regression models included farm ID as a random effect, no outputs were obtained at this level. The Bayesian model, which incorporated both serological results and clinical data to make an inference of an animal's conditional probability of 'true' infection (CPI), was applicable in this respect. Although this model did not define a cut-off point, it tends to classify animals as either infected or non-infected. This is reflected by the gradient of the PPI curve given in 3.5, which is quite steep; for an ideal test, it would be vertical. The model's predicted levels of infection are higher than the clinical prevalence, as the model may identify animals as being latently infected. Almost all young stock are assigned a low CPI; it is only in the cow groups that substantial numbers of animals are identified with a high CPI. The pattern of infection is comparable with both the clinical prevalence and the serological distributions (see Figures 4.1, 4.4 and 4.7). Stratifying the farms by prevalence level shows that the medium and high prevalence farms have a higher level of infection; a proportion of the young stock are evidently exposed to infection on these farms. This is consistent with our clinical findings.

Specific interpretation is required of the Bayesian model results. From our data, the prevalence of lesions appears to decrease with age, but the antibody titre tends to remain high. This suggests that there is a more or less constant level of exposure to the causative pathogens in the cow groups, and that older animals develop a degree of immunity to developing lesions. On the basis of the high titre, these animals will be identified as 'infected' by the Bayesian model. The model therefore implies that these animals are infected with BDD-associated pathogens, rather than saying that an animal has BDD, which is by its definition a clinical condition. A more correct terminology would be that animals with high titres but no BDD are 'exposed', where this is defined as 'infected but not diseased' (this is discussed more fully in Chapter 6). We have not distinguished between these states in the Bayesian model.

If older animals do not develop clinical BDD after exposure, investigation of serol-

ogy may be useful for epidemiological investigation of the distribution, transmission and immunology, but has limited meaning for clinical investigation of BDD. A low titre indicates absence of infection; a high titre may indicate infection, but disease status can only be determined by clinical inspection. Hence, we still require more detailed knowledge on strain variation and pathogenic mechanisms of BDD associated *Treponema* spp.

This is further highlighted by our findings on the negative control farm. This farm had been closed to importing stock for over twenty years, had high levels of management and biosecurity and could be said to be clinically BDD negative with a high degree of confidence. No BDD lesions were observed during the sampling of this farm. However, of 217 lactating and dry cows sampled, 74 had a serologic PP greater than 30 (identified by the Bayesian models as having a CPI of 80% or greater of being infected), of which 14 cows had a PP greater than 50 (greater than 90% probability). The serological frequency distribution was comparable to other farms which did have clinical BDD. Accordingly, the Bayesian model also showed high levels of infection in the lactating and dry cow groups. Despite our improved understanding of the aetiology and humoral mechanisms, interpretation of this is still subject to conjecture. It is possible that BDD-associated *Treponema* spp. (i.e. the 'necessary' cause as outlined in the section on causality, Chapter 1.3) are present on this farm, but that the levels of hygiene and management are such that the preconditions for development of disease (i.e. a 'sufficient' cause) are not met. Alternatively, other, non-BDD related or less pathogenic *Treponema* spp. bacteria (of unknown origin) could effectuate seroconversion. Thirdly, the ELISA may cross-react with other antibodies. Finally, it is possible that clinical BDD is present on this farm at low prevalence, but has not been observed.

4.4.2 Association of BDD with age

Clinical findings No lesions were observed in any animals less than a year old, and only a few in the 1 to 2 year category. In contrast to other studies, the highest prevalence was not found in the first parity group (age 2 to 3), but in the second parity group. Some studies have concluded that first lactation cows are specifically at risk (Brentrup and Adams, 1990; Frankena et al., 1991), although others have not found any effect (Green et al., 2002). The prevalence decreases slightly with age; a finding that was also made in recent study by Holzhauer et al. (2006). This may indicate development of partial immunity.

Serology Assessing the age group boxplots, it is apparent that titres are low, rising gradually up to the age of 2 to 3 years (which corresponds to the time of first exposure in milking cow groups); hence this age category was stratified into non-lactating and lactating. The titres of the lactating sub-category were significantly higher than

the non-lactating sub-category. Once animals entered the cow environment, the titres remained more or less uniformly high, presumably due to repeated exposure in the housing environment. It is interesting to compare this with the clinical prevalence, which shows a decreasing trend. Insofar as there is development of partial immunity, it would therefore appear that this is not through humoral mechanisms – a conclusion shared by other authors (Walker et al., 1997; Demirkan, Walker, Murray, Blowey and Carter, 1999). From our data, we found that on the individual level, titres fall rapidly during and after regression of lesions; the fact that median titres remain high with increasing age categories indicates that serology is a good marker of repeated exposure to infection.

Statistical modelling For the outcome of clinical BDD, one of the GLMMs with farm as random effect included age as a fixed effect. The highest odds of BDD were in the 2-3 year category; animals >6 years had substantially lower odds. For the mixed effects models for the serological outcome, the estimates rose gradually, showed a sharp rise for the 3-4 year category, and continued to rise slightly with increasing age. The model outputs therefore mirror the EDA.

4.4.3 Association of BDD with management group

Clinical findings No clinical BDD was observed in the unweaned calves, weaned calves and bulling heifer groups on any of the farms. Only a few lesions were seen among in-calf heifers; these were from high or medium prevalence farms. The disease primarily appears in the cow groups. The prevalence decreases slightly for the dry cows. This can possibly be explained by a combination of cleaner housing, lower exposure levels and cessation of milk production.

Serology From the management group boxplot, a similar gradual rise in titres is seen until the start of lactation, followed by a sharp rise; titres remain high in the dry cow group. Clinically, a lower prevalence was observed in this group. It is possible that the duration of the drying-off period is too short for a significant decline in titres to take place; although this would appear to be in conflict with the finding that animals with regressing lesions tend to have lower titres.

Statistical modelling When management group was included as a fixed effect in the GLMM for clinical BDD (with farm as a random effect), the odds ratio for lactating cows was more than double that of dry cows. For the LME models for serology, the two models in which management group was specified as a fixed effect (both with farm as a random effect) gave differing results, depending on the second explanatory variable. With body hygiene score as the second covariate, the estimate was similar for lactating and dry cows; with age as the second covariate, the estimate was lower for dry cows.

When the individual CPIs from the Bayesian model were stratified by management group and graphed, it appeared that the model tended to dichotomize quite strongly (in comparison to the PPI plot given by Figure 3.5, most CPIs cluster below 10% and above 80%). Nearly all calves and heifers are assigned a low CPI; roughly half as many cows are likely to be uninfected than infected. While relatively few animals fall in the CPI range from 10% – 80% (which corresponds to the ELISA PP range of $\sim$ 17% to $\sim$ 30%), those that do are nearly all cows; in infectious disease terms, these animals appear to be transiting from uninfected to infected states, or vice-versa.

The boxplots per management group show a very high median CPI for the cow groups, and a negligible mean CPI for the young stock groups (albeit with high CPI outliers). The means (given in the barplot) are lower for the cow groups and higher for the young stock groups, respectively. Given the underlying frequency distributions (strongly skewed for the young stock groups and strongly bimodal for the cow groups), this is to be expected. As noted in 4.3.1 above, cows with high titres were identified on the clinically BDD free farm; this is consistent with the model, which assigns high mean CPIs to cows from this farm.

4.4.4 Association of BDD with lactation stage and parity

The serological boxplots did not show any discernible trend for lactation number and stage; the potential confounding effect of season could not be studied. As the serological boxplots for age showed comparable titres for cows of increasing age, and parity is a function of age, the absence of a trend is consistent.

Neither lactation stage nor parity were significant in the univariable GLMMs for clinical BDD (see Appendix G). This is possibly a consequence of the reduced number of observations (the data came from 609 animal records). Lactation stage and parity did remain significant for the univariable LMEs for serology, but they could not be included in multivariable models. The model estimates showed an overall rising trend with increasing parity and advancing lactation stage.

4.4.5 Risk factors for BDD

Hygiene in the housing is the risk factor that has been most commonly associated with BDD. We were not able to confirm this, as the management group level explanatory variables (housing hygiene, housing comfort, BDD treatment and footbath protocols) were not significant in the univariable analysis, for either of the statistical models for clinical BDD or serology.

Quantitative estimation of these variables is a complex task. In particular housing hygiene is a highly changeable variable, and it is difficult to obtain a representative estimation from a single inspection. The management group variables were compound indices of relevant information derived from the questionnaire interview and subse-

quent inspection of the farm premises; simplification of the many various variables is inevitable, and derivation of such indices is inevitably a subjective procedure. It is therefore possible that these indices were incorrectly specified; alternatively, they could have been insufficiently detailed to statistically identify relevant trends.

The individual-level hygiene scores FHS and BHS can, however, be considered proxies for environmental hygiene, and these were more simply and repeatably assessed. The statistical models did not associate high FHS with clinical BDD or high serology; it appeared that within management groups, foot hygiene scores tended to be uniform. However, BHS was significantly identified as a fixed effect by both the final GLMM and the final LME.

Considering the farm distribution of BDD, as indicated by the EDA, it was not surprising that management group and / or age were also significant risk factors. The final models incorporated management group in the random effects structure (nested within farm).

Chapter 5

Investigating temporal patterns of BDD: a longitudinal study

5.1 Introduction

Very little scientific work has been done on the investigation of temporal trends of BDD. Various studies have described the development and progression of clinical lesions over time (Döpfer and Willemen, 1998; Blowey, 1992; Brizzi, 1993); others have mentioned the seasonal element of the disease (Blowey and Sharp, 1988; Blowey, 1993; Nutter and Moffitt, 1990; Peterse et al., 1992). Döpfer (1994) performed a descriptive repeated cross-sectional study on two dairy farms in the Netherlands over a period of six to eight months, which resulted in a detailed classification system of BDD lesions; however, it did not address disease transmission or rates of infection.

No longitudinal studies specifically investigating BDD incidence rates and temporal disease trends have been published. Whereas there have been advances in recent years in our knowledge of the microbial aetiology and pathogenesis (see Chapters 1.2 and 2.4), our understanding of the factors influencing the transmission at the group and farm level, and hence the 'force of infection', is still mostly hypothetical. Risk-based studies are difficult to carry out in the absence of knowledge about exposure to the infection. Consequently, we still do not have a good understanding of the infection dynamics of the disease.

Knowledge of temporal patterns in the epidemiology of BDD is a prerequisite for the development of practical and effective control and intervention strategies. Risk factors for BDD have been studied in some detail by various authors in the cross-sectional study setting (see Chapter 4). Fixed risk factors (e.g. age and sex) enable high-risk groups to be identified. However, some of the variables associated with BDD are time-varying (e.g. environmental hygiene), and it is therefore likely that these exert a direct influence on the seasonal nature of the disease. Such effects or relationships have not been investigated or quantified. An understanding of this may determine the impact of various strategies, and whether efforts can be concentrated on the housing period only,

or whether continuous measures are required for effective control.

Our objectives were to study temporal patterns of infection and disease and their relationships with animal level risk factors. This included trends in BDD prevalence, seasonal changes in presentation of lesions, and the association of incidence of lesions with the serologic response. For lactating cows, we also aimed to assess the relationship of BDD with production characteristics, i.e. lactation stage and parity. By including high, medium and low clinical prevalence farms that had previously participated in the cross-sectional study (see Chapter 4), background information was already available. As with the cross-sectional study, we utilized clinical inspection as well as serology to describe the patterns of disease. The relationship between these outcomes and time-varying and static variables were examined using random effects regression models, formulated to explore longitudinal data. These enabled the relative importance of specific risk groups and risk factors as determinants of BDD to be identified.

Finally, this study provided data for calculation of the rates of incidence of new clinical cases, recovery and serological status for the parameterization of mathematical SEIR (Susceptible – Exposed – Infected – Recovered) type models (Chapter 6).

5.2 Materials and methods

5.2.1 Study farm selection and sampling populations

Study farms

This prospective longitudinal study was carried out on four dairy farms in Cheshire, UK. These farms were recruited from the cross-sectional study; this was advantageous as baseline data on BDD prevalence, housing conditions and management practices were already available. The study farms included two high-prevalence farms (06 and 08), a medium-prevalence farm (05) and a low-prevalence farm (07); refer to Tables 4.1 and 4.5 in Chapter 4 and Appendix B for relevant details. As with the cross-sectional study, these farms were held to be representative in terms of production and general management practices.

Farms 06 and 07 were sold in September 2004. Farm 06 dropped out of the study. Farm 07 was leased to the previous farm manager, who consented to continued participation; however, the cohort sizes were halved as the farm was partially de-stocked.

Cohort structure

Three cohorts per farm were selected, in such a way that all management groups would be represented over the study duration:

1. Newborn calves or calves of < 8 weeks of age, which were followed through weaning and bulling, and were midway into the first gestation at the study's conclusion.

2. In-calf heifers of age 18 to 24 months, which were followed through the first calving and first lactation, and were towards or at the end of the first lactation at the study's conclusion. This group was proportionally over-represented, as it was identified from the literature as a specific risk group.

3. Cows (lactating and dry), consisting of a mixture of ages / parities and lactation stages.

For practical and logistic reasons, the cohort sizes were initially set at 20 – 30 animals per farm, broken down into approximately 20% calves, 30% heifers and 50% cows. Herd lists (including animal ID, date of birth and management group) were available from the study farms. These lists were stratified by management group. Within each management group, the animals were sorted by date of birth. The lactating cows group, which was the largest, was split into two equal blocks; a spread of ages of the study animals was thus incorporated. Study animals were then selected from each management group by random sampling.

To avoid confounding by age, group and season, recruitment of animals was performed at several time points, i.e. was staggered, specifically for the unweaned calves and in-calf heifer cohorts. This was not necessary for the cows, as these animals are in a lactating – dry cycle, and there was no marked seasonality of calving for the study farms. As loss to follow-up was inevitable, particularly in the cow groups, the cohorts were dynamic to adjust for this, i.e. replacement was performed to keep the cohort of a constant size. In practice, regular sampling of the young stock and dry cows was problematic due to housing and logistic constraints (e.g. limitations of the farmers' time and capacity to collect these animals; during the summer, grazing on distant pastures). Table 5.1 lists the total number of animals that participated in the study; the 109 individual animals represent a total of 120 animal years of study.

Farm ID	No. in cohort			
	calves	heifers	cows	total
05	4	16	15	35
06	6	5	18	29
07	8	0	15	23
08	6	5	11	22
All	24	26	59	109

Table 5.1: Cohort sizes of the longitudinal study

5.2.2 Data collection and sampling protocol

Sampling commenced shortly after the completion of the cross-sectional study in February / March 2004, and continued to December 2005; the study duration was therefore

approximately 22 months. This period was extended from 18 months to include the transitional periods of housing to grazing, and subsequent grazing to housing, for two successive years. This was desirable as empirical knowledge indicates that the changes in disease rates are particularly large in these periods. Up to 30 observations per individual animal were made.

Sampling was performed on a fortnightly basis during the housing season (maximum risk period) and every four weeks during the grazing season (low risk period). All field work was done by the author. The inspection protocol was similar to that performed during the cross-sectional study (see Chapter 4.2.3). At each sampling point, foot hygiene scoring and body hygiene scoring were performed; individual hygiene scores on a 1-4 ordinal scale were calculated. Blood samples were obtained for serological analysis. The feet were inspected for lesions using the borescope, which were scored as described in Chapter 3 and Appendix C.

Additionally, the conformation of the feet was recorded by visual assessment of the length of the claws, the symmetry of the claws and the heel height; it was scored as normal, overgrown (long toes, shallow angle) or abnormal (irregular or uneven). The stance of the cow was assessed as a binary variable, i.e. whether the hocks of the hind legs were turned in ('cow hocked'); this is often a consequence of overgrown, neglected feet (Toussaint Raven, 1989). A description of all observed variables is included in Table 5.2.

Every foot identified by borescope inspection as having a BDD lesion was lifted in a crush to better characterize the lesion. Images were taken of the lesions identified, for future reference. The feet of, on average, every sixth cow were lifted regardless of whether lesions were observed with the borescope or not, as part of a validation exercise (see Chapter 3 for more details).

5.2.3 Laboratory analysis

The serological protocol was identical to that used for the cross-sectional study (Chapter 4). Serum samples were analysed with the 'cocktail' ELISA as described in Chapters 2 and 4, and by Demirkan, Walker, Murray, Blowey and Carter (1999).

5.2.4 Statistical analysis

Data handling and validation

All data collection and entry was performed by the author. The data were checked for errors and reorganized and recategorized as appropriate. Compound indices were derived for foot hygiene score (FHS), body hygiene score (BHS), foot conformation and BDD lesion presentation. Another index was calculated for lesion severity: a product of a score for the presentation (with erosive lesions assigned a higher score than granulomatous and regressing lesions) and the diameter of the lesion (in cm). In

addition, the date of sampling was split into month categories so that rolling monthly averages for the study period could be calculated. Environmental hygiene and comfort data (similar to that collected in Chapter 4) were felt to be insufficiently accurate, due to the difficulty of making representative observations, the complexity of the assessment and the difficulty of obtaining a suitable index. No management group- or farm-level variables were considered for further analysis.

Specific attention was given to the issue of missing values, which may influence or bias the outcomes of analysis, and result in a loss of power. Missing data are common, and indeed inevitable, in longitudinal analysis (Horton and Laird, 1998; Diggle et al., 2000; Engels and Diehr, 2003); a good review on the subject is given by Carpenter and Kenward (2005).

Three scenarios for the occurrence of missing values can be identified:

1. *Missing values within records.* This corresponds to observations that were forgotten to be recorded, observations that are unclear or ambiguous, or missing results due to breakage, loss or test failure of a serum sample. These were less than 1% of the total number of observations, and occured at one timepoint only. The probability of these missing values occurring is assumed not to depend on the other observations, and hence they are 'missing completely at random' (MCAR).

2. *Missing records.* This corresponds to animals that should have been inspected at a sampling point, but were not. This could be a consequence of animals not being presented by the farmer (missed out, moved to a different management group, turned out to pasture, ill or not available for other reasons) or alternatively animals not being sampled by the author (unmanageable animals, lack of capacity or time). These missing values are defined as 'missing not at random' (MNAR) and may influence the results. Cows from the cubicle housing were consistently sampled, but animals in the loose housing (young stock and dry cows) tended to be undersampled for the reasons mentioned above. Also, these problems were encountered when sampling during the grazing season. Statistical methods can account for unequal intervals between sampling dates (irregular time series), but cannot compensate for this missingness.

3. *Dropout / loss to follow-up.* Removal of animals from the cohorts was due to production-related performance (poor milk production or mastitis, reproductive failure and related causes), and to the author's best knowledge no dropout was specifically associated with BDD or related lameness. It is therefore unlikely that this was a a source of bias.

Several methods are available for dealing with missing data, the simplest employing *ad-hoc* methods which create a single, 'complete' dataset which is analysed as if it were

the fully observed data. Removing all records containing missing values is an inefficient option; if different explanatory variables are included in the dataset, the resulting dataset may be very small, and if the mechanisms of missingness are not completely random, bias is introduced. Mean imputation can be performed for missing continuous variables, where the mean or median of the variable is used to replace missing values; alternatively, the mean of the previous and next values can be used (but only if the time intervals are constant). For categorical variables, mean imputation is not an option and 'last observation carried forward' (LOCF) or 'next observation carried back' (NOCB) methods may be considered. Information from the joint distribution of known variables can be used by applying regression techniques to predict estimates for the missing values. All these methods will to an extent influence the means and covariance structure (Engels and Diehr, 2003; Carpenter and Kenward, 2005). Other, more rigorous methods such as multiple imputation or a method of weights in generalized linear models strive to minimise this (Schafer, 1999; Horton and Laird, 1998).

The longitudinal study dataset was relatively detailed, with many observations per record, a short between-sampling interval (2 or 4 weeks) and a relatively large number of observations per animal over the duration of the study (up to 30). The total proportion of MCAR values was calculated to be <1%. Moreover, many observations such as FHS, BHS, foot conformation and ELISA PP tended to be slow-changing. As it was unlikely that *ad-hoc* methods would significantly affect, hence bias, the outcomes of statistical analysis, these were felt to be valid for completing these records. For categorical variables, the NOCB method was used; for the continuous variable (ELISA PP), the mean of the previous and next samples was taken. The completed records were then included in the analysis.

Imputing missing clinical BDD observations was more problematic, because lesion scores were more changeable than the other variables. As these scores were categorical, taking a mean of previous and next scores was not an option, and variability in lesion presentation is not necessarily a linear process. Employing LOCF or NOCB methods could therefore over- or underestimate the imputed scores. To minimise this, the inspection scores of the record closest in date to the one containing the missing values were adopted. If the interval with these records was >3 weeks, the ELISA PP was compared to previous and next records to impute the most reasonable scores. It is likely that this technique does introduce some bias; however, it was felt to be preferable to deleting the records altogether, and this method was only considered as the number of missing values was small. In three cases, it was felt that *ad-hoc* imputation was not defensible, and the records were removed from further analysis. No adequate technique was used to account for the NMAR data (missed records).

Table 5.2 lists the variables available for possible inclusion in the statistical models.

Variable	Type	Categories
Lesion presence	binary	0: absent
		1: present
Lesion presentation	ordinal	1: acute
		2: chronic
		3: regressing
Lesion severity index	continuous	n.a.
Foot conformation	categorical	1: normal
		2: overgrown
		3: abnormal
Foot hygiene score	categorical	1: very clean
		2: mostly clean, fresh soiling
		3: mostly soiled
		4: deeply caked-on soiling
Body hygiene score	categorical	1: very clean
		2: mostly clean, fresh soiling
		3: mostly soiled
		4: deeply caked-on soiling
Serology	continuous	n.a.
Age	continuous	n.a.
	OR categorical	1: 0-1 years
		2: >1-2 years
		3: >2-3 year
		4: >3-4 years
		5: >4-5 years
		6: >5 years
Management group	categorical	1: calves
		2: heifers
		3: lactating cows
		4: dry cows
Lactation stage	continuous	n.a.
	OR categorical	1: 0-50 days in milk (DIM)
		2: 51-100 days in milk (DIM)
		3: 101-150 days in milk (DIM)
		4: 151-200 days in milk (DIM)
		5: 201-250 days in milk (DIM)
		6: 251-300 days in milk (DIM)
		7: >300 days in milk (DIM)
Parity	categorical	1: first
		2: second
		3: third
		4: fourth
		5: fifth and higher
Date of sampling	continuous	n.a.
Farm-Group-Time	nominal	composite variable of management group, farm and sampling date

Table 5.2: Overview of variables available for inclusion in statistical models of the BDD longitudinal study

Exploratory data analysis (EDA)

While longitudinal and cross-sectional studies share common characteristics in that they address relationships between a mean response and a set of explanatory variables, longitudinal data include an extra covariate, namely time; the EDA attempts to uncover trends in time which cannot be determined in the cross-sectional setting (Diggle et al., 2000). Changes over time in individual animals can be identified, as well as differences between individuals when grouped into categories or cohorts. The time variable can be applied in several different ways:

- Continuous time, i.e. study duration;
- Period, season or calendar date; this allows rolling averages to be displayed, which smooth out fluctuations in trends;
- Animal time, i.e. age, days in milk, lactation stage, parity.

Only the third variable can be studied in cross-sectional studies, as it is not recorded in continuous time. For longitudinal studies, univariate summary statistics may be useful for exploratory analysis of multiple measurements which can be used in the modelling phase of the analysis; but awareness of the confounding effects due to clustering is essential – for example, assessing the effects of lactation stage and parity. This is adjusted for by using appropriate statistical models.

EDA was performed using the same graphical methods as in Chapter 4 (barcharts, histograms, boxplots) as well as techniques aimed specifically at identifying temporal trends and correlation structure (scatterplots, sample variograms and lorelograms).

Scatterplots. These can be used to display the relationships between a continuous response (y-axis) and continuous covariates (x-axis), which can be time or another covariate, e.g. age or days in milk. They can be plotted in trellis plots to allow relevant stratification of the dataset, e.g. by farm or lactation number.

Sample variograms. These are useful for describing the association between values when sampling intervals are irregular, whereas other methods require artificially rounded measurement times to be defined; they enable the degree of autocorrelation to be investigated (Diggle et al., 2000). It is calculated from a relevant regression model. The residuals are extracted from the model for all points i; the observed half-squared differences between pairs of residuals at times j and k (where $j < k$) is calculated:

$$v_{ijk} = \frac{1}{2}(r_{ij} - r_{ik})^2$$

The corresponding time intervals are calculated, i.e.

$$u_{ijk} = t_{ij} - t_{ik}$$

As multiple observations were made on the sample dates, there will be multiple pairs of residuals with the same interval u_{ijk}. We let $\hat{\gamma}(u)$ be the mean of the υ_{ijk} for all of these and this is what we call the sample variogram. We plot υ_{ijk} against u_{ijk} and then fit $\hat{\gamma}(u)$ as a smoothed curve. We also plot the 'process variance', which is the mean of all of half-squared differences, and is therefore a constant. The sample variogram increases with lag, corresponding to decreasing correlation as observations are separated in time. Once the sample variogram exceeds the process variance, the autocorrelation is less than the variance in the data, and may therefore be assumed to have decayed to zero.

Lorelograms. This graphical technique was developed by Heagerty and Zeger (1998) to explore association amongst binary or multinomial data. A correlation-based approach is not feasible as the range of correlation is constrained by the means. However, association between binary variables can be modelled using the pairwise odds ratio Ψ, which is not constrained (Diggle et al., 2000); for two observations Y_j and Y_k made at times t_j and t_k this is given by

$$\Psi(Y_j, Y_k) = \left[\frac{\Pr(Y_j = 1, Y_k = 1)\Pr(Y_j = 0, Y_k = 0)}{\Pr(Y_j = 1, Y_k = 0)\Pr(Y_j = 0, Y_k = 1)}\right]$$

For paired observations close together in time, i.e. if the lag $|t_k - t_j|$ is small, we would expect the numerator to be high and the denominator to be low; as the interval between observations increases, the numerator decreases and the denominator increases. The odds ratio is therefore strictly positive and unbounded. The logarithm is taken of this to yield the entire real line as the range of possible outcomes. Another advantage is that for binary responses, the logit link function induces the regression parameters to be measured on this scale; this facilitates direct comparison and interpretation (Heagerty and Zeger, 1998). The general definition of the lorelogram for a longitudinal sequence $Y_{i1}, ..., Y_{in}$ with measurement times $t_{i1}, ..., t_{in}$ is therefore given by

$$\mathrm{LOR}(t_j, t_k) = \log\ \Psi(Y_j, Y_k)$$

This can be easily graphed.

5.2.5 Statistical modelling

Background and approach

We wished to investigate associations between a set of explanatory variables and an outcome or response (clinical BDD or serology). As with the cross-sectional study, the explanatory variables can be regressed on the outcome as the linear predictor in linear

models. The dataset had the same hierarchical structure as for the cross-sectional study (i.e. animals within management groups on different farms); a similar random effects modelling approach could therefore be taken to account for clustering in the data (see Chapter 4.2.6).

In addition, however, serial observations or measurements on the same study subject were correlated; thus the data were clustered not just within management groups nested within farms, but also within animals. Specific approaches were required to account for this dependence. As a single measurement per individual animal was now replaced by a vector of successive measurements, the data represent a multivariate, rather than a univariate, distribution (Lindsey, 1997; Diggle et al., 2000). The biological mechanisms which bring about the dependence between these measurements cannot be observed or quantified; we assumed that these will differ between animals, and it was hence appropriate to include the individual animal identifiers as a random effect in our models. Techniques for exploring the degree of autocorrelation include the lorelogram and sample variogram (see above). Appropriate methods compensating for autocorrelation in the statistical models were applied.

It may therefore be assumed that there were three elements of random variation that needed to be considered (Diggle et al., 2000):

1. Random effects (heterogeneity between individuals);
2. Autocorrelation (with decreasing correlation as the time lag increases);
3. Measurement error.

Measurement error includes factors such as variation in the ELISA procedure or inconsistencies in observations of study animals' feet. Such errors are inevitable. Through assessment of the repeatability of the ELISA (Chapter 2 and Appendix A) and validation of the borescope (Chapter 3), we have indicated that measurement error was unlikely to affect the results. As we lacked sufficient information to parameterize this in our models, it was not further considered.

As with the cross-sectional study models, the modelling process was stepwise, and could be subdivided into *formulation*, *estimation* and *inference*.

Formulation. This was essentially dependent on the study design and the primary objectives of study, the outcome of interest and the structure of the dataset, and was also guided by the EDA; hence, it flexibly allowed incorporation of biological information and certain assumptions, e.g. choice of appropriate model and fixed / random effects structure. For the clinical outcome of BDD lesions (binary outcome), generalised linear mixed models (GLMMs) were appropriate. For BDD serology (continuous outcome), linear mixed-effects models (LMEs) were appropriate. As with the cross-sectional study, the GLMMs were estimated using the Laplace algorithm, and the

LMEs were estimated using maximum likelihood (ML). For a more detailed treatment of these families of models, see Chapter 4.2.6 or Dohoo et al. (2003).

Estimation. Various combinations of likelihood estimation algorithms and random effects structures were investigated to identify the best model formulation. As mentioned above, the unique animal identifier was included as a random effect. In addition, we hypothesized that there may be a unobservable clustering effect for animals sampled within a specific management group on a particular farm on a sample date; we created a Farm - Group - Time (FGT) index, which was included as another random effects variable. Thus, the various hierarchies in the data were represented in the random effects term. As animal ID and FGT are not nested variables, they should be specified as separate or crossed random effects (in contrast to the cross-sectional study models, where management group was nested within farm) (Bates, 2005). As the number of study farms was half that of the cross-sectional study, farm ID was not fitted as a random effect.

Covariance structure was assessed, specifically the influence of seasonal trends on the infection. Detrending was performed if appropriate, to remove this source of variability.

Finally, the correlation structure was taken into account. As the outcomes of the models were on different scales (binary and continuous), different techniques were used.

For the clinical BDD model, a transition (or Markov) model was the most straightforward and suitable approach. As mentioned above, the range of the correlation between a pair of binary variables is constrained by their means. The transition model assumes that a variable Y_{ij} is explicitly influenced by its previous values, which are denoted as its history, H_{ij}. Hence, for q previous responses,

$$\Pr(Y_{ij}|H_{ij}) = \Pr(Y_{ij}|Y_{ij-1}, ..., Y_{ij-q})$$

where q represents the order. The previous outcomes are then included in the model as additional explanatory variables. The probability of having a lesion at time t_{ij} therefore depends on the explanatory variables as well as the lagged outcomes, which are included in the model as new explanatory variables. Although the lorelogram indicated serial correlation of a higher order, we utilized a first-order Markov chain for simplicity and convenience, i.e. $\Pr(Y_{ij}|H_{ij}) = \Pr(Y_{ij}|Y_{ij-1})$. A drawback of using a transition model is that the first outcome must be dropped (as there is no outcome for $t-1$ in this case); this was a relatively minor problem with our dataset as it included a relatively large number of observations per animal. As higher orders are modelled, more observations will need to be excluded, leading to loss of power. Also, the transition model assumes equally-spaced time intervals, which was not always the case; a first-order Markov chain minimises the degree to which this assumption is not met.

For the linear mixed-effects model for BDD serology, there are more than two states: we cannot make use of a similar transition matrix, and therefore resort to a different

method to model the dependence between serological test results. We applied a first order autoregressive model, which is a member of the group of models variously described as autoregressive models, exponential correlation models, moving average models or Box and Jenkins models (Pinheiro and Bates, 2000; Diggle et al., 2000).

The assumption is made that the correlation between two results, ϵ_j and ϵ_k, decreases exponentially towards zero as the lag, $(|t_j - t_k|)$, between the results increases:

$$\text{Cov}(\epsilon_j, \epsilon_k) \;=\; \sigma^2 \text{exp}(-\phi \mid t_j - t_k|)$$

where ϕ is a correlation parameter (Diggle et al., 2000). The exponential function is defined as the autoregressive model; its order is defined as the number of observations between j and k. In the first-order model $AR(1)$, ϕ represents the correlation of a serological result with the previous result, and takes values between -1 and 1. The $AR(1)$ assumes that the data are observed at equally spaced intervals, but this case can be generalized to continuous time measurements (i.e. days in study) – which is referred to as the continuous time $AR(1)$, or $CAR(1)$. This model was used. Higher order autoregressive models are more complex to specify (Pinheiro and Bates, 2000).

Inference. The same method was followed as for the cross- sectional study (see Chapter 4.2.6). Univariable analysis was first performed using the explanatory variables listed in Table 5.2; those that were significant at a liberal p-value (0.25) were considered for inclusion in multivariable models. Multivariable models were iteratively constructed. The models were compared and selected on the basis of likelihood ratio tests (LRTs) and ANOVA.

More details on the methodology of the statistical models, including the relevant code, are given in Appendix I.

5.2.6 Association between clinical disease and serology

In the EDA, group-level trends in the clinical incidence and serologic response were examined. For the formal analysis of the data, appropriate techniques were used to model the correlation structure caused by dependence between serial observations or samples, and periodicity was investigated and de-trended if appropriate for the estimation of risk factors. However, neither approach directly provides information on how the serologic response is associated with the incidence of clinical BDD on an individual level.

Due to the variability in clinical presentation and humoral response, it was difficult to designate a discrete timepoint at which clinical disease could be considered to begin or end – which is a requirement for investigation of such patterns. Plots were made of the serologic profiles of individual animals over the study duration, and these were related to the incidence of lesions expressed as a lesion severity score (which was an

index derived from the presentation and size of the lesion); however, such plots were qualitative and did not yield clear information.

5.3 Results

5.3.1 Exploratory data analysis

In this section, the data are presented for a large part as univariate (i.e. one observation per animal), whereas in reality they are multivariate (multiple observations per individual). The dependency in the data due to repeated sampling of individuals is not taken into account. However, the objective of the EDA is to use simple graphical relationships between variables measured on individual cows at each time point to identify broad trends.

Clinical data

This was analysed for the cow groups only, as very few lesions were observed in the in-calf heifer groups, and none in the young stock.

Seasonal trends in prevalence. A strong seasonal effect was observed, with a peak in the prevalence between November and January, and minimum prevalence occurring in June and July. Figure 5.1 shows these trends. In both years, the incidence appeared to decrease before the end of the housing period; it started to rise at the end of the grazing season when the cows started coming in for night-time housing, before they were housed day and night. The plot shows that the prevalence decreased further over the summer of 2005 than it did in 2004: the patterns over the two years are otherwise largely comparable. Minor variations in farm-level prevalence were observed (data not shown).

Seasonal trends in lesion presentation and severity. Figure 5.2 shows a barplot and spinogram of the lesion prevalence and a breakdown by presentation of lesions (acute, chronic and regressing), for all farms. This shows that all lesion types occurred all year round; the proportions of acute lesions, which represent new cases of disease, was more or less constant. The relative proportion of regressing lesions increased towards the end of the housing period, and decreased at the beginning of the housing period. The widths of the spinogram bars are proportional to the number of cases observed; as expected, the bars are narrower during the summer months. There is an artefact in the spinogram in that only one January month (2005) was included, and roughly one and a half February and December months. Therefore the bars for these months are narrower than would really be the case.

Figure 5.1: Mean clinical BDD prevalence (all animals, all study farms) over the study duration (top) and the rolling average over the study period (bottom). The shaded periods roughly correspond to the grazing season

Age and management group trends. The incidence of clinical BDD was positively associated with age. No lesions were seen in animals younger than one year old on the date of sampling. Lesions were seen in seven in-calf heifers; in six cases, they were small, acute, did not persist for more than two weeks, and did not lead to seroconversion. In the seventh case, lesions persisted for three months and seroconversion did take place. The disease therefore was therefore almost exclusively observed in the cow groups.

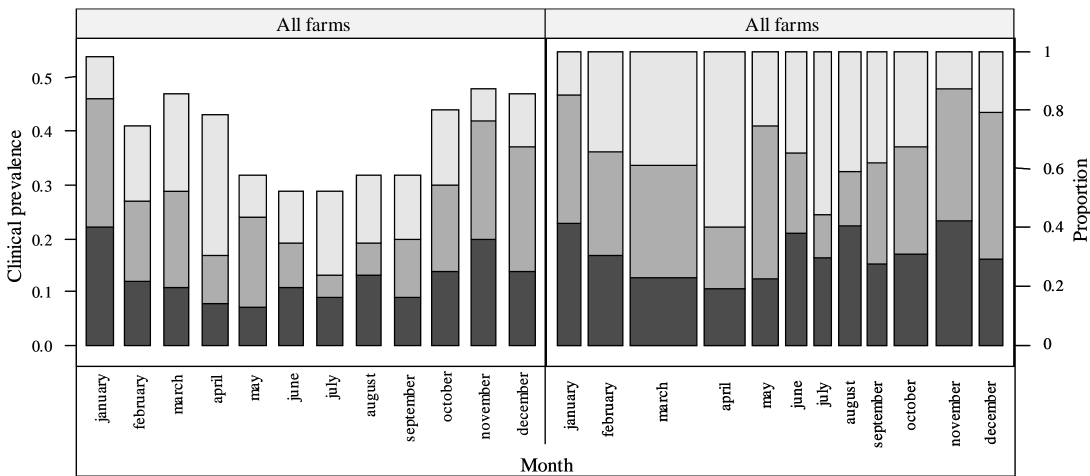


Figure 5.2: The barplot on the left shows the mean clinical BDD prevalence on all farms per calendar month (rolling average 2004 - 2005). The spinogram on the right is an extension of a histogram, showing the number of counts in each bar not by proportional height but by proportional width; this highlights trends within the proportions not immediately obvious from the barplot. Dark shading: acute lesions; medium shading: chronic lesions; pale shading: regressing lesions

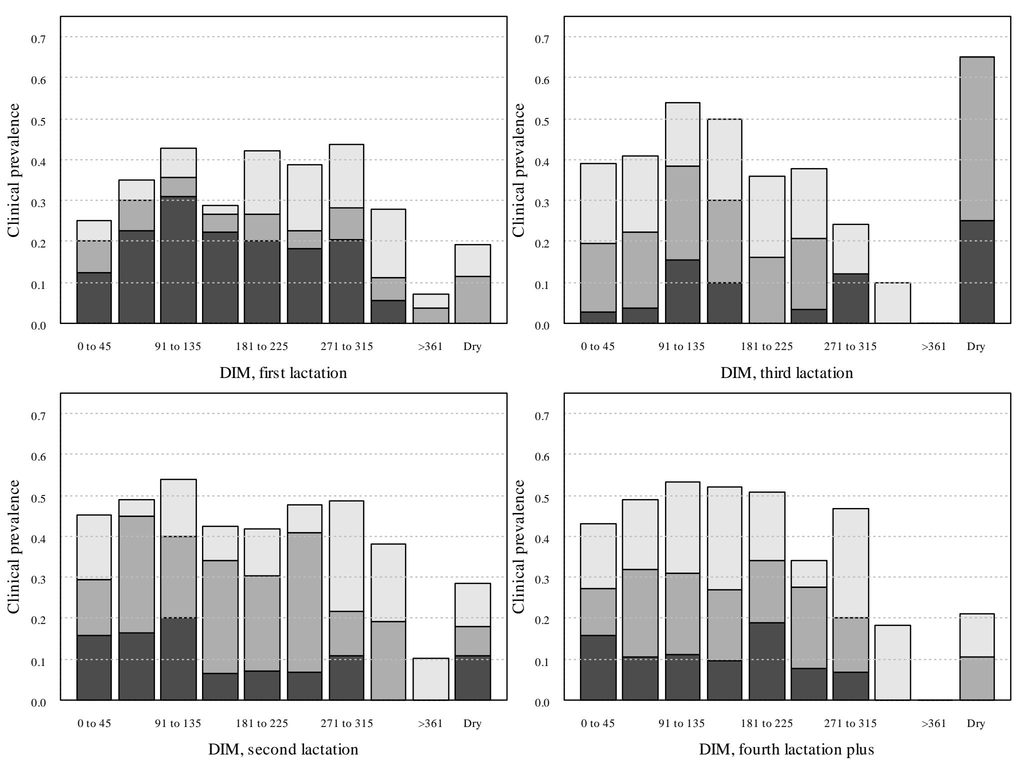


Figure 5.3: Clinical BDD prevalence of different parity cows, broken down by lactation stage (days in milk, DIM) and including dry cows (n=1346 observations). Dark shading: acute lesions; medium shading: chronic lesions; pale shading: regressing lesions

Parity and lactation stage trends in prevalence and lesion presentation. As shown in Figure 5.3, a general trend for all parities can be seen: from the start of the lactation, there was an increase in the clinical prevalence; peak prevalence corresponded with the peak of lactation (91 to 135 day category). The prevalence remained reasonably constant thereafter, but decreased towards the end of the lactation.

Second parity and higher cows appeared to have higher clinical prevalence than heifers calving for the first time. However, there is a marked difference in the type of lesions observed; first calving heifers had a relatively much higher proportion of acute lesions. Another finding is that the dry cows seemed to have a higher clinical prevalence than the cows at the end of their lactation; there is a possible bias here, as the dry cows could not be routinely sampled on two of the study farms (of which one was a high prevalence farm, and the other was a low prevalence farm).

Lorelogram. Figure 5.4 shows the lorelogram for the longitudinal study data. This is for the binary outcome of BDD lesions, and therefore does not differentiate, or show the autocorrelation, between the stages or severity of the lesions. As expected, the lorelogram declines rapidly with increasing lag between observations, to about 7 months. It then continues to fluctuate; it does not decay to 0, nor do any 95% confidence intervals include 0. This indicates that there is a long-term dependence between BDD observations. The confidence intervals increase as the lag between the observations increases.

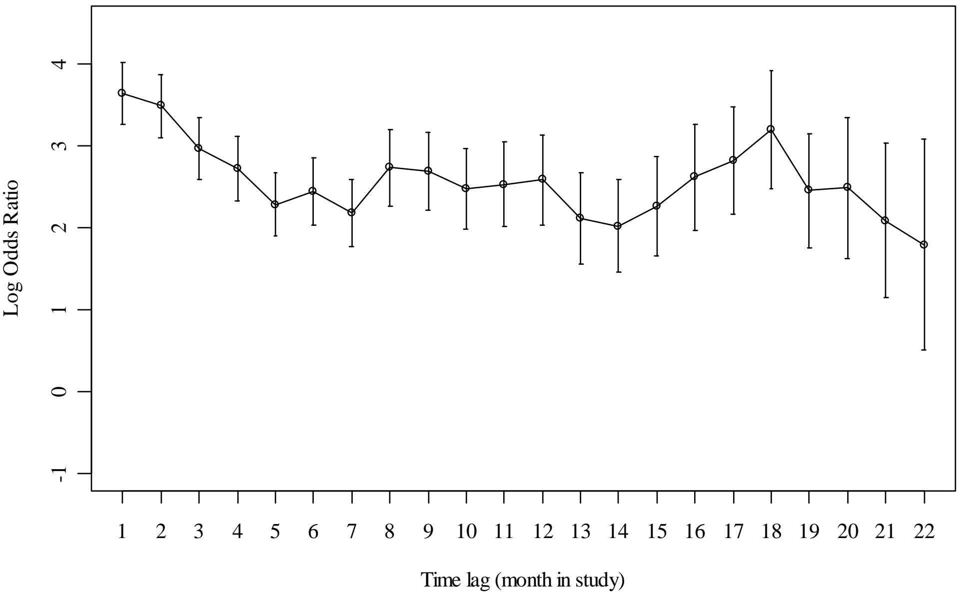


Figure 5.4: Lorelogram of BDD lesions, plotting the log of the odds ratios for two observations Y_j and Y_k made at times t_j and t_k; the bars represent 95% confidence intervals

Serological data

Serological distributions. Frequency histograms and density plots of all tested animals, clinical BDD positive animals and clinical BDD negative animals were made; this was performed for all management groups and the subset of lactating and dry cows. The results are given in Figure 5.5. Compared to the cross-sectional study serological distributions (Figure 4.2 in Chapter 4), the positive response is much stronger; the distribution is more clearly bimodal, although the degree of overlap is still very large.

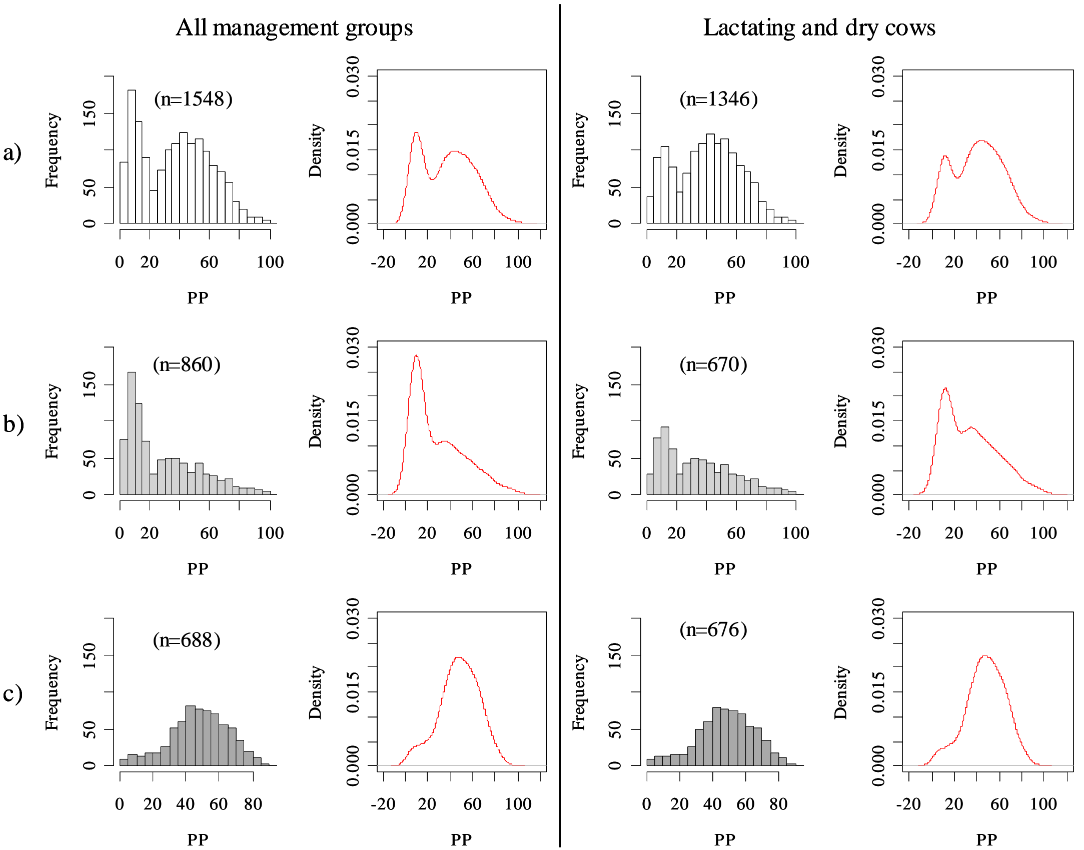


Figure 5.5: Serological frequency distribution histograms and density plots. a) all animals, b) the subset of clinical BDD negatives, c) the subset of clinical BDD positives; n refers to the number of samples

Seasonal trends in serology. In marked contrast to the strong seasonal trend for incidence of BDD lesions, no seasonal trend was identified at all for the treponemal serology (Figure 5.6), although animals with clinical BDD had significantly higher titres.

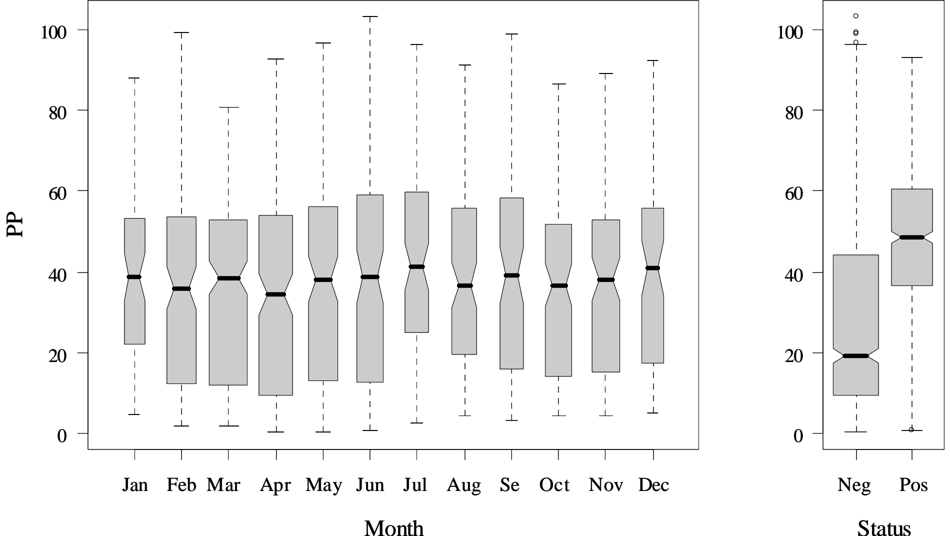


Figure 5.6: Boxplots of ELISA titres per calendar month (rolling average 2004 - 2005), and boxplots of clinical negatives and positives over the same period (n=1565 samples)

As the scatterplots in Figures 5.7 and 5.8 show, this lack of trend was consistent for all study farms; there was a decrease in the mean titre for farm 07, but this was not seasonal, and was possibly management-related. When stratification by age was performed, the only discernable pattern was a rise in the mean titres with increasing age, which is consistent with other results. Subsequent analysis showed that while lesion regression resulted in a decrease of clinical prevalence over the grazing period, many animals retained high titres (this is presented in detail in Chapter 6).

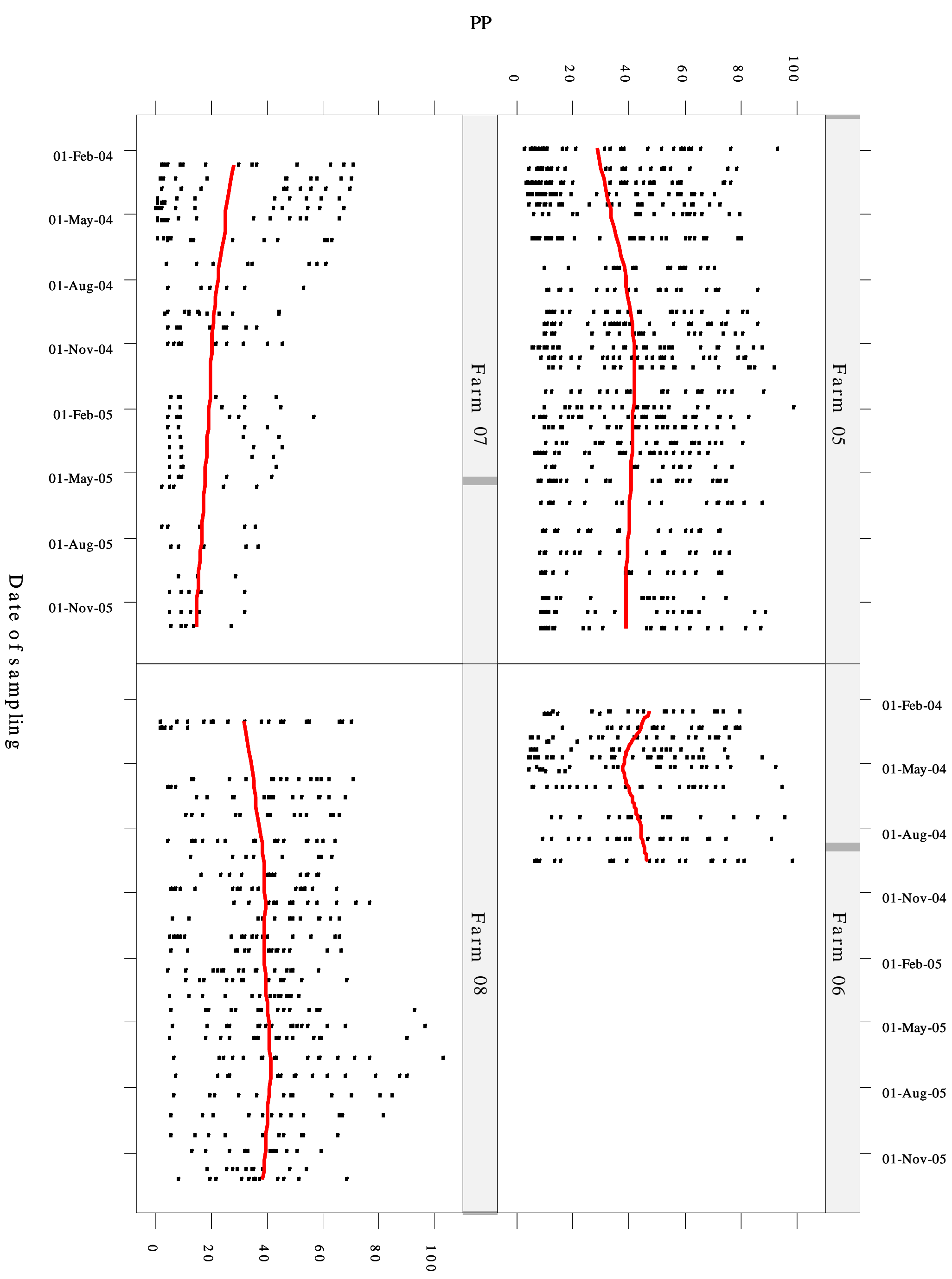


Figure 5.7: Scatterplots of ELISA titres per calendar month (rolling average 2004 - 2005, n=1565 samples) for the study farms (note that Farm 06 left the study after 6 months). The smoothed line was fitted using the loess technique

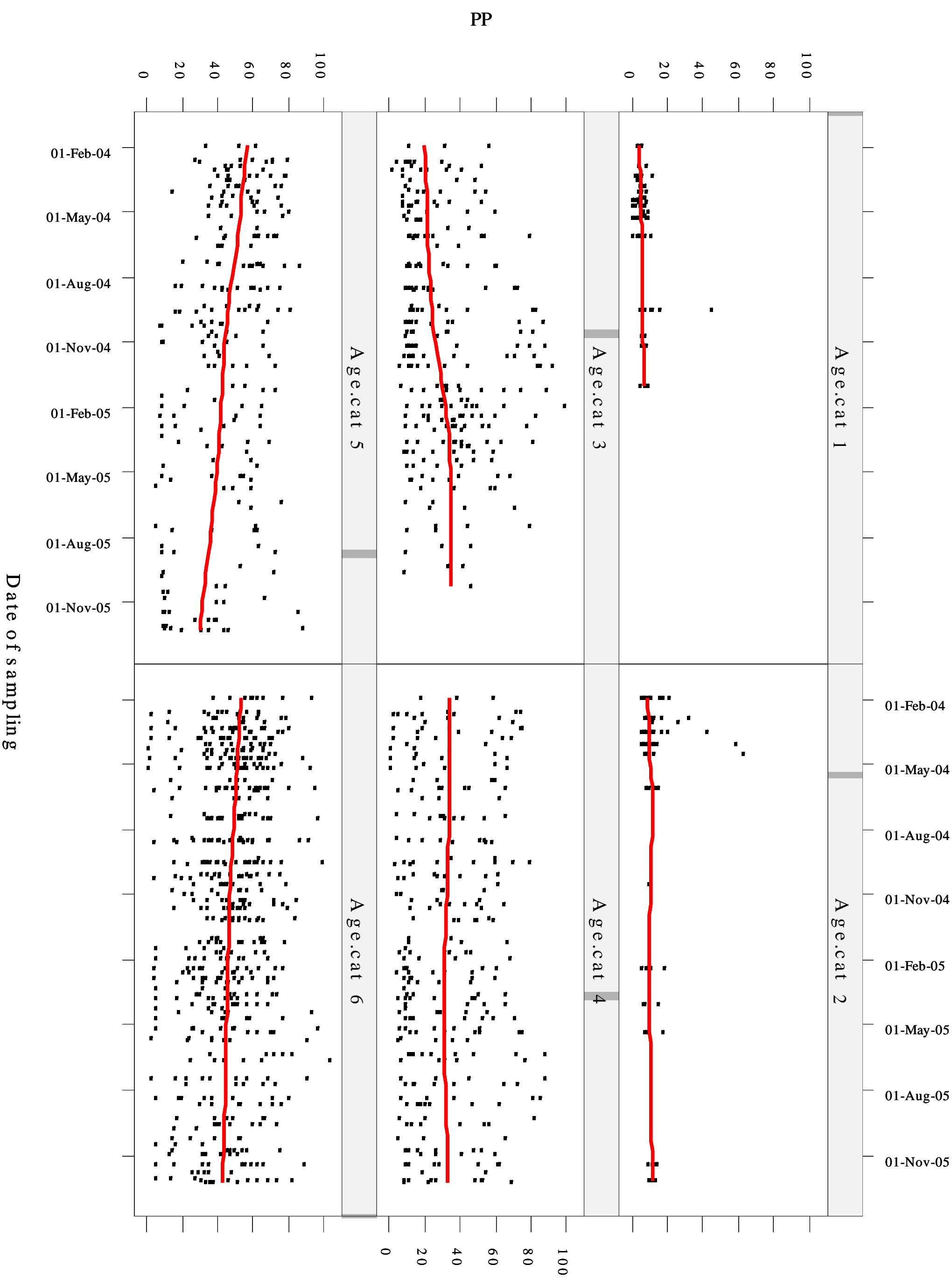


Figure 5.8: Scatterplots of ELISA titres per calendar month (rolling average 2004 - 2005, n=1565 samples) for the age categories (1: 0-1y; 2: 1-2y; 3: 2-3y; 4: 3-4y; 5: 4-5y; 6: >5y). The smoothed line was fitted using the loess technique

Age trends in serology. An increase in titres is apparent with increasing age on all farms (Figure 5.9). On farm 06, the loess smoothed line shows a downturn; this is a consequence of a single older animal, and not an actual trend in the data. Qualitatively, the points fan out from about two years of age, with some animals developing high titres and others remaining low.

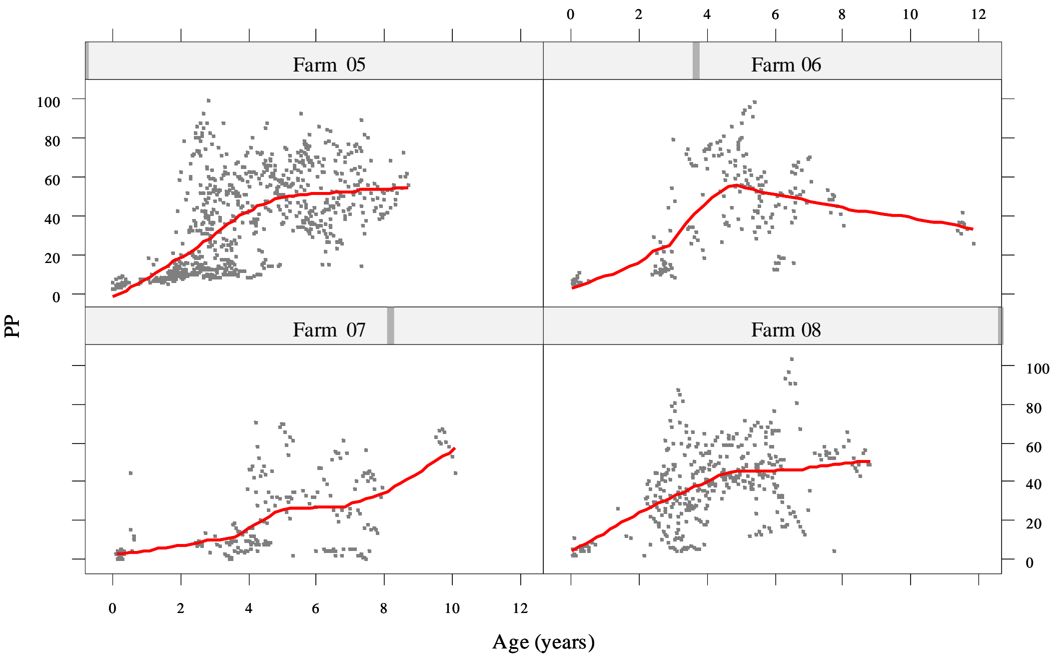


Figure 5.9: Scatterplots of ELISA titres versus age for the study farms (n=1565 samples). The smoothed line was fitted using the loess technique

Lactation stage and parity trends in serology. No clear relationships emerged between the incidence of BDD and the stage of lactation (or days in milk, DIM). The scatterplots (Figure 5.10) appear to show an increasing trend over the duration of the lactation for first and second lactation cows, and a stationary or slightly decreasing trend for older parity cows. The level of the tires appears to rise slightly with advancing lactation number. The boxplots (Figure 5.11) show more clearly that the titre is initially low for first-calving heifers, but rises up to about 200 days, and subsequently declines. For subsequent parities, the PP is reasonably constant at a median of about 40.

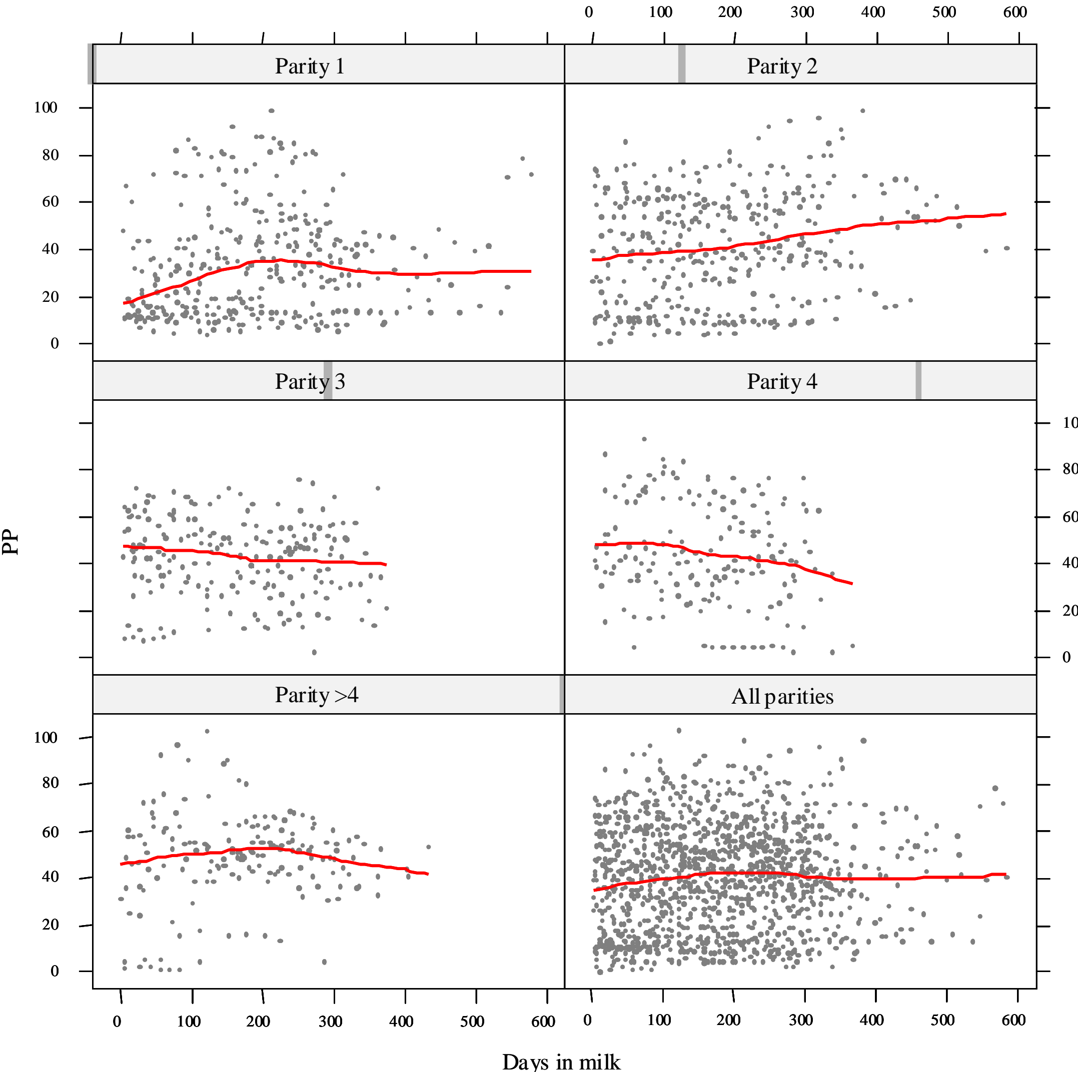


Figure 5.10: Scatterplots of ELISA titres versus lactation stage (days in milk) for cows stratified by lactation number (n=1253 samples). The smoothed line was fitted using the loess technique

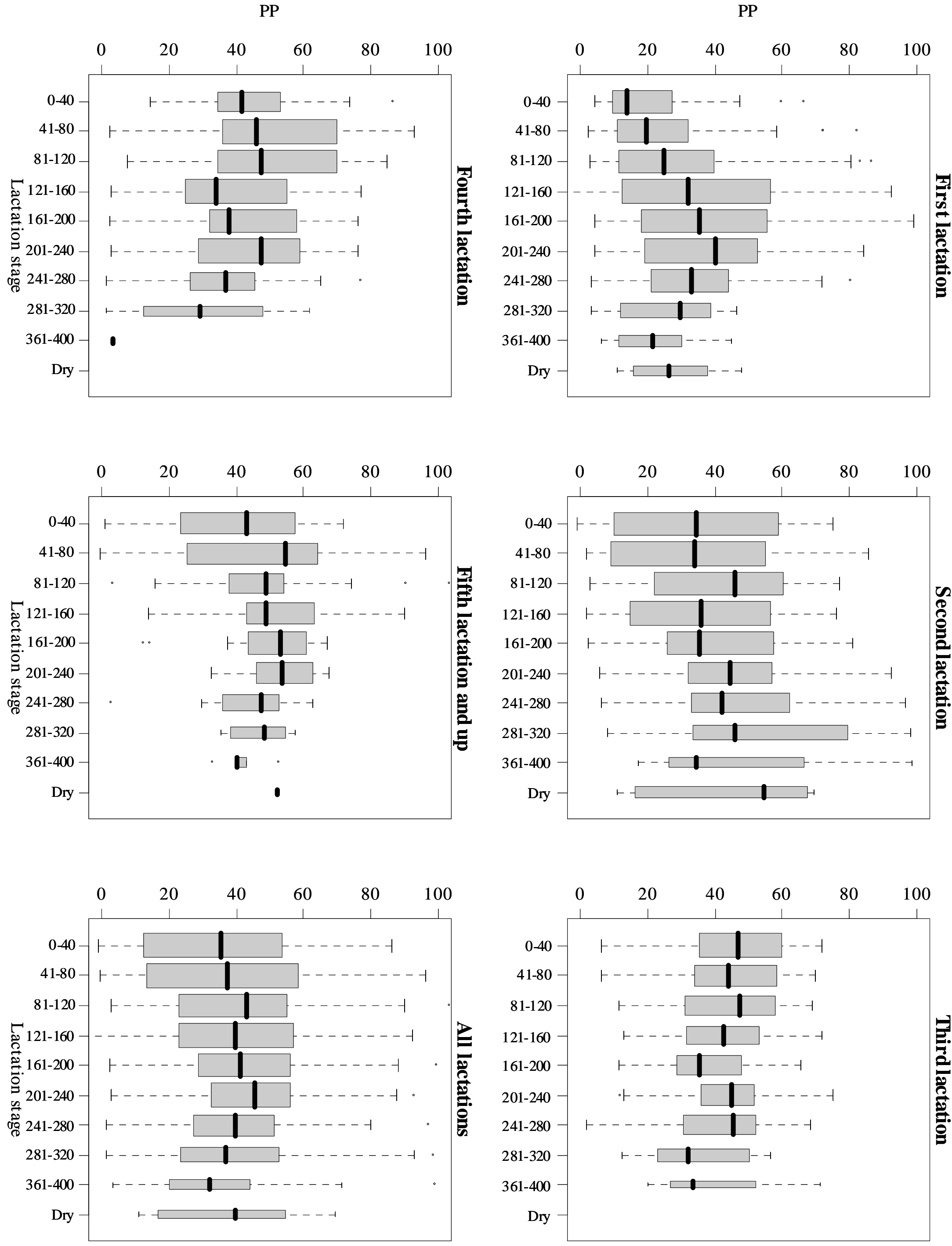


Figure 5.11: Boxplots of ELISA titres versus lactation stage (days in milk) for cows stratified by lactation number (n=1253 samples)

Sample variogram. Figure 5.12 was plotted by extracting the residuals from the LME model presented in 5.3.2 below. The horizontal dashed line represents the 'process variance'; when it is crossed by the kernel smoothed line representing the sample variogram, the correlation between residuals is less than the variance in the data, and the autocorrelation may therefore be assumed to have decayed to zero. For BDD serology, this is a duration of approximately 230 days. As the density of points is maximal at the beginning of the study and minimal at the end of the study (due to dropout), it is likely that there are some edge effects.

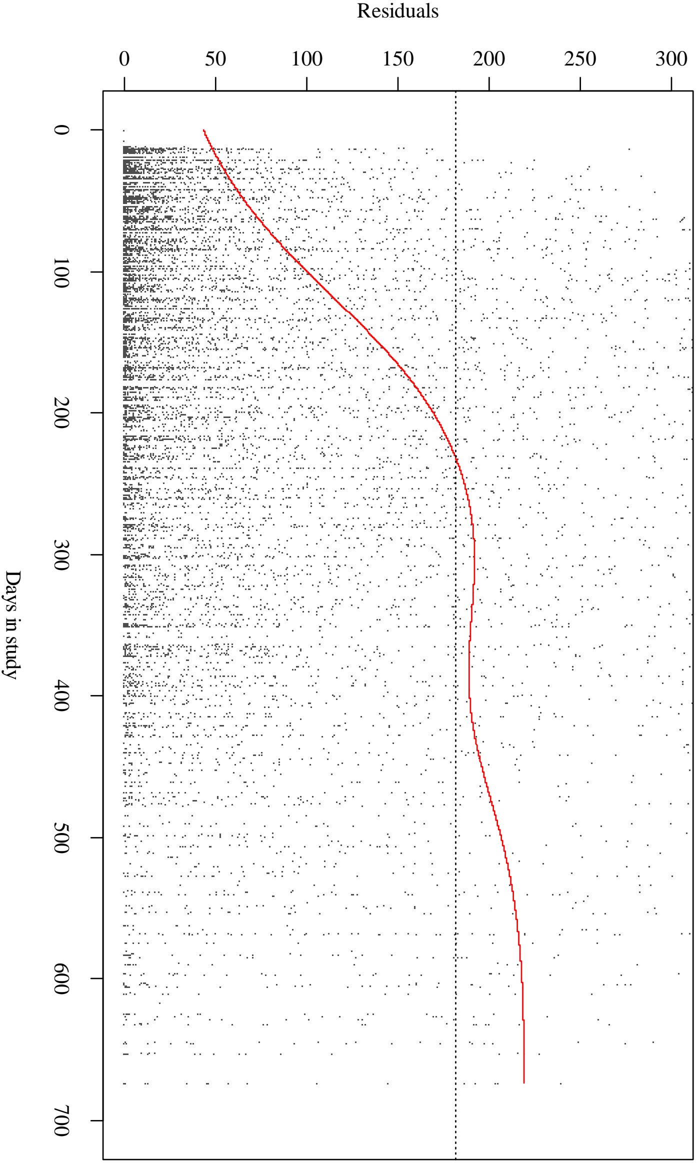


Figure 5.12: Sample variogram of residuals from the BDD serology LME model. Each point represents the half-squared difference between a pair of residuals, v_{ijk}, plotted for all time intervals $u_{ijk} = t_{ij} - t_{ik}$. The red line is kernel smoothed with a bandwidth of 150 days; the dotted line is the 'process variance' (refer to text for details)

5.3.2 Statistical models

The univariable analyses are not presented. Results of the final transition model for BDD lesions and CAR(1) model for BDD serology are given below. Separate models were fitted for the productivity-related parameters, i.e. parity and lactation stage, as these variables were only available for a subset of the data.

Transition model for BDD lesion outcome

Season of year and housing (as a binary variable, housed or grazed) were significant before, but not after, removing the seasonal component and de-trending the data. Table 5.3 shows the output of the final model. The likelihood ratio test (LRT) of this transition model compared to the final GLMM (i.e. before fitting the lag term as an additional fixed effect) was 192.85 (corresponding to a χ^2 with 5df of p <0.0001). The LRT of the GLMM compared to the null model was 51.06 (corresponding to a χ^2 with 5df of p <0.0001). The final GLMM was therefore significantly better than the null model, and the transition model was a significant improvement on the GLMM.

Variable		Coefficient	SE	OR	95% CI	P-value
Sine function	$\sin(days/365 * 2 * \pi)$	-0.53	0.21	0.59	0.39 – 0.88	0.01
	$\cos(days/365 * 2 * \pi)$	0.69	0.24	2.00	1.25 – 3.22	< 0.01
Age (categorical)	0-2y	Referent				
	>2-3y	2.13	0.78	8.39	1.83 – 38.52	< 0.01
	>3-4y	2.31	0.81	10.06	2.06 – 49.14	< 0.01
	>4-5y	2.43	0.86	11.35	2.09 – 61.63	< 0.01
	> 5y	2.52	0.86	12.48	2.33 – 66.71	< 0.01
Body hygiene score	1	Referent				
	2	1.75	0.75	5.74	1.32 – 24.89	0.02
	3	1.84	0.79	6.32	1.36 – 29.45	0.03
	4	1.64	0.83	5.14	1.02 – 25.96	0.05
BDD lesion$_{(t-1)}$		3.15	0.25	23.36	14.24 – 38.31	<0.01

Table 5.3: Output of the final longitudinal study transition model with clinical BDD as binary outcome. Note the sine function added to detrend seasonal effects, and inclusion of the lag term. Animal ID and the farm - group - time (FGT) variable were fitted as random effects

CAR(1) model for serologic outcome

Table 5.4 shows the output of the final model. The LRT of this CAR(1) model compared to the final LME (i.e. before fitting the CAR(1) function) was 1107.33 (corresponding to a χ^2 with 5df of p <0.0001). The LRT of the LME compared to the null model was 263.21 (corresponding to a χ^2 with 5df of p <0.0001). The final LME was significantly

better than the null model, and CAR(1) model was a significant improvement on the LME.

Variable		Value	SE	95% CI	t-value	P-value
Age (categorical)	0-2y	Referent				
	>2-3y	5.94	2.27	1.50 – 10.39	2.62	< 0.01
	>3-4y	8.79	2.65	3.59 – 14.00	3.31	< 0.01
	>4-5y	13.21	2.96	7.41 – 19.02	4.46	< 0.01
	>5-6y	17.07	3.21	10.77 – 23.37	5.31	< 0.01
	>6-7y	12.81	3.45	6.06 – 19.57	3.72	< 0.01
	>7-8y	15.85	3.92	8.18 – 23.53	4.05	< 0.01
	> 8y	20.63	5.20	10.43 – 30.82	3.96	< 0.01
Management group	Unweaned calves	Referent				
	Weaned calves	1.00	1.91	-2.73 – 4.74	0.53	0.60
	Heifers	10.77	4.00	2.92 – 18.62	2.69	< 0.01
	Lactating: pre-peak	19.49	4.16	11.33 – 27.64	4.68	< 0.01
	Lactating: peak	20.67	4.18	12.47 – 28.86	4.94	< 0.01
	Lactating: post-peak	20.77	4.19	12.55 – 28.99	4.95	< 0.01
	Dry	20.37	4.26	12.02 – 28.72	4.78	< 0.01
Body hygiene score	1	Referent				
	2	-0.13	0.98	-2.06 – 1.79	-0.13	0.89
	3	0.02	1.06	-2.06 – 2.10	0.02	0.99
	4	-0.41	1.20	-2.77 – 1.95	-0.34	0.73

Table 5.4: Output of the final longitudinal study CAR(1) model with BDD serology as outcome. Animal ID was fitted as the random effect

The value of ϕ estimated by the model was 0.992. This implies that for an animal sampled with an interval of 14 days, the correlation between the samples will be $(0.992^{14}) = 0.89$; for an interval of 28 days, this will be $(0.992^{28}) = 0.80$.

Models investigating association of BDD with lactation parameters

Models were fitted for both outcomes (BDD lesions and BDD serology) following the same methodology as described above. All records from lactating cows (n=1253 from 91 cows) were included.

Transition model. As shown by Table 5.5, neither lactation stage nor parity were significant in the model, or showed any clear trends. As above, the transition model was a significant improvement (p <0.0001) on the GLMM.

Variable		Coefficient	SE	OR	95% CI	P-value
Sine function	sin(days/365*2*π)	-0.50	0.23	0.61	0.38 – 0.95	0.03
	cos(days/365*2*π)	0.79	0.26	2.21	1.33 – 3.66	< 0.01
Lactation stage	0-50 DIM	Referent				
	51-100 DIM	0.47	0.44	1.59	0.67 – 3.79	0.29
	101-150 DIM	-0.06	0.44	0.94	0.39 – 2.23	0.88
	151-200 DIM	0.11	0.44	1.12	0.47 – 2.67	0.80
	201-250 DIM	-0.07	0.45	0.93	0.39 – 2.25	0.87
	251-300 DIM	0.02	0.46	1.02	0.42 – 2.49	0.97
	>300 DIM	-0.88	0.48	0.42	0.16 – 1.08	0.07
Parity	1	Referent				
	2	0.72	0.43	2.05	0.89 – 4.74	0.09
	3	0.56	0.64	1.76	0.50 – 6.16	0.38
	4	0.41	0.72	1.51	0.37 – 6.24	0.57
	≥5	0.82	0.78	2.28	0.49 – 10.56	0.29
BDD lesion$_{(t-1)}$		2.75	0.28	15.64	9.08 – 26.92	< 0.01

Table 5.5: Output of a transition model with BDD lesions as binary outcome, for the lactating cows subset. Note the sine function added to detrend seasonal effects, and inclusion of the lag term. Animal ID and the farm - group - time (FGT) variable were fitted as random effects

CAR(1) model. Lactation stage did not show a clear trend (Table 5.6). Parity is significantly associated with BDD; the titres rise in successive lactations. As above, the CAR(1) model was a significant improvement (p <0.0001) on the LME.

Variable		Value	SE	95% CI	t-value	P-value
Lactation stage	0-50 DIM	Referent				
	51-100 DIM	0.37	0.87	-1.35 – 2.08	0.42	0.67
	101-150 DIM	1.54	1.15	-0.72 – 3.80	1.34	0.18
	151-200 DIM	2.74	1.29	0.21 – 5.27	2.12	0.03
	201-250 DIM	2.99	1.39	0.27 – 5.70	2.15	0.03
	251-300 DIM	2.24	1.45	-0.60 – 5.09	1.54	0.12
	>300 DIM	3.02	1.62	-0.16 – 6.19	1.86	0.06
Parity	1	Referent				
	2	4.70	2.32	0.15 – 9.25	2.02	0.04
	3	7.63	3.33	1.09 – 14.16	2.29	0.02
	4	8.73	4.02	0.85 – 16.60	2.17	0.03
	≥5	11.22	4.67	2.07 – 20.36	2.40	0.02

Table 5.6: Output of a CAR(1) model with serology as outcome, for the lactating cows subset. Animal ID was fitted as the random effect

5.3.3 Association between clinical disease and serology

Figure 5.13 shows plots of the serologic profiles of four randomly chosen cows over the study duration, and the corresponding lesion severity score over the same period; plots were made for more animals, but are not included. Qualitatively, the incidence of lesions was frequently accompanied by a rise in the antibody titre, although high titres without evidence of a lesion were common (e.g. top right plot). The bottom left plot represents an animal with fluctuating lesions over the study period (it was considered to be lesion negative on only two occasions); the titre was uniformly high. The bottom right plot also shows an animal with chronic lesions for the majority of the study period; decay of the antibody titre follows shortly after regression of the lesion(s). Less commonly, lesions were found in animals that had not developed a high titre.

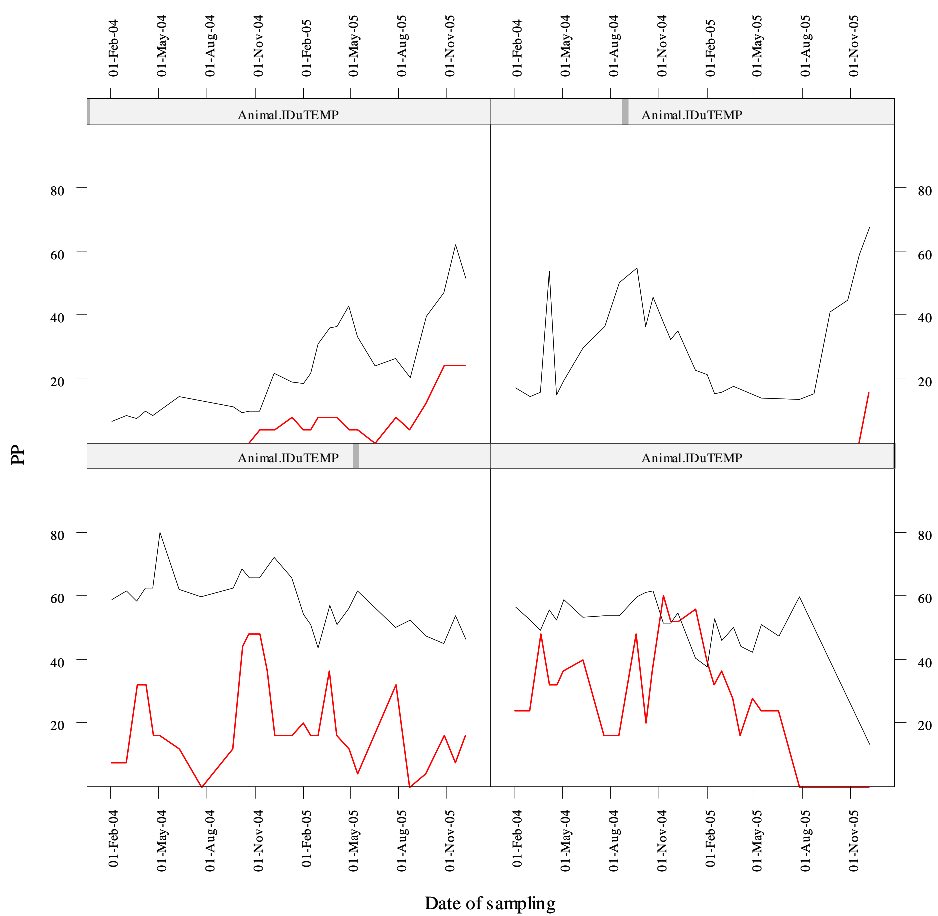


Figure 5.13: Serologic profiles (black line) and BDD lesion severity scores (red line) of four randomly selected animals over the study duration. A lesion severity score of 0 indicates no lesion; all higher values represent a lesion, where increasing severity is determined by size and presentation of the lesion

5.4 Discussion

The dynamics of BDD are poorly understood and have not been subject to rigorous scientific investigation. This study describes trends and associations in the incidence, development and progression of disease, for different ages, lactation stages and parities. By relating the serology to the clinical presentation over the study duration, we gained further insights into the humoral immune response. We specifically investigated the seasonal patterns in the dynamics of infection.

5.4.1 Seasonality in the incidence of BDD

Laven and Lawrence (2006) analysed the seasonality of a total of 3299 BDD reports originating from a database of veterinary treatments recorded by a network of 40 veterinary practitioners; they found that the morbidity of BDD had a significant seasonal component, with fewer reports being made from June to October. Comparing their findings to those of Murray et al. (1996), they inferred that the overall seasonality of BDD had decreased since the early 1990s; they attribute this to the continued spread of BDD, leading to an endemic disease pattern on affected farms, where the disease persists into the grazing period. This is consistent with Holzhauer et al. (2006), who compared the results of their cross-sectional study with those of Frankena et al. (1991) and conclude that the morbidity of BDD had increased in the intervening 15 years. They suggest that this could possibly be associated with prolonged housing periods.

Our results show a similar seasonal distribution (Figure 5.1). There was some variability in clinical manifestation and prevalence between farms, which was possibly related to farm management practices such as footbathing, or climactic / environmental conditions, time of turning out and re-housing, and farm-specific BDD infection dynamics. There was also some variation in BDD incidence per year, with the incidence in 2005 being lower. Interestingly, the incidence rate of lesions started declining before the end of the housing period, and continued into the grazing period. However, the incidence over the grazing period remained relatively high (resulting in a prevalence no lower than 0.2 to 0.3), and acute lesions were identified all year round (Figure 5.2). If, as Laven and Lawrence (2006) postulate, the seasonality of BDD has indeed decreased, our data would suggest that this might be due to increased incidence over the grazing period, rather than reduced incidence during the housing period.

We may look to the serology of BDD-associated *Treponema* spp. for further clues. Contrary to expectation, we found no evidence of seasonality. From the cross-sectional study (Chapter 4), we had established that lesion positives had significantly higher titres than lesion negatives; and the serological frequency distribution showed evidence of bimodality, which indicated the existence of serologically negative and positive subpopulations corresponding to clinical negatives and positives. This was confirmed by the

longitudinal study data. Moreover, and in agreement with other results (Walker et al., 1997), our results indicated that the half-life of the IgG_2 antibodies is short, decaying during or soon after regression of the lesions. Therefore, the lack of a seasonal pattern in the serology was contrary to expectation. A possible explanation relates to the relatively high incidence of new lesions observed during the grazing period, which indicates that there may be continuous exposure to the causative organisms. Applying the hypothetic causal web described in Chapter 1.3, the lesions could regress during the grazing period due to dry and cleaner underfoot conditions; however, continued exposure to the *Treponema* spp. could still be sufficient to maintain substantial antibody titres, and in some cases bring about development of new lesions.

5.4.2 Association of BDD with age

The incidence of clinical BDD in the young stock groups implies that it does occasionally occur in these groups, but seldom leads to chronic lesions; it does not spread rapidly or widely. The EDA and the transition model both indicated that there is a sharp increase in the clinical incidence after heifers calve and enter the milking cow group (associated with an odds ratio of 8.39, as determined by the transition model). Age was positively associated with BDD lesions in this model, rising to an OR of 12.48 for animals ≥ 5 years.

We modelled lesion score as a binary, rather than a multinomial variable, for simplicity and ease. It is possible that information was lost this way, as the EDA indicated that there was an observable shift in the presentation of lesions with advancing age. The proportion of acute lesions was much larger for first calving heifers; it therefore seems that these animals are more susceptible when first exposed to the higher force of infection in the lactating cows group. With increasing age, the proportion of acute lesions decreases while that of chronic lesions increases; the cows in the highest age category have a higher proportion of regressing lesions. This agrees with evidence indicating development of resistance after long-term exposure.

The EDA of the serology showed a gradual rise in titres up to an age of two to three years, after which the range of values increased drastically. This timepoint coincides roughly with the age of first calving. In the scatterplot (Figure 5.9) this is evident as a fanning out of points; this was presumably attributable to some animals seroconverting after infection, while other animals remain uninfected. The mean serologic titres continue to rise. This is reflected by the CAR(1) model, which also shows a sharp rise upon entering cow group, after which there is a gradual rise to about 4-5 years, after which the titre fluctuates somewhat, remaining more or less constant.

5.4.3 Association of BDD with lactation stage and parity

Figure 5.3 indicated that BDD prevalence rose from the start of lactation, reaching a maximum at the peak of milk production; it remained reasonably constant thereafter, but decreased towards the end of the lactation. Assuming constant exposure of all cows regardless of lactation stage, this suggests development of some degree of resistance. However, the transition model for lactation stage and parity (Table 5.5) did not indicate that either lactation stage or parity were significantly associated with BDD. It is therefore possible that, due to the relatively small number of animals in the higher lactation stage and dry cow categories, the trends identified by the barplots were spurious.

The lactation stage boxplots (Figure 5.11) show that first calving heifers tended to be immunologically naive upon first entering the lactating cows group (low median titre); this rose to a peak in mid-lactation and then decreased. There was some variability for subsequent lactations, but no clear trend (as also reflected by the scatterplot in Figure 5.10). The mean titre rose with increasing parity. The lack of a trend between serology and lactation stage, and elevation of titres in successive lactations, suggest that exposure to the causative pathogens may be continuous during lactation. As calving occurred year round on all the study farms, no confounding by season is likely. The CAR(1) model for lactation stage and parity does not show a clear association of serology with lactation stage, but shows that serology is associated with increasing age.

5.4.4 Association of BDD with hygiene-related risk factors

A limitation of the statistical models is that we were unable to incorporate group- and farm-level variables, particularly housing hygiene. This was partly due to the small number of observations made on these levels. There was great variability in the housing hygiene, depending on when it had previously been cleaned or scraped out; therefore, observations represented a snapshot and were unlikely to be representative. Also, evaluating these variables is complex, time-consuming and subjective. Considering that hygiene is considered to be a primary risk factor for BDD, it was highly desirable to include this in our analysis. We therefore performed body hygiene scoring and foot hygiene scoring; these can be considered to be proxies for environmental hygiene which are more easily and repeatably observed. We chose to include body hygiene score (BHS) in our models; this was significantly associated with incidence of clinical BDD in the transition model. Before fitting the CAR(1) model, BHS was significant, but after accounting for autoregression it became non-significant.

5.4.5 Association between clinical disease and serology

Information on the relationship between the serologic response and development of clinical BDD on an individual level would be useful to inform our understanding of disease

dynamics and humoral immunity, and assist our interpretation of the data. We do not yet know whether antibody titres rise before, during or after clinical morbidity; likewise, whether they decay before, during or after regression. Nor do we have biological explanations for the occurrence of false positive and false negative titres.

It was hoped that the data collected during this study would help to address these issues. Unfortunately, the nature of the study design and the disease were such that this was difficult to perform. While the sampling interval of two or four weeks was relatively short, experience has shown that lesion presentation may change markedly within this period, and the seroprofiles may also show fluctuations within this period. Moreover, progression of the disease was not necessarily linear, where this is defined as transition from acute lesions to chronic lesions, which then regress. In many instances, acute lesions regressed spontaneously, while in others new foci of infection developed out of chronic or regressing lesions. The duration of persistence of the lesions was also highly variable. Recurrence or relapses were common. This variability is presumably related to multiple complex host and environment factors such as immunity, hygiene, BDD treatment, season etc.

As a consequence, it was difficult to designate a discrete timepoint at which clinical disease could be defined as beginning or ending – in this respect, BDD could possibly be considered as a chronic condition in which transitions between the states may occur. This makes investigation of the relationship between serology and lesion incidence problematic. The individual plots of seroprofiles related to lesion severity were illustrative, but too qualitative to provide clear information; further analysis of the dataset (e.g. time series analysis) might give a clearer picture. However, a case-control type design where cases are intensively followed would probably be a more appropriate approach.

Chapter 6

Development of a mathematical model for BDD

6.1 Introduction

In Chapters and 2 and 3, we developed and assessed the usefulness of serology for the epidemiological investigation of BDD; Chapters 4 and 5 describe observational field studies in which we combined inspection of feet for BDD lesions with serology. We examined associations between serology and clinical BDD, including serological frequency distributions of clinical positives and clinical negatives, changes in this association per season, age and lesion presentation. We concluded that serology was a valuable tool for epidemiological investigation of BDD.

We therefore extended the case definition from clinical inspection for BDD lesions alone, to include serology of BDD-associated *Treponema* spp. as a second criterion. We developed a model based on Bayesian techniques which defined infection as a latent variable; as the 'true' infection status was not observed, the model distinguished between 'infection' and 'disease' (see Chapter 3). This enabled us to estimate an animal's conditional probability of infection given the serologic result and presence or absence of BDD lesions. The model utilized data from the cross-sectional study; hence, it did not incorporate any temporal components.

For a better understanding of the infection dynamics of BDD, mathematical simulation models are useful. Such models provide a theoretical framework within which the underlying disease mechanisms and processes are specified, including contact patterns, transmission dynamics and pathogenesis. They are analytic tools for identifying the information needed to improve understanding, and hence guide the collection of data and provide a framework for data analysis; furthermore, they allow the effects of putative intervention strategies to be assessed.

Development of such models in the dairy farm setting is facilitated by the existence of discrete management groups. Where loose housing is used, animals within the management groups are assumed to mix homogeneously (this obviously does not

apply where animals are housed individually, e.g. in pens or in tie stalls); no mixing is assumed between groups. The infection dynamics can therefore be specified for each group separately. This enables the infectious disease to be modelled more accurately on the farm level. The multigroup management system is comparable and consistent across UK dairy farms, and mathematical models have been developed incorporating this structure (Turner et al., 2003; Xiao et al., 2005; Turner et al., 2006; Xiao et al., 2006). We will apply a similar modelling approach.

A complex condition such as BDD will require a complex model to accurately simulate it. However, the lack of information on fundamental biological disease determinants requires too many assumptions to be made for reliable formulation and parameterization of such a model.

Our approach was therefore to formulate a simplified deterministic model, based on our current understanding. The design of the model was driven by data from our observational studies. This had the advantages that the group dynamics and transitions on the farm level could be parameterized with a higher degree of reliability. The model was hence more robust (even if it did not yet capture the entire biological complexity of the condition). The longitudinal data could be applied to test model fits.

6.2 Development of a compartmental model for BDD

Compartmental models were developed within the context of epidemic modelling. The target population was subdivided into classes which represented discrete disease states; individuals in the population were classified into one of these states. Standard convention defines four such classes, depending on an individual's experience with respect to the disease: S (for susceptible hosts), E (for exposed, i.e. infected but not infectious), I (for infectious) and R (for recovered). The formulation of the SEIR model depends on the biology of a specific infectious disease. At its simplest, susceptible individuals become infected and remain infected for life; this is referred to as an SI model. If lasting immunity develops after infection, an SIR model is appropriate. If recovery does not lead to lasting immunity, the recovered individuals eventually become susceptible again: this is an SIRS model. Other permutations (including those incorporating the E state) are determined by the specific biological properties of the disease in question.

As the composition of each target population will be unique (depending on natural birth and death rates, previous exposure to the disease etc.), the numbers of individuals in each state should be specified for time $t = 0$. Also, as disease is a dynamic process, the number of individuals in each compartment will vary over time. Therefore, these numbers are a function of time t: $\mathrm{S}(t)$, $\mathrm{E}(t)$, $\mathrm{I}(t)$, $\mathrm{R}(t)$.

The transition of individuals from one state to another is governed by rates, which must be specified by the model. Hence, the mathematical model determines the numbers of individuals in each compartment at each time.

The rate of transmission from S to I (i.e. the rate at which susceptible individuals become infected) is referred to as the 'force of infection', λ. The rate of recovery from I to R is given by δ. The SIR system can then be expressed as a set of ordinary differential equations (ODEs):

$$\frac{dS}{dt} = -\lambda SI$$

$$\frac{dI}{dt} = \lambda SI - \delta I$$

$$\frac{dR}{dt} = \delta I$$

The basic reproductive ratio R_0 for the system, which is given by

$$R_0 = -\frac{\lambda}{\delta}$$

is defined as the average number of secondary cases caused by one infectious individual in a completely susceptible population. If $R_0 > 1$, the infection will spread; if it is less than one, it will fade out. On the farm level, BDD is endemic in nature, i.e. it persists in the population without the need for external inputs. In this case, a steady state is achieved; the overall $R_0 = 1$. The seasonal nature of the infection means that periodic epidemics occur, during which $R_0 > 1$, and periods of subsidence, during which $R_0 < 1$.

Assuming a constant population size, the model must include births to compensate for deaths (due to disease, culling etc.); we assume that newborn calves are immunologically naive and hence are susceptible. A multigroup population such as cows on a dairy farm are effectively partitioned into the management groups; as animals age, they move from one group to another, until they eventually enter the lactating cows group. Thereafter, they cycle between lactating and dry cow groups.

It is clear that accuracy of the model is dependent on its formulation and the classification of 'realistic' numbers of animals into the respective compartments is of basic importance. This depends on the case definition and the assumptions made about the infection dynamics of BDD; the information to perform this was derived from the literature as well as prior analysis of our field studies.

6.2.1 Definition of states

As a precursor to model formulation, we determined which model states (S, E, I, R) were appropriate, and defined criteria for the classification of individuals into these compartments. We utilized the analysis performed on our observational studies data and its interpretation. Of particular relevance was the work done on the validation of the ELISA using the Bayesian model (see Chapter 3); here, a distinction was made

between disease and infection, and the application of serology as an adjunct to clinical inspection was discussed. However, the output of the Bayesian model was not directly applicable here for three fundamental reasons:

1. In keeping with probabilistic analysis, it did not dichotomize or classify individuals into categories, but instead assigned probabilities of infection. However, the designation of individuals into relevant compartments is a prerequisite for the deterministic model.

2. It defined infection as an unobserved ('latent') variable, which could not be determined; for the deterministic model, explicit assumptions need to be made for classification of infected animals.

3. It was based on the cross-sectional study data, and did not incorporate temporal components.

Clinical inspection for BDD lesions

BDD is by definition a clinical disease. In Chapter 3, the very high estimated specificity of inspection for lesions effectively implied that if routine observation identified BDD lesions, the animal was infected and diseased. For this model, we accordingly assumed that presence of lesions defined infection, regardless of serologic result.

Given that protective immunity does not develop, and recurrent infection may occur regardless of duration and level of exposure, it was reasonable to suggest that clinically uninfected animals were susceptible, resulting in an SIS model. However, our results indicated that in the older age categories, the prevalence of BDD lesions decreased, the lesions tended to be more regressing rather than acute in presentation, and serologic titres tended to be higher. Therefore, it appeared that a degree of immunity did develop. In Chapter 3, we specified a lower prior sensitivity for the Bayesian model, implying that a clinically uninfected animal cannot automatically be classified as susceptible. For the mathematical model, we used the serology to further differentiate between states.

Applying serology

The Bayesian model gave a conditional probability of infection (CPI) for every animal tested by ELISA given the serologic result and the lesion status (i.e. disease). This resulted in a curve which is reproduced in Figure 6.1. Presence or absence of lesions hardly affected the outcome. ELISA PP values >30 and <18 were relatively easy to interpret, as these corresponded to CPIs of >80% and <10%, respectively. However, outcomes in the intervening range were difficult to interpret; fortunately, these were relatively scarce. This model did not dichotomize the results to avoid misclassification of results in this range.

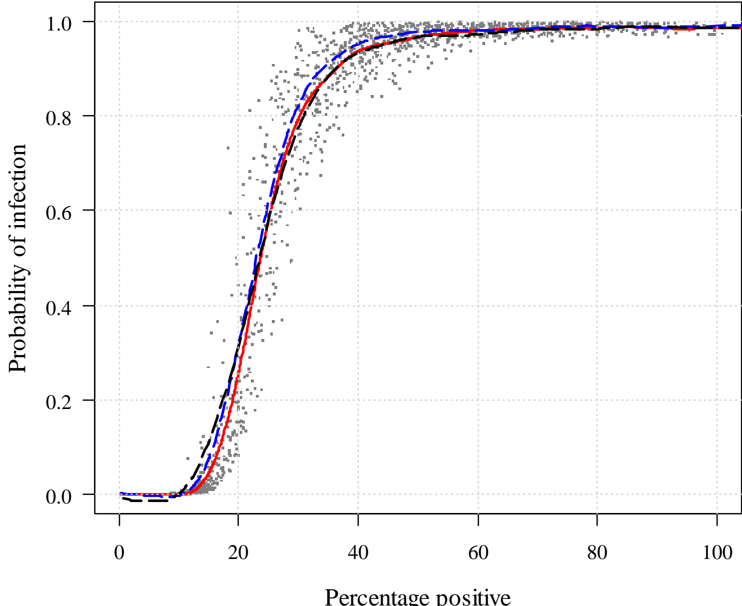


Figure 6.1: Probability of infection plot as a function of serology, given the data. The blue dashed line represents the predictive probability of infection (PPI) of lesion negative animals (i.e. $P(I|S, L = 0,\ \text{data})$; n=376); the dashed black line represents the PPI of lesion positive animals ($P(I|S, L = 1,\ \text{data})$; n=208). The red line represents the PPI of all animals, regardless of lesion status ($P(I|S,\ \text{data})$; n=2160); the grey points represent the conditional probability of infection given its serologic test result for every individual animal

This model utilized data from the cross-sectional dataset, but for the mathematical model we utilized the longitudinal study data. The serological frequency distributions of the longitudinal study dataset were similar (see Figure 6.2); we emphasize again that by presenting these data as univariate, we ignore the dependency in the observations due to repeated sampling of individuals. The distributions of all inspected animals showed a degree of bimodality; when differentiating by clinical status, BDD negatives showed a right-skewed distribution and BDD positives showed a more normal distribution.

To make use of serology for the classification of animals' infection status, we had to dichotomize the test. From the probability of infection plot in Figure 6.1 as well as the serological frequency distributions given in Figure 6.2, a PP of 25 represented a sensible cut-off. Figure 6.2 shows that the young stock largely contributed to the right-skewing. Of the clinically inspected cows, the BDD negative distribution was right-skewed while the BDD positive distribution was slightly left-skewed; the range of PP values was similar. The density plots indicated that serologically, the clinical negatives consisted of a mixed population: it showed a bimodal effect. The BDD positive plot showed a 'shoulder' of seronegatives. The degree of false seropositivity of clinically negative animals was more substantial than the degree of false seronegativity

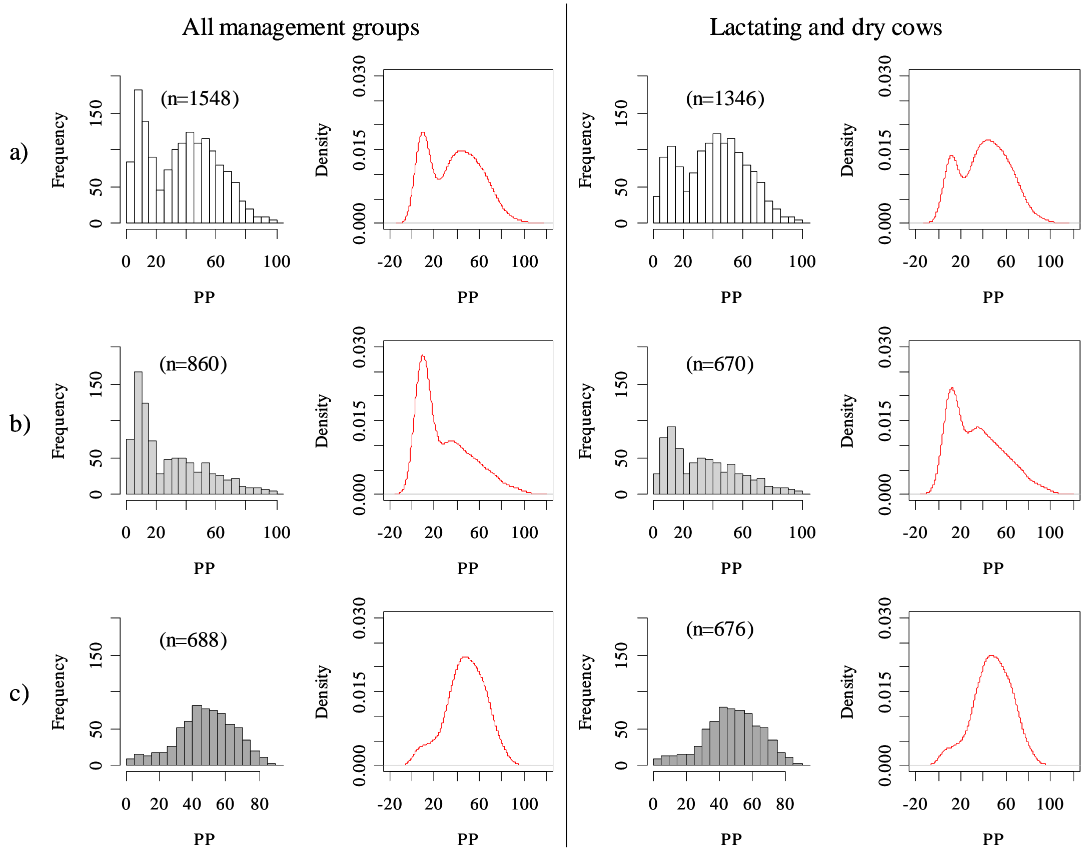


Figure 6.2: Serological frequency distribution histograms and density plots for all inspected animals (left) and lactating and dry cows only (right). a) all animals, b) clinical BDD negatives, c) clinical BDD positives

of clinically positive animals.

Designating a PP of 25 as a cut-off point with the longitudinal study dataset, Table 6.1 confirms that the number of serologic false positives was proportionally very high; consequently, the kappa statistic was rather low: 0.45, which signifies moderate agreement.

		Serology		
		Positive	Negative	Total
BDD lesion	Positive	615	73	688
	Negative	394	466	860
	Total	1009	539	1548

Table 6.1: 2x2 table of BDD inspection versus serology, using the longitudinal study data with a serologic cut-off PP of 25

The serologic false negatives could be due to test error, or failure of the animals to seroconvert. The 73 samples came from 26 animals, of which 9 were false negative at repeated sampling points. Of the 73 samples, 25 corresponded to acute lesions and 30 corresponded to regressing lesions, i.e. 75% of false negatives were accounted for by early lesions (which might not yet have led to seroconversion) or late lesions (the titres of which may have already decayed to below the cut-off point). Measurement error may have contributed to a number of these false negatives. All animals with evident BDD lesions were considered to be infected, regardless of serologic titre.

The high number of clinically uninfected, seropositive animals required investigation. The 394 samples came from 59 animals, of which 32 were false negative at repeated sampling points. There was no clear farm effect, i.e. the farm infection level did not influence the proportion of serological false positives.

Analysis of the field study data indicated that serological titres remain high with increasing age, while the prevalence of clinical BDD decreased correspondingly. To assess the age effect of the longitudinal study, all lesion negative observations were stratified into age categories; per age category, the number of serologic false positives was recorded and expressed proportionally. Table 6.2 shows that this proportion rose per age category up to 87% of animals over 5 years old. Older animals therefore had markedly higher titres without developing clinical BDD.

Age category	Total №. lesion negatives		№. false seropositives		Propⁿ. false seropositives	
	samples	animals	samples	animals	samples	animals
0-1 year	99	28	1	1	0.01	0.04
>1-2 years	72	16	1	1	0.01	0.06
>2-3 years	156	24	51	13	0.33	0.54
>3-4 years	154	33	57	19	0.37	0.58
>4-5 years	105	22	64	16	0.61	0.73
>5 years	275	39	220	34	0.80	0.87
All	860	162	394	84	0.46	0.52

Table 6.2: Total numbers of lesion negative samples and animals these were taken from, and numbers and proportion of clinically BDD negative observations that corresponded with an ELISA PP of >25, per age group. Data were derived from the longitudinal study dataset

Figure 6.3 provides a graphical representation of the number of animals in the longitudinal study that were classified using the criteria of clinical inspection and serology over the duration of the study, stratifying the cows into two age groups. Evidently, the proportions of exposed and infected animals in the young stock groups was very low. For simplicity, these groups were excluded from the model. While the lactating and dry cows were housed in different areas and should perhaps be modelled separately, the relative frequency plot showed that comparing the profiles of the lactating and dry

cows for the two corresponding age groups, the relative frequencies were comparable. To further simplify, we therefore modelled the lactating and dry cows as a single group, incorporating the age effect by dividing this group into two subsets: animals $\leq$5 years of age and animals >5 years of age.

As a highly seasonal trend was observed in the incidence of BDD lesions (Chapter 5), we defined two discrete six-month periods in the model: the housing period (October to March) and the grazing period (April to September).

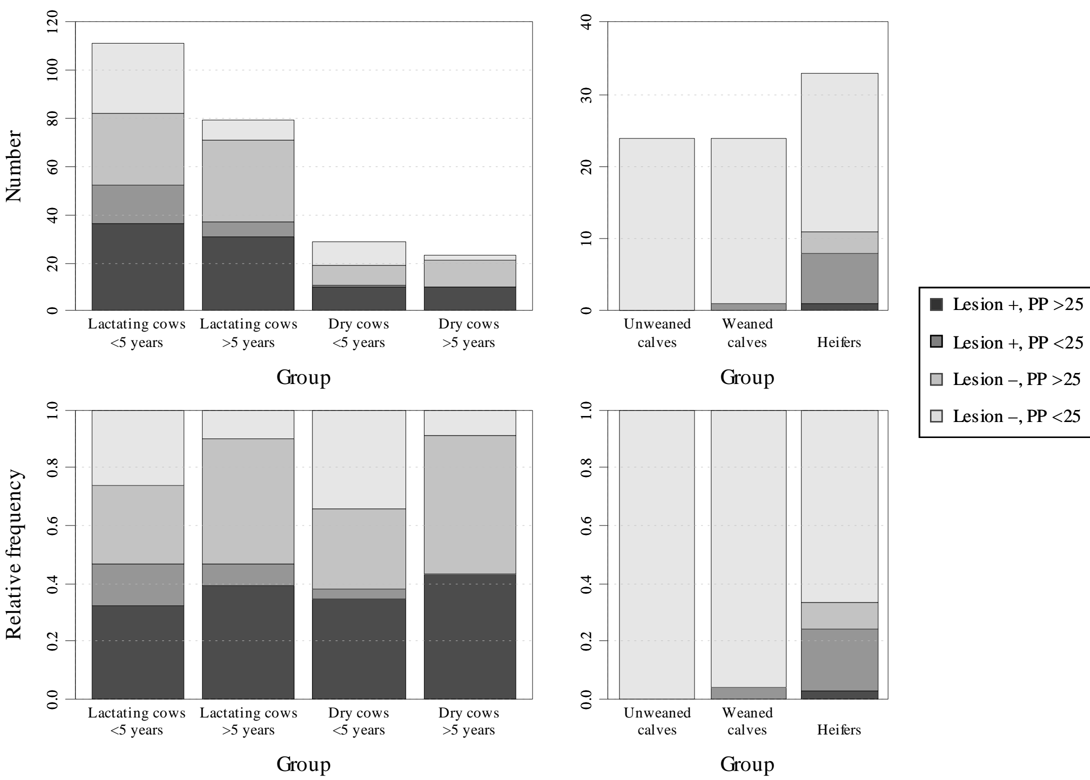


Figure 6.3: Number (top) and relative frequency (bottom) of animals from the longitudinal study, classified by clinical inspection and serology (using a cut-off PP of 25), stratified by management group and age

6.2.2 Model formulation

Model compartments

Assuming that no protective immunity developed, the simplest model was an SIS model, where case definition was based on clinical diagnosis alone (S = absence of clinical lesions, I = presence of clinical lesions). Augmenting the diagnostic procedure by serology, the model could be extended. This presupposed an understanding of the serological response, which is still incomplete; we based the assumptions made on the best available information.

We assumed that animals with clinical lesions were always infected, regardless of serologic status. For animals without clinical lesions, we used serology as an additional test. It was reasonable to assume that animals without lesions that were seronegative were susceptible. The most problematic category was the animals that were clinically negative and seropositive; these could either be designated as exposed or as recovered.

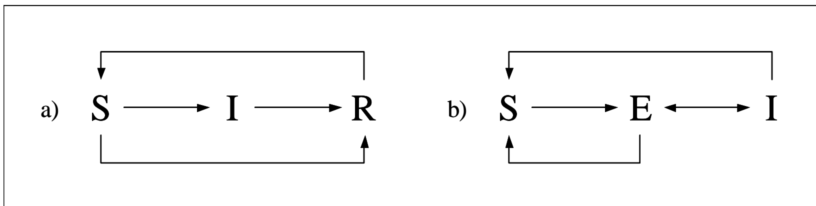


Figure 6.4: Two putative compartmental models for BDD, based on information from field studies: a) SIRS model, where animals can progress from S to R, either via I, or directly; b) SEIS, where animals progress from S to E and in some cases on to I; return to S is possible from either state, and I can also return to E

Two possible flow diagrams are given in Figure 6.4. The first assumes that a susceptible animal (S) becomes infected (I) and subsequently recovers (R), with a residual positive titre that decays, whereupon the animal returns to being susceptible. As we recorded numerous instances of titres rising spontaneously without clinical disease being observed, we also made provision for an animal passing directly from susceptible to recovered, possibly after experiencing a latent or 'subclinical' infection. The second scenario assumes that exposure necessarily occurs before clinical infection can follow. A susceptible animal is exposed to the causal treponemes (E) and develops antibodies; clinical infection may subsequently develop, or alternatively, the serologic response may be transient, and the animal may return to susceptible status. After regression of lesions, infected animals return to being susceptible (i.e. become seronegative) or exposed (i.e. remain seropositive).

Both scenarios in Figure 6.4 are consistent with current hypotheses of the pathogenesis and infection dynamics, although both may eventually prove to be wrong. Flow diagrams incorporating S, E, I and R could easily be defined, but there is currently no way of differentiating between E and R. The difficulty of defining these states is that the 'classic' definitions of the epidemic model do not hold in this case. The humoral response does not confer protective immunity, which is the convention for the R state. Therefore, defining an exposed state (animals without lesions but with a high titre) may be more appropriate. This definition is also consistent with the Bayesian model, which estimated the probability of infection given the serologic outcome – it did not differentiate between states, and hence considered animals both exposed and infected to have a high probability of being latently infected. On the other hand, defining a

recovered state would contradict the Bayesian model, as the definition of 'latent infection' would encompass the mathematical model states of I plus R. Preference was therefore given to the SEIS model.

Model flows

We assumed that the group environment was contiguous and that there was homogeneous mixing of individuals; we also assumed that the size of the groups was constant. Therefore, the force of infection, λ, was the same for all susceptible animals. This is the intuitively logical product of the rate of contacts between animals, c, the probability that these contacts are in fact with infected individuals, p, and the probability that such a contact leads to infection, υ (McCallum et al., 2001; Begon et al., 2002). In our SEIS model, this contact led first to exposure; this terminology is slightly misleading as E is conventionally defined as 'infected but not infectious', and is used to represent an incubating phase. In this specific setting, we considered it to be 'infected but not diseased', and denoted it as a prodromal state. Hence,

$$\frac{dE}{dt} = \lambda S = Scp\upsilon$$

The probability that contact of a susceptible animal is with an exposed animal is given by E/N, where N is the total number of animals in the group, i.e.

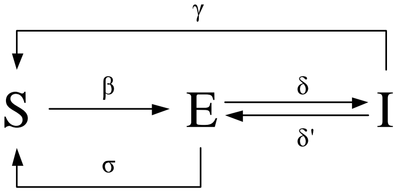
$$\frac{dE}{dt} = \frac{SEc\upsilon}{N}$$

There are two alternative ways of specifying c. The first assumes that this is dependent on the density of the population, and includes a term for the area occupied by the population; this is referred to a density-dependent transmission. The second assumes that c is a constant, in which case transmission does not depend on the area but on the number of contacts between individuals; this is referred to as frequency-dependent transmission (Begon et al., 2002). The surface area of the environment occupied by the cows was discrete (either the cubicle house, or the pasture field), and the stocking density was generally comparable between dairy farms; frequency dependence was therefore more appropriate. Since we also assumed that υ was a fixed property of the disease, the product υc was therefore also constant; this is usually referred to as β, the transmission coefficient:

$$\frac{dE}{dt} = \frac{\beta SE}{N}$$

This transmission coefficient is of fundamental importance; it must be estimated, as it is a product of constants which cannot be objectively measured.

We denoted the rate at which exposed animals became infected as δ, and the rate at which infected animals returned to being exposed as δ'. Exposed animals returned to being susceptible at a rate of σ. Finally, infected animals became susceptible at a rate of γ.



The environmental compartment

We do not yet have any information on the distribution of the causative bacteria in the environment. As the only location in which BDD-associated treponemes have been identified is the actual lesions, we assumed that infected animals dissipated infectious material from the BDD lesions into the environment, and that the transmission mechanism of the infection was 'foot to foot'. We defined a separate model compartment for the environment. Assuming that the bacteria were uniformly distributed, all animals had an equal probability of being exposed; contact with the bacteria might result in transition from S to E, and E to I.

The lack of knowledge of reservoirs of the BDD-associated treponemes could be a serious impediment to the model's accuracy. Should other sources be identified than the lesions, it is possible that susceptible and exposed animals also contribute to this environmental compartment.

6.2.3 Estimation of model parameters

Figure 6.5 shows the flow diagram of the model. It includes the two age category groups: animals $\leq$5 years of age (Y, mean = 3.50 years) and animals >5 years of age (O, mean = 6.46 years). We assumed that the population size is constant, i.e. there was no growth or decline; this was reasonable for dairy farms, where the size of the managed milking herd is kept quite constant. Gain terms into the model compartment included heifers calving (c), and aging of cows from Y to O (a); we have assumed the farm to be closed, and did not include purchase of cows. Loss terms included natural death and culling (d). These were estimated from the data and literature.

The rate of shedding of infectious material is represented by p; the rate of environmental removal of the bacteria is given by q and the rate at which infectious material causes exposure or infection is given by r (we assume equivalent exposure and rates).

Transmission parameter, β

As the transmission dynamics differed between the specified age groups (Y and O) and the periods (housing and grazing), estimation of separate β transmission coefficients was required. This was performed as described by Monti et al. (2006). Given the SEIS model, we defined the rate at which new infections develop as $\beta SE/N$ (see above); we

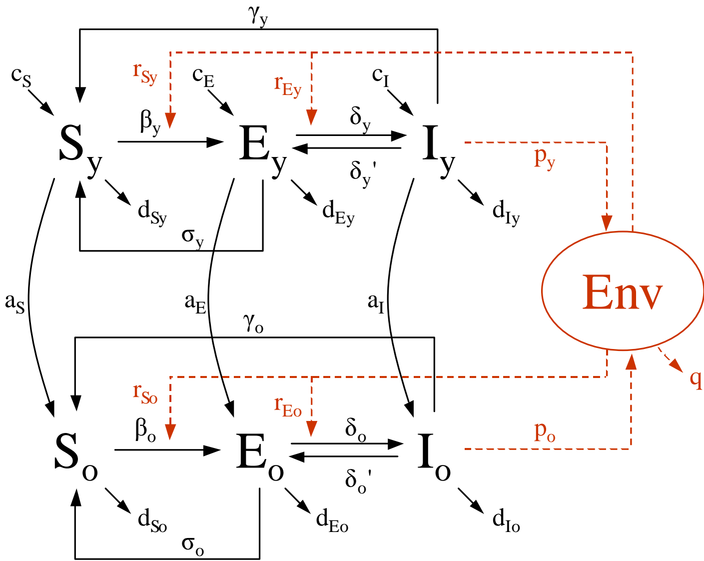


Figure 6.5: Flow diagram of a compartmental BDD model, incorporating two age categories of cows (Y: ≤ 5 years and O: >5 years) and an environmental compartment of infectious *Treponema* spp. bacteria. Solid lines show the movement of animals; dashed red lines show the movement of BDD-associated *Treponema* spp. For definition of parameters, refer to text

assumed that the number of new infections follows a Poisson distribution.

To estimate β, we considered the model in discrete time, as an approximation to continuous time. We specified a fixed time interval (month). From our data, we could determine the number of new infections, which we call Z, per month. We also knew the numbers of the corresponding S, E and N. For a given time t, we therefore knew Z (the number of new infections since $(t-1)$) and could calculate SE/N, which was the average of $S_t E_t / N_t$ and $S_{(t-1)} E_{(t-1)} / N_{(t-1)}$:

$$Z = \beta SE/N = \beta * 1/2 * \{(S_t E_t / N_t) + (S_{(t-1)} E_{(t-1)} / N_{(t-1)})\}$$

The transmission parameter β was estimated using a generalized linear model (GLM); we took logs to get

$$\log(Z) = \log(\beta) + \log(SE/N)$$

This was fitted in a GLM with a log link function, with Z the response variable and $\log(SE/N)$ as an offset variable. The estimate of β was obtained by exponentiation; 95% confidence intervals were calculated from the standard errors. Table I.1 and I.2 in Appendix I show the data used. Estimates were obtained for Y, O and all cows combined, and for the housing period, grazing period and overall (Table 6.3).

Period	Transmission parameter, β (month^{-1})		
	Y	O	All
Housing	0.55 (0.30-2.44)	1.05 (0.61-2.27)	0.74 (0.50-1.83)
Grazing	0.73 (0.48-1.91)	0.55 (0.28-2.68)	0.67 (0.47-1.72)
Overall	0.66 (0.47-1.69)	0.76 (0.50-1.88)	0.70 (0.53-1.50)

Table 6.3: Estimated transmission parameter (with 95% confidence intervals) of cows $\leq$5 years of age (Y), cows >5 years of age (O), and all cows combined, for the housing period, grazing period, and overall

Other coefficients: δ, δ', σ and γ

These rates were determined from the data. The number of animals in the model at any given time t was given by $N_t = N_{tY} + N_{tO}$, where $N_{tY} = S_{tY} + E_{tY} + I_{tY}$ and $N_{tO} = S_{tO} + E_{tO} + I_{tO}$. From Figure 6.3, the proportion of infected animals, I/N, was approximately 0.4 for both age groups (this is not the same as the prevalence, as we are using longitudinal study data); we would expect this proportion to be the same. However, the proportions of S/N and E/N differed: for N_Y, these were 0.3 and 0.3 respectively, and for N_O, these were 0.1 and 0.5 respectively. This reflected the age effect.

We distinguished between the housing period and the grazing period, as the dynamics of infection are likely to differ significantly. From the longitudinal study data, we determined the number of cows per age group, per period, in each compartment. Also from the data, we identified the number of transitions between compartments. Given that these occurred in a six-month period, the monthly rates of transition could be calculated. These data are given in Table 6.4.

Husbandry-related parameters

Loss terms included natural death, culling, and removal for sale. These were estimated from the data (including questionnaires) and literature. As there was no information on which to base any parameter estimates for the environmental compartment, values were iteratively assumed.

6.2.4 Formulation of model equations

A series of ordinary differential equations (ODEs) was formulated to represent the transition rates between the SEIS states. These also incorporated the rates of shedding of, and infection by, treponemes; and rates of environmental survival of these treponemes. Finally, the rates of transfer of individuals from Y to O were included. These ODEs were coupled to merge them into a single mathematical model. The model was run using the specified model parameters. The model code is included in Appendix I.

		Housing period		Grazing period	
		Y	O	Y	O
Nº. animals	S	20	8	26	5
	E	25	22	25	32
	I	47	32	40	33
Nº. transitions	$S\rightarrow E$	4	4	1	7
	$E\rightarrow S$	2	2	3	7
	$E\rightarrow I$	8	2	7	13
	$I\rightarrow E$	7	5	10	8
	$I\rightarrow S$	0	2	3	0
Rates of transition	$\sigma^{\S}$	0.013	0.015	0.020	0.037
	δ	0.053	0.015	0.047	0.068
	δ'	0.025	0.026	0.042	0.040
	γ	0	0.010	0.013	0

Table 6.4: Numbers of animals in the disease states, and number of transitions between these states, per period and age category (Y represents animals $\leq$5 years old, O represents animals $>$5 years old, S is suceptible, E is exposed, and I is infected). § Calculation: 2 out of 25 E animals $\leq$ 5 years old pass from $E\rightarrow S$ during the housing period, hence the rate is 2/(25*6) = 0.013 month^{-1}

6.3 Model outputs

Figure 6.6 gives a graphical output of the fully-parameterised model.

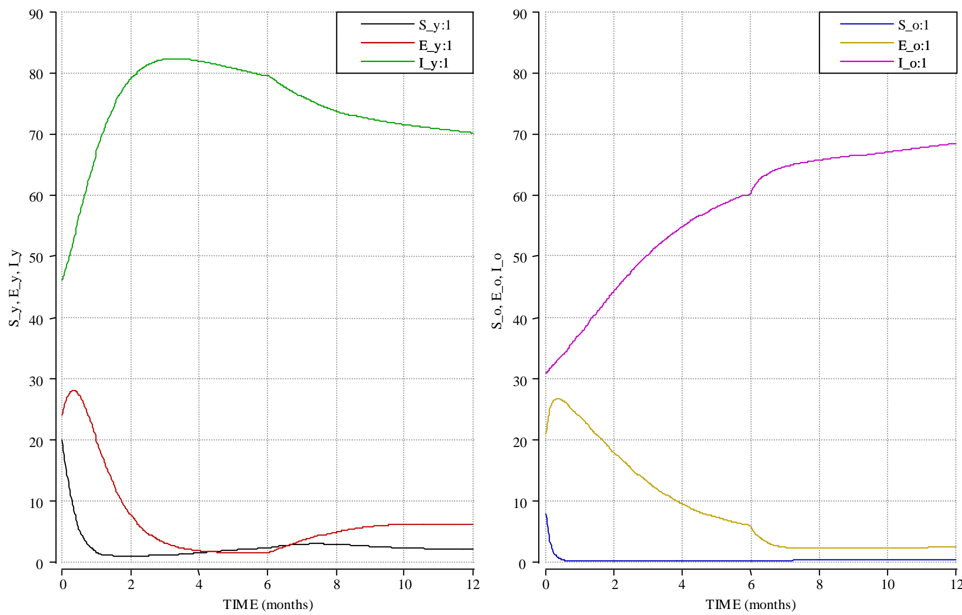


Figure 6.6: Graphical output of the SEIS model, showing the numbers of animals in each state over a 12-month period, for animals $\leq$5 years old (left) and animals $>$5 years old (right), where t=0 represents that start of the housing period, and t=6 represents the start of the grazing period

At the beginning of the housing period, the number of infected younger cows (I_y) rose more quickly than infected older cows (I_o), and correspondingly, the number of exposed younger cows (E_y) declined more rapidly than exposed older cows (E_o). In both cases, the number of susceptibles (S_y and S_o) dropped off quickly, and remained very low. The number of I_y rose to a maximum about midway through the housing period, and then decreased slightly to the end of the housing period; the number of I_o continued to rise up to the end of the housing period. The number of I_y was consistently greater than I_o, whereas the number of E_y tended to be lower than E_o. This was consistent with analysis of our observational studies data.

At the beginning of the grazing period, the number of I_y decreased, which was accompanied by a rise in E_y and S_y. However, the number of I_o continued to rise, which was a consequence of development of lesions from E_o animals. This was unexpected – it could be explained by the δ coefficient of the older cows in the grazing period (see Table 6.4), which was relatively high.

The pattern shown by the young animals was as expected, although the number of I_y animals was overestimated; this was possibly due to an overestimation of the β coefficient and the δ parameter. The data showed very few transitions of animals with lesions directly back to the susceptible state, hence γ was very low. This was not surprising; although the half-life of the antibody response is short, most animals retain a 'positive' titre for some time after regression of lesions, and will therefore pass back to the exposed state. They could thereafter return to the susceptible state. However, the low numbers of exposed and susceptible animals compared to infected animals, as determined by the model, indicates that of the bidirectional flows, the backward flows are relatively minor; it therefore seems likely that the coefficients σ and δ' were underestimated. For an endemically stable condition, the numbers of S, E, and I would be expected to be comparable at t=0 and t=12.

Figure 6.7 shows the variation within the environmental compartment for the corresponding time period.

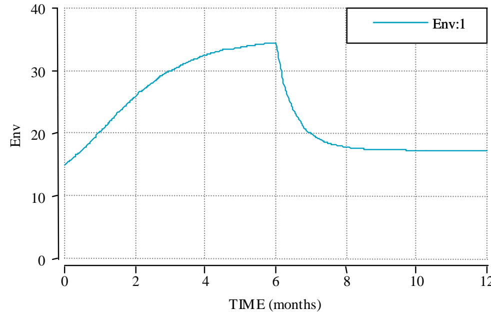


Figure 6.7: Graphical output of the SEIS model, showing the variation in the environmental compartment over a 12-month period, where t=0 represents that start of the housing period, and t=6 represents the start of the grazing period

The rates of shedding from infected animals and removal of the treponemes (through death of the organisms as well as cleaning of the cubicle house) were iteratively determined in the absence of suitable information for parameterisation. The curve shows the expected pattern, with the environmental exposure rising gradually throughout the housing period. This assumes there is an accumulation of infectious material over this period; this is plausible due to the increase in the number of infected animals in the corresponding time. At the start of the grazing period, the level of exposure drops to a lower level; it remains constant throughout the grazing period, at the same level as the beginning of the housing period.

6.4 Discussion

The model developed here should be considered a preliminary effort at exploring the use of SEIR-type models. BDD is a complex condition; a highly simplified model is unlikely to accurately capture the infection dynamics. Arguably, it is possible to formulate models with any level of complexity and assumed transmission parameters, which can be specified in such a way that the results are consistent with empirical or described patterns of disease. The inherent danger of doing so is that the many assumptions which must be made for formulating and fitting these models could easily lead to spurious results.

Investigation and application of observed data reduces this risk. Our longitudinal study has given us the required data to examine alternative model formulations, and specify the most appropriate structure; we have attempted to define a parsimonious model, i.e. reduced to the greatest degree without affecting its ability to capture the relevant dynamics. This has reduced the number of transmission coefficients to be determined. The data have also enabled us to directly calculate or estimate these coefficients. Even so, we have had to make several assumptions, particularly with respect to the environmental compartment.

The model output shows general patterns that are as expected from our data and empirical experience, but there are several discrepancies. These could be due to over- or underestimation of transmission parameters, or they could be a result of inappropriate model formulation. Further exploration, analysis and model fitting is required. Although the model's transition parameters were calculated or estimated from the data, it is possible that the dataset was not large enough to perform this accurately. Fluctuations in the observed data are random in nature; however, this model was purely deterministic, and thus could not incorporate any such stochastic effects. Fitting a similar model as a stochastic process could prove to be more appropriate.

From the data as well as the model, an obvious finding is that when utilising serology in addition to clinical inspection for our case definition, the infection is highly prevalent in the cow groups – far more so than currently appreciated. The outputs of

the Bayesian model, when applied to investigate levels of infection on the group level (Figure 4.7) already indicated this, so our models are consistent. Of course, we make the fundamental assumption that the serology of BDD-associated *Treponema* spp. is directly associated with clinical BDD. This cannot be determined with certainty; however, the results of our observational studies strongly support this assumption.

Consequently, it appears that the proportion of cows 'latently' infected (to use the Bayesian model definition) or 'exposed' (to use the SEIS model definition) is very high. This indicates that the organisms causing BDD are ubiquitous in the cows' environment, and the 'force of infection' is high. Not all cows exposed to the treponemes develop clinical BDD; we do not yet understand the component causes required in addition to exposure with this probable 'necessary cause'.

We have not yet been able to calculate the reproductive ratio, R_0. This is not straightforward from our model, as we have effectively partitioned the process of becoming infected into two steps, i.e. animals moving from S to I via E. For an endemic disease with a seasonal distribution such as BDD, we would expect R_0 to be >1 during the housing period, <1 during the grazing period, and 1 overall.

Referring to Figure 6.8, we are currently at the stage of refining the model, which will enable the parameters to be redefined. Other points of attention include performing diagnostics such as sensitivity analysis, and including stochastic effects.

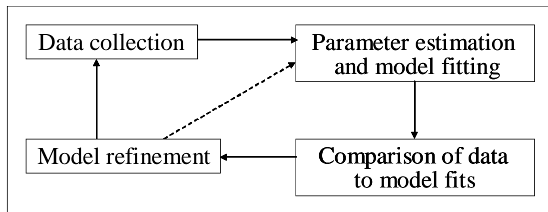


Figure 6.8: Process of iterative refinement of model

A mathematical model that effectively simulates the condition can be used predictively to assess the effect of interventions, and hence to investigate putative control strategies. Such effects might include the following:

1. Improvement of environmental hygiene. This increases the removal of treponemes from environmental compartment, reducing exposure and the force of infection.

2. Effect of treatments. This has two effects: it accelerates the flows back to the S and E states, from I (increase γ, δ' and possibly σ). Secondly, it reduces the amount of infectious material disseminated into the environment by infected animals.

3. Husbandry-related factors. Reducing stocking density will reduce β (by reducing c and p); and different age structures (i.e. proportion of 'young' to 'old' animals) can be investigated; further refinements to the model could incorporate factors such as the seasonality of calving, and the increased risk associated with buying in stock.

This is an important step for making recommendations about the most effective approaches for BDD control, and may lead towards the development of preventive measures.

Chapter 7

Perspectives for BDD: current knowledge and research priorities

7.1 Introduction

In the 20 years since BDD was first observed in the UK, the majority of UK dairy farms have become infected, and there are indications that the infection is still spreading further (Laven, 2003). BDD is currently considered to be the leading infectious cause of lameness in the dairy sector in the UK. Given the intense painfulness of the disease, it is a leading welfare concern, which seems bound to further increase in significance.

Studies over the past decade have showed a trend of rising prevalence (Somers et al., 2005; Holzhauer et al., 2006), both in terms of the proportion of farms infected as the morbidity on the farm level; Holzhauer et al. (2006) suggest that this may be due to prolonged housing periods. Laven and Lawrence (2006) found that the seasonality of BDD incidence has decreased since 1997, ascribing this to the disease having become endemic on most farms, and persisting into the grazing period. Our studies show that new infections occur all year round, albeit at a lower rate of incidence during the grazing period.

BDD is a complex condition. The current understanding of the aetiology, pathogenesis and epidemiology of BDD is far from complete. The rapid and pervasive spread of BDD may have altered the infection dynamics of the disease, posing new challenges for investigation and the design of practical and effective control measures. The objective of our research was to provide scientific information on the current farm-level distribution and dynamics of the disease, culminating in the identification of intervention measures.

7.2 Aetiology, causation, diagnosis and case definition

From an analysis of the published literature (Chapter 1), the putative causative mechanisms and risk factors were discussed within the framework of the component-cause

model. The evidence that BDD-associated *Treponema* spp. bacteria represent the 'necessary cause' is becoming increasingly strong (Demirkan et al., 2006; Edwards et al., 2003; Stamm et al., 2002; Trott et al., 2003; Walker et al., 1995); we have assumed this to be the case. However, it is clear that this is a highly multifactorial disease, and hence a complex of other component causes has been identified. These consist of demonstrated as well as hypothetical ('biologically plausible') factors. It is currently unknown which of these causes combine with the 'necessary' cause to result in a 'sufficient' cause which will bring about clinical BDD. A set of component causes was incorporated into a causal web, which exhibits a hierarchical structure, from proximate (physiological) causes to more distal effects (management factors). This causal web was useful for directing our thinking about the formulation of hypotheses (e.g. regarding aetiology, pathogenesis and transmission), the definition of variables of interest, and identifying possible confounders and sources of interaction. As the causal web had BDD as the outcome and explicitly defined a hierarchical structure, it also lent itself to the fitting of multivariable multilevel models.

We performed detailed microbiological studies investigating the diversity of BDD-associated *Treponema* spp., obtaining 23 cultured isolates from tissue biopsies taken from BDD lesions on nine farms (Chapter 2). Three distinct antigenic groups were consistently identified when performing electron microscopy, 16S rRNA sequence analysis, flagellin gene analysis, enzyme activity profiling, and serology. The groups were closely phylogenetically related to *T. medium*, *T. phagedenis* and *T. denticola*, three human treponemes. These findings were consistent with, albeit much more detailed than, other microbiological studies performed in the past ten years (Choi et al., 1997; Collighan and Woodward, 1997; Collighan et al., 2000; Stamm et al., 2002; Trott et al., 2003; Edwards et al., 2003). We cannot yet relate the isolates to specific clinical findings or farm-level morbidity of BDD. Trott et al. (2003) postulate about the relative importance of the treponemal groups as 'primary' and 'secondary' invaders, but as we currently lack information on predominating treponemal morphotypes and possible sequential shifts of different species over the course of the infection (Edwards et al., 2003b), we are unable as yet to make any inferences. Research is ongoing: the development of phylotype-specific PCRs is uncovering further antigenic diversity in the cultured isolates. These results have not yet been presented.

T. phagedenis-like treponemes were most commonly identified; *T. denticola*-like treponemes were found less frequently, and *T. medium*-like treponemes were identified on only one farm. More than one treponemal species was cultured from biopsies on four of the nine farms; this suggests that mixed populations are common on farms. Furthermore, isolates from two species were cultured from single lesion biopsies from two different animals on two different farms, indicating that simultaneous infections with treponemes from different species are possible. We do not yet know whether all

treponemes have pathological significance.

Although treponemes were successfully cultured from BDD lesion tissue, there is no information on the distribution of the BDD-associated *Treponema* spp. in the farm environment. There were indications that these bacteria may have been present on the clinically negative farm included in the cross-sectional study (seroconversion of clinically negative animals), but until we can identify and sample from reservoirs of infection, this cannot be confirmed. Although the treponemes are closely related to treponemal species infecting humans, it seems likely that dairy cattle are the only host species or 'target population' (although contagious ovine digital dermatitis, CODD, appears to have a common or shared aetiology (Dhawi et al., 2005)). It therefore seems likely that either the environment constitutes a reservoir, or the target population is itself the source population, or both. Long-term persistence of the treponemes in the environment seems unlikely due to their fastidiously anaerobic nature. Over the summer grazing period, the cubicle houses are usually cleaned out; yet within a short period of re-housing, the incidence of clinical infection rises. This evidential information suggests that the source of infective treponemes is more likely to be animal-related. Various locations could be suggested; further advances in microbiological techniques may provide more information in the near future. Such information would enable more accurate epidemiological modelling of the disease to be performed. Exploratory work performed by us has failed to locate treponemes related to those associated with BDD in any part of the gastrointestinal tract. The skin or urinary tract are other locations we have sampled, but have consistently failed to identify treponemes in. BDD lesions are currently the only known reservoirs of the treponemes; our current hypothesis therefore is that transmission occurs by dissemination of treponemes into the underfoot environment and 'foot to foot' infection.

In order to apply the ELISA for routine analysis of serum samples taken on the study farms, we investigated the serological reactivity patterns of test sera with the 23 treponemal antigens. The results indicated the existence of at least three serogroups / -types, consistent with the phylogenetic divisions. The ELISA results for representatives of each group showed marked similarity. The *T. phagedenis*-like isolates, which were most commonly identified on the farms of origin, solicited the highest response, regardless of clinical status. These antigens were related to treponemes that Moter et al. (1998) demonstrated using an *in situ* hybridization technique to penetrate most deeply into the lesions. It is therefore possible that this group has the highest pathogenic significance.

There was, however, substantial cross-reactivity. This could be a consequence of a lack of antigenic specificity of the IgG_2 antibodies. Alternatively, it could be due to mixed infections with treponemes from multiple groups – which is plausible considering the mixed treponemal populations identified on farm level. We were unable to clarify

this, which impeded interpretation of the serology; our results indicate that the existence of serogroups is likely, but we are currently unable to distinguish between these. Serological diversity is well documented for *Brachyspira hyodysenteriae*, to which the treponemes are related.

For the application of a 'general purpose' ELISA, we combined five antigens, including representatives of each of the three groups, into a 'cocktail'. The performance and repeatability of this ELISA was assessed. The variability of the test was small and it was found to be precise and hence consistent (Dohoo et al., 2003; Thrusfield, 1997). It was therefore considered to be a suitable procedure for routine analysis, and an improvement on the ELISA described by Demirkan, Walker, Murray, Blowey and Carter (1999); it was applied in subsequent observational studies. A disadvantage of this ELISA was that it could not identify potential predominance of certain antigenic strains over others on the farm level.

Although investigation of serology has indicated that antibody titres against BDD-associated *Treponema* spp. are elevated in BDD-affected animals compared to non-diseased animals, case definition is currently limited to clinical inspection of feet. Although efforts have been made to standardize the description of lesions (Döpfer and Willemen, 1998), the variability in presentation is such that most authors in the published literature have applied their own system.

On the basis of our serologic results, and in the absence of other detailed information on the underlying infection processes which eventually result in clinical manifestation of BDD lesions, we assumed that animals were exposed to the causative organisms and were infected prior to clinical disease becoming manifest. Under this assumption, clinical inspection is not a 'Gold Standard' diagnostic test – and such a test is currently not available. We therefore defined infection with BDD-associated treponemes as a latent variable, meaning that given the current tools at our disposal, an animal's 'true' infection status cannot be directly measured. We investigated whether serology was applicable as an additional, possibly more sensitive and specific, diagnostic criterion. This was performed using Bayesian techniques; we chose not to dichotomize the test outcome to prevent the inherent loss of information. Rather, we estimated the conditional probability of infection (CPI) of an animal given its serologic test result and BDD lesion status. This was very similar regardless of whether disease was present or absent; the model produced a set of curves which, assuming that the study population was representative, can be used predictively in other studies.

The relationship between the humoral response and clinical disease is complex, and probably relies on pathogenic and immunogenic mechanisms we have not yet uncovered. The large overlap in the serological distributions of non-diseased and diseased animals implies that substantial proportions of BDD-negative animals have high titres, or conversely, that BDD-positive animals have low titres. Our data indicated that this

was predominantly due to the former, i.e. animals with high titres that had regressing lesions or that were clinically negative. These animals were classified as infected by the Bayesian model, and were considered to be exposed in the deterministic mathematical model. A more detailed discussion follows in sections 7.4 and 7.5.

Basing our case definition on clinical status as well as serology allowed us to improve the case definition of BDD, and formulate different models to assess infection status and dynamics. It is clear that while serology has great potential for prospective epidemiological study of BDD, interpretation is not straightforward; further study and refinement of the models developed here is required before they can be practically applied.

7.3 Spatial distribution of BDD on the farm level

The morbidity, prevalence and risk factors for BDD have been relatively well investigated and published in various cross-sectional studies and surveys. These studies concurrently investigated different infectious conditions causing lameness (Frankena et al., 1991; Somers et al., 2005)), and / or were primarily interested in management, husbandry and production effects across all herds (the same authors, and Rodriguez-Lainz et al., (1996); Rodriguez-Lainz et al. (1998, 1999); Wells et al. (1997); Holzhauer et al. (2006)).

The objective of our study was to investigate the distribution of, and risk factors for, BDD within herds (i.e. within and between management groups) and between farms. Hence, we elected to perform a detailed study on a small number of farms, rather than a less detailed study on a larger number of farms. We did not limit our investigation to the milking herd, but sampled every animal within all management groups. We combined clinical inspection of BDD with serology of BDD-associated treponemes, applying statistical methods to characterize the farm-level prevalence, distribution and risk factors for BDD.

We stratified the study population into high, medium and low prevalence farms on the basis of prior clinical information. As expected, high prevalence farms had higher clinical prevalence in the lactating and dry cow groups (35-60%); they also had a higher proportion of acute lesions, i.e. recent infections. The mean prevalence was approximately 40%, which is comparable to published figures (Laven, 2003).

No clinical BDD was observed in the unweaned calves, weaned calves and bulling heifer groups on any of the farms. Only a few lesions were seen among in-calf heifers; these were from high or medium prevalence farms. Of the lactating cows, the highest prevalence was found in the second parity group. The prevalence was decreased for animals over 5 years of age. This may indicate development of partial immunity.

These findings are indicative of an epidemic disease pattern on high prevalence farms, corresponding to a higher level of exposure. The disease pattern was more

endemic on lower prevalence farms. Anecdotal information from farmers and veterinarians suggests that fluctuations of these patterns occur within farms over time; these may be partly due to seasonal effects, but are also due to unexplained effects.

Serology was useful for further exploring the farm-level dispersion of the infection. While the ELISA was developed using a 'cocktail' of BDD-associated *Treponema* spp. antigens, we could not unequivocally assert that the IgG_2 titres measured were elicited by BDD-related challenge with these bacteria. However, the serological frequency distributions were consistent with an infectious disease, showing evidence of a bimodal response; this effect was stronger for high prevalence farms. Comparison of the distributions of the clinical positives to the clinical negatives showed more clearly that the ELISA distinguishes between positive and negative sub-populations. However, there was substantial overlap, although the difference between the mean titres of these sub-populations was significant. Animals with acute or chronic lesions had significantly higher titres than those with regressing lesions. This indicated that the antibody half-life was short, which was consistent with previously published research (Walker et al., 1997; Trott et al., 2003).

Antibody titres were low for the young stock, rising gradually up to the age of first calving. Subsequently, they rose sharply; thereafter, they remained high, presumably due to repeated exposure in the housing environment. No reduction was found for animals over 5 years of age, as was the case with clinical disease; this indicated that serology was a good marker for repeated exposure to infection. Insofar as this led to development of partial immunity, it would appear that this was not through humoral mechanisms – a conclusion shared by other authors (Walker et al., 1997; Demirkan, Walker, Murray, Blowey and Carter, 1999).

Bayesian techniques have been increasingly applied in recent years for diagnostic test validation, particularly in the absence of a 'Gold Standard' (Enøe et al., 2000; Johnson et al., 2001; Branscum et al., 2004, 2005). We applied a recently-developed approach by estimating the probability of latent infection without imposing a cut-off (Choi et al., 2006); we elected to perform this as the serological distributions showed substantial overlap. The outputs of the model were consistent with our clinical and serologic findings. The estimated conditional probability of infection (CPI) was low for almost all young stock; it was only in the cow groups that substantial numbers of animals were identified with a high CPI. The model showed that medium and high prevalence farms had a higher level of infection; a proportion of the young stock was also exposed to infection on these farms. As the CPI was an expression of the 'hidden' infection given the serologic outcome, animals with a high titre were identified as being infected regardless of lesion status. The model therefore did not distinguish between infection with BDD-associated pathogens and clinical BDD. A more accurate terminology would be that animals with high titres that are BDD negative are 'exposed', where

this is defined as 'infected but not diseased' (this is further discussed in 7.5).

An interesting and relevant case was presented by the negative control farm; this farm had been closed to importing stock for over twenty years, had high levels of management and biosecurity and could be said to be clinically BDD negative with a high degree of confidence. No BDD lesions were observed during the sampling of this farm. However, the serological frequency distribution of the farm population was comparable to other farms which did have clinical BDD, and dozens of lactating and dry cows had high titres – accordingly, the Bayesian model also showed high levels of infection in these groups. In the near future, we may be able to ascertain whether BDD-associated *Treponema* spp. are present on this farm.

The statistical models quantitatively confirmed the patterns identified by the EDA. As two outcomes were possible (clinical inspection and serology), different models were formulated. Comparison of different random effects structures indicated that fitting management group nested within farm was most appropriate. For the outcome of clinical BDD, a generalized linear mixed model (GLMM) was applied; for the outcome of serology, a linear mixed effects model (LME) was used. Unfortunately, no management group-level explanatory variables (housing hygiene, housing comfort, BDD treatment and footbath protocols) were significant in the univariable analysis, for either of the statistical models; therefore only individual-level variables remained in the final multivariable models.

The highest odds of clinical BDD were in the 2-3 year category; animals >6 years had substantially lower odds. The odds ratio for lactating cows was more than double that of dry cows. For the serological outcome, the estimates rose gradually, showed a sharp rise for the 3-4 year category, and continued to rise slightly with increasing age.

Hygiene in the housing is the risk factor that has been most commonly associated with BDD. The individual-level foot hygiene scores (FHS) and body hygiene scores (BHS) could be considered to be proxies for environmental hygiene. The statistical models did not associate high FHS with clinical BDD or high serology; however, BHS was significantly identified as a risk factor by both the GLMM and the LME. This was surprising, as FHS might be expected to exert a stronger effect. An empirical observation is that within management groups, these scores tended to be quite uniform; it is possible that effects on the foot level could be masked by a lack of observable variability in FHS.

7.4 Temporal aspects of BDD on the farm level

The infection dynamics of BDD are poorly understood and have not been subject to rigorous scientific investigation. Our understanding of the factors governing the transmission at the group and farm level is still mostly hypothetical; as some of these factors are time-varying (e.g. environmental hygiene), they are likely to directly influence the

seasonal variability of the disease. For the stated objective of developing effective intervention strategies, such knowledge is a prerequisite.

Few studies have been published specifically investigating BDD incidence rates and temporal disease trends. Laven and Lawrence (2006) analysed a database of veterinary treatments over an eight-year period recorded by a network of 40 veterinary practitioners; they found that the morbidity of BDD had a significant seasonal component, with fewer reports being made from June to October. However, they inferred that the overall seasonality of BDD had decreased since the early 1990s. They attributed this to the continued spread of BDD, leading to an endemic disease pattern on affected farms, where the disease persists into the grazing period. Holzhauer et al. (2006) compared the results of their cross-sectional study with those of Frankena et al. (1991) and concluded that the morbidity of BDD had increased in the intervening 15 years; they suggested that this could possibly be associated with prolonged housing periods.

Our longitudinal study results broadly agree with these studies. We identified a strong seasonal trend in the clinical incidence of BDD, with a maximum between November and January; interestingly, the incidence rate started declining before the end of the housing period, and continued into the grazing period. Minimum incidence occurred between June and July. However, it remained substantial throughout the grazing period, and newly-developing lesions were observed all year round. There was some variability in clinical manifestation and prevalence between farms, which was possibly related to farm management practices such as footbathing, or climactic and environmental conditions, time of turning out and re-housing, and farm-specific BDD infection dynamics. There was also some variation in BDD incidence per year, with the incidence in 2005 being lower.

Combining the information from Laven and Lawrence (2006), Holzhauer et al. (2006) and our study, it can be speculated that observed increases in morbidity are due to the disease having become endemic on most farms (possibly due to a prolongation of housing periods), as a result of which new lesions continue to develop throughout the grazing period.

The incidence of clinical BDD was positively associated with age. No lesions were seen in animals younger than one year old on the date of sampling, and in few in-calf heifers; in all but one case, those lesions that were observed were small, acute, did not persist for more than two weeks, and did not lead to seroconversion. This implies that clinical BDD occasionally occurred in the in-calf heifers group, but did not lead to chronic lesions; it was not observed to spread rapidly or widely. There was a sharp increase in the clinical incidence after the first calving. Compared to older cows, the proportion of acute lesions was much larger for these heifers. With increasing age, the proportion of acute lesions decreased while that of chronic and regressing lesions increased. BDD prevalence rose from the start of lactation, reaching a maximum at the

peak of milk production; it remained reasonably constant thereafter, but decreased towards the end of the lactation. This suggests development of some degree of resistance over the duration of the lactation. However, neither lactation stage, nor parity were significantly associated with BDD by the transition model. Body hygiene score (BHS) was significantly associated with incidence of clinical BDD. We were unable to incorporate group- and farm-level variables, particularly housing hygiene, into our statistical models. This was because inspection of the housing was unlikely to be representative due to the variability in the housing hygiene, depending on when it had previously been cleaned or scraped out. Also, creating compound indices for these variables is complex and subjective.

Serology provided further clues on the exposure to BDD-associated *Treponema* spp. As determined both by the EDA and the CAR(1) model, the IgG_2 titres were very low at birth, showing a gradual rise up to the age of two to three years, which coincides roughly with the age of first calving. Subsequently, the range of PP values increased markedly, which was presumably due to some animals seroconverting after infection, while other animals remained uninfected. The mean serologic titre continued to rise up to an age of four to five years, after which it remained more or less constant. There was no discernable lactation stage effect, suggesting that exposure may be continuous.

These results are consistent with an endemic disease pattern and provide evidence of development of partial immunity after long-term exposure. Currently, we do not have the microbiological tools which enable us to identify sources of the infection. Our serologic results suggest that young stock are exposed to the infection at a low level, possibly as a consequence of spread of infectious material by movement of farm materials, vehicles and personnel. The 'force of infection' appears to rise in the heifer groups; this may be related to the relatively poorer hygiene in these groups. However, most animals were immunologically naive upon first entering the lactating cows group. The level of exposure was probably much higher in these groups.

The serology showed no evidence of seasonality. This was surprising, since we had established that BDD lesion positive animals had significantly higher titres than lesion negative animals. Also, our results indicated that the half-life of the IgG_2 antibodies was short, decaying during or soon after regression of the lesions. It was therefore logical to expect that the serology would show a similar seasonal pattern as the incidence of clinical lesions. Two possible explanations can be advanced for this.

Firstly, the relatively high incidence of new lesions observed during the grazing period indicates that there may be continuous exposure to the causative organisms. In accordance with the causal web presented in Chapter 1.3, the lesions could regress during the grazing period due to drier and cleaner underfoot conditions; however, the continued exposure could still be sufficient to maintain substantial antibody titres, and in some cases bring about development of new lesions.

The second explanation relates to the association between the humoral response and clinical disease. From the serological distributions of clinical negatives and positives, it was observed that the number of clinical negatives with high IgG_2 titres was more substantial than the number of clinical positives with low titres. Further investigation showed a clear age effect, with older cows having a much higher proportion of such serologic false positives. It is therefore likely that older animals, who have presumably had a prolonged exposure to the organisms, are more likely to seroconvert without developing clinical BDD.

Inspection of plots of individual animals relating the serological profile to clinical disease were difficult to interpret due to the variability of the lesion presentation between sampling observations. Progression of the disease was not necessarily linear, where this is defined as transition from acute lesions to chronic lesions, which then regress. In many instances, acute lesions regressed spontaneously, while in others new foci of infection developed out of chronic or regressing lesions. The duration of persistence of the lesions was also highly variable. Recurrence or relapses were common.

In initial studies (Walker et al., 1997; Demirkan, Walker, Murray, Blowey and Carter, 1999), the serology was strongly associated with clinical BDD, and very few inconsistent results were reported. It is possible that in this period, animals had only been relatively recently exposed to the infection, and hence showed a pattern consistent with primary infection; no degree of immunity (the mechanisms of which are unknown) had yet developed. As the disease became endemic in successive years and animals were repeatedly challenged, the development of a degree of immunity has led to older animals seroconverting without developing clinical BDD.

Another explanation of the findings of these and more recent studies, such as that of Trott et al. (2003), is that they were experimental case-control studies rather than population-based in design; and smaller numbers of animals were studied. It is therefore possible that the extent of these inconsistent serologic results could simply not be determined.

As a consequence of our serologic findings, we assumed existence of 'latent' infection, during which the animal is infected without developing clinical disease. This assumption was applied in the formulation of the Bayesian model and the mathematical SEIS model.

7.5 Infection dynamics of BDD

We utilized the longitudinal study dataset to define an appropriate deterministic compartmental model. As the disease almost exclusively occurred in the lactating and dry cow groups, the young stock was excluded from the model. For the definition of disease states, observations of clinical BDD status were combined with serologic results. As BDD is defined as a clinical disease, we assumed that presence of lesions constituted infection, I, regardless of serologic status. The seasonal nature of BDD incidence has

been discussed above. Investigating the serological distributions, a cut-off PP of 25 was considered appropriate. It was shown that few clinical positives are serologically negative; on the other hand, a large proportion of clinical negatives were serologically positive. We therefore defined two additional states: clinical negatives that were seronegative, which were considered susceptible (S), and clinical negatives that were seropositive, which were considered exposed (E). Further investigation showed that there was a strong age effect, with cows ≤ 5 years of age having substantially fewer exposed animals than cows >5 years of age. The lactating and dry cows were similar, and were hence considered together. We formulated an SEIS model, where the transitions from S to E and E to I were bidirectional, and transitions from I to S were also possible. We defined the two age groups to capture the age effect, and specified the housing and grazing periods separately to account for the seasonality. An environmental component, representing the reservoir of BDD-associated *Treponema* spp., was also defined.

From the longitudinal study data, we could determine the number of cows in each state at a given time; we also knew the numbers of cows that had transferred from another compartment since the previous time point, including from S to E, which were defined as 'new' infections. Using this information, we estimated the transmission parameter, β, which determines the rate at which S animals move to E, following the methodology of Monti et al. (2006). Separate βs were estimated for younger and older cows, during the housing and grazing periods. We also calculated the transmission coefficients governing the rates of cows between the other compartments. The model was formulated using a series of coupled differential equations, and run using the parameter estimates. The environmental compartment was iteratively parameterised, in the absence of suitable information to assist with this.

The model output was generally consistent with our data and empirical experience, but there were several discrepancies, which were possibly due to over- or underestimation of transmission parameters, or a result of inappropriate model formulation. Further exploration, analysis and model fitting is required. This model was deterministic, and thus did not incorporate any stochastic effects; incorporation of stochastic processes could prove to be more effective.

The model confirmed our findings from the observational studies and the Bayesian model that use of serology in addition to clinical inspection for BDD identifies a very high level of exposure to the infection in the cow groups. Use of serology assumes a direct association with infection by BDD-associated *Treponema* spp. This cannot be determined with certainty; however, the results of our observational studies strongly support this assumption. This indicates that the organisms causing BDD are ubiquitous in the cows' environment, and that the 'force of infection' is high. Not all cows exposed to the treponemes develop clinical BDD; we do not yet understand the component

causes required to constitute a ‘sufficient cause’.

The model presented in this thesis should be considered a preliminary effort at exploring the use of SEIR-type models; further refinement and diagnostics are required. The benefits of a mathematical model that effectively simulates the condition are great: it can be used predictively to assess the effect of interventions, and hence to inform putative control strategies. For instance, the effects of improving environmental hygiene can be assessed (by increasing the removal of treponemes from the environment), the likely effect of treatments can be estimated, and the effectiveness of husbandry-related factors can be explored.

7.6 Current research priorities and future perspectives

The combination of microbiological research with epidemiological investigation has contributed to an improvement of our understanding of BDD. Possible directions for future research are discussed below.

7.6.1 Microbiological research

Work on establishing the antigenic heterogeneity of BDD-associated *Treponema* spp. is ongoing. This consists of two components. Firstly, existing techniques such as culturing of treponemes, PCR, immunohistochemistry, Western blotting and serology (ELISA), can be further developed and/or refined. For instance, the development of phylotype-specific PCRs has recently shown that the 23 original cultures were not in fact pure, but constitute over 50 different antigens. Secondly, the collection of more lesion biopsies from other farms over a larger geographic area will serve to further characterize the heterogeneity of the population of BDD-associated *Treponema* spp.

Another priority for microbiological research is to identify reservoirs of infection and the spatial distribution on the animal and farm level of the BDD-associated treponemes; this would also provide information on the mechanisms of transmission of infection. Given the recently developed tools, this is now a realistic feasibility. This would enable the disease to be more accurately epidemiologically investigated and modelled, both statistically and mathematically. It would also inform the design of effective intervention and control measures. The investigation of patterns of antimicrobial resistance could also contribute to this.

7.6.2 Epidemiological research

The applied epidemiological research within this project has concentrated on in-depth, detailed studies to elucidate the farm-level distribution and dynamics of BDD. The finite resources available have necessitated this to be carried out on a relatively small number of farms. Nevertheless, the detailed findings provide information on which to

base future investigation.

The findings and outcomes could be investigated on a larger number of representative dairy farms using loose-housed Holstein Friesian cows, over a larger geographic area. Appropriate sample sizes can be calculated based on parameters provided by our studies. As with our studies, a combination of clinical inspection and serology is appropriate. Such a study could easily be combined with microbiological research, such as taking lesion biopsies and environmental samples on study farms. The results would provide information for further parameterizing and refining the statistical and mathematical models.

Our longitudinal study enabled us to identify trends and risk factors, but it was insufficiently detailed to investigate the dynamics and progression of the disease on the individual animal level; hence, we have not yet elucidated the relationship between serology and clinical disease. To further study this, a case-control study could be considered, where a discrete number of cases are compared with controls, for a predetermined period. The animals would need to be intensively monitored, e.g. on a bi-weekly basis; lesion biopsies and other samples could be taken.

7.6.3 Intervention studies

Attempts at eradication of BDD on affected farms have been unsuccessful to date. Control measures have primarily concentrated on treatment, strengthening biosecurity and improving environmental hygiene. These have been applied empirically, and are highly divergent; hence few reliable reports of their efficacy (which is likely to be equally variable) exist. The number, scope and design of clinical trials is limited; Laven and Logue (2006) comment that few 'meaningful' and repeatable studies have been performed.

Epidemiological and infectious disease research is essential to determine the aetiology, pathogenesis, distribution and dynamics of diseases; with BDD, this could be said to be a specific priority due to our poor understanding of these elements. However, while such research may be useful for identifying key risk factors and developing recommendations for elements of control and prevention strategies, they cannot actually determine the effectiveness of such strategies. Such practical, effective and successful interventions are a prerequisite for the future control and prevention of BDD. In this respect, practical intervention studies are an essential component of the research field as a whole; even when their design must be guided by empirical or assumed knowledge.

Treatment and control of BDD. Possible treatment studies would include appropriately designed and implemented clinical trials (possibly in combination with antimicrobial resistance studies, see above). It is essential to consider the practical aspects, i.e. consider safety and ease of administration, cost, disposal, development of resistance and so forth. A review by Laven and Logue (2006) covers current treatment options

for BDD; no further suggestions will be made here.

Studies investigating control measures on the management group or farm level are more difficult to implement. Barring investigation of treatment, these would assess the effects of exposure of study animals to the pathogens causing BDD; this requires cohorts to be selected on case farms on which the interventions are carried out, and on control farms on which they are not. For comparison of results, the conditions on these farms must be standardised as far as possible. Prospective interventions directed at improving hygiene in the environment can be impractical and costly as they may necessitate alterations being made to the existing facilities.

Prevention of BDD. The development of effective prevention strategies is probably a long way off. Incidence of BDD lesions can presumably be prevented by eliminating exposure to the causative pathogens, or alternatively by inducing a protective immune response. Given that BDD is highly contagious and infected farms have never successfully eliminated the infection, the first option does not seem feasible, particularly if it should transpire that the reservoirs of infection are animal-related or widely distributed in the environment. There has been interest and research in vaccine development, but no effective vaccine currently exists. This is hardly suprising, considering that we do not yet understand the immunologic mechanisms related to BDD – the humoral response certainly does not confer protective immunity.

7.7 Towards prevention: utilizing knowledge and making recommendations for interventions

The emphasis of our studies lay on elucidating the disease distribution and infection dynamics of BDD, and they were not designed to provide or recommend treatment and control strategies; as described above, suitable intervention studies are required for this. However, our results do provide science-based and detailed information to suggest that the following measures should be considered for control programmes (most of which are already known and applied):

1. Our clinical observations indicated that periodic reactivation of chronic lesions was common. Serology did not show the same seasonal pattern as incidence of clinical lesions. This indicates that exposure is continuous. If this is the case, treatments of BDD lesions will need to be regular and repeated to have an effect; a single treatment will not be effective.

2. Exposure of the young stock is low, and few heifers develop the disease. Control measures should therefore be concentrated on the cow groups.

3. The treatment options currently available to farmers basically consist of individual treatments with an oxytetracycline aerosol, or footbaths (see Chapter 1.2.6

or Laven and Logue (2006) for details). Individual treatments are indicated for BDD cases; however, considering that exposure to BDD seems widespread, footbaths (when utilized effectively) can certainly be useful for limiting the infection pressure.

4. The seasonal nature of the disease means that the most intensive efforts should be concentrated on the housing period, particularly in the early period, when the incidence of BDD rises to its maximum, i.e. October to January. If footbaths are applied on the farm, they should be performed most frequently in this period; if antibiotic footbaths are used, this is the best time to do so.

5. However, the year-round incidence implies that control measures should be continued throughout the grazing season, for example by vigilance and treatment of 'new' lesions in the milking parlour.

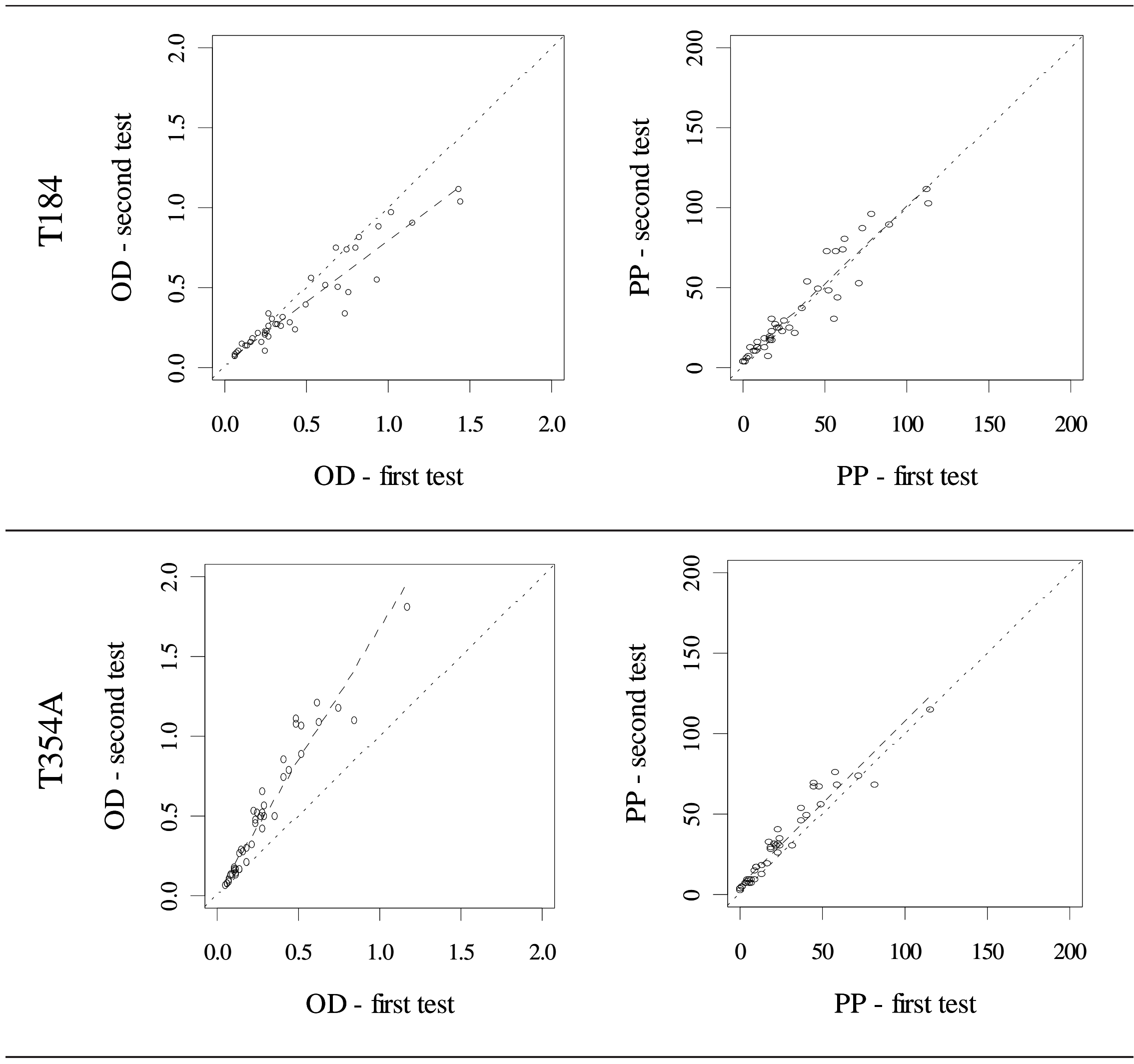
6. First lactation heifers have not previously been exposed to a high force of infection, and although they do not necessarily have a higher rate of incidence of lesions, they tend to develop painful acute lesions; although we did not perform lameness scoring, it is possible that the incidence of lameness caused by BDD is highest in this group. Farmers should be aware of this, and would be recommended to treat these animals individually. The welfare importance of BDD may be greatest for this group. Again, the milking parlour is a good opportunity to monitor these animals, and acute lesions should respond well to repeated treatment with topical oxytetracycline spray.

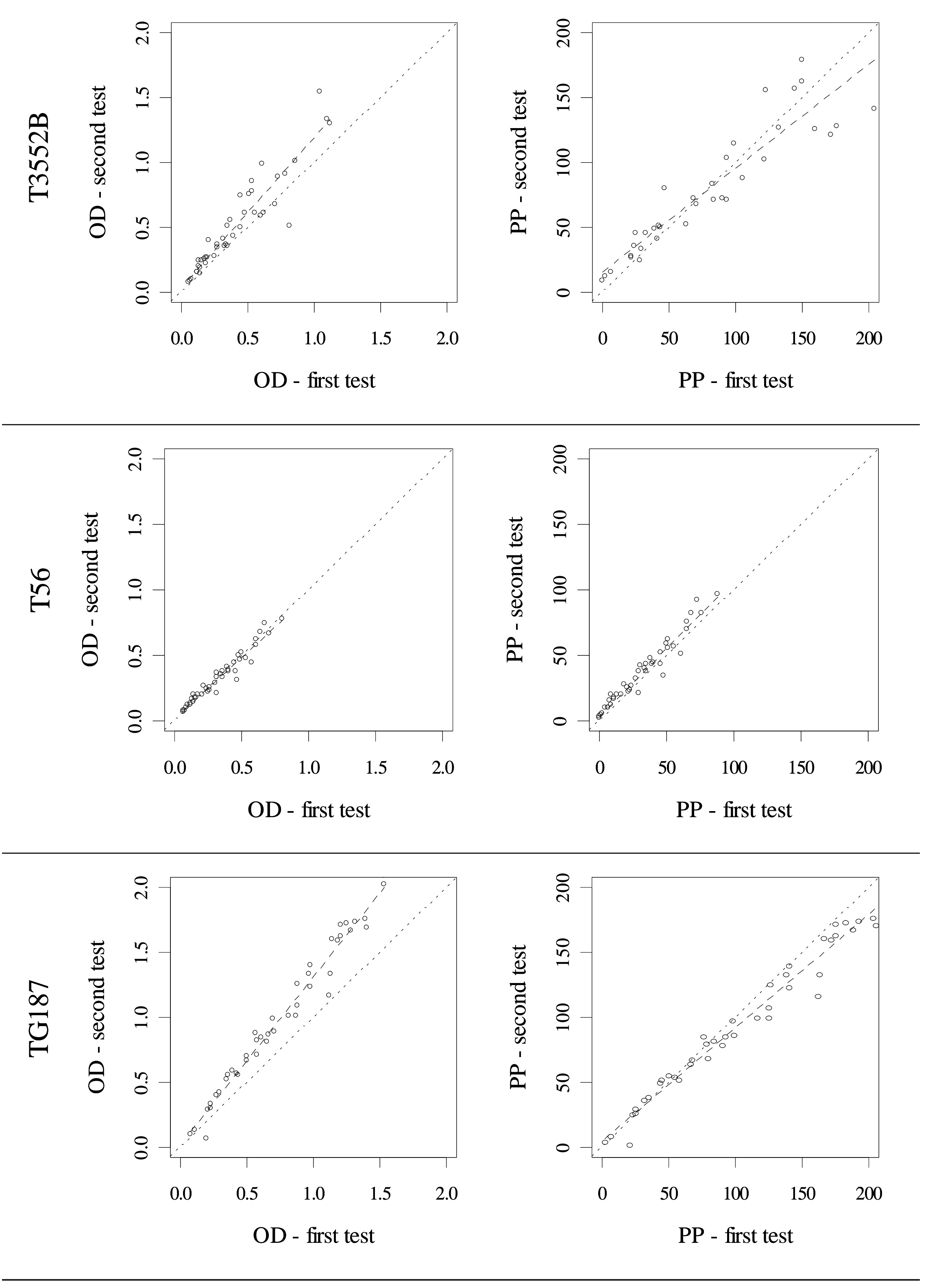
7. Our results show that second and third lactation cows have equally high prevalence, and that these lesions are more chronic in nature. These lesions may not respond as well to treatment, as they are more keratinous and prominent; repeated treatment is certainly required in these cases. It appears that older cows do not develop as many acute or chronic lesions.

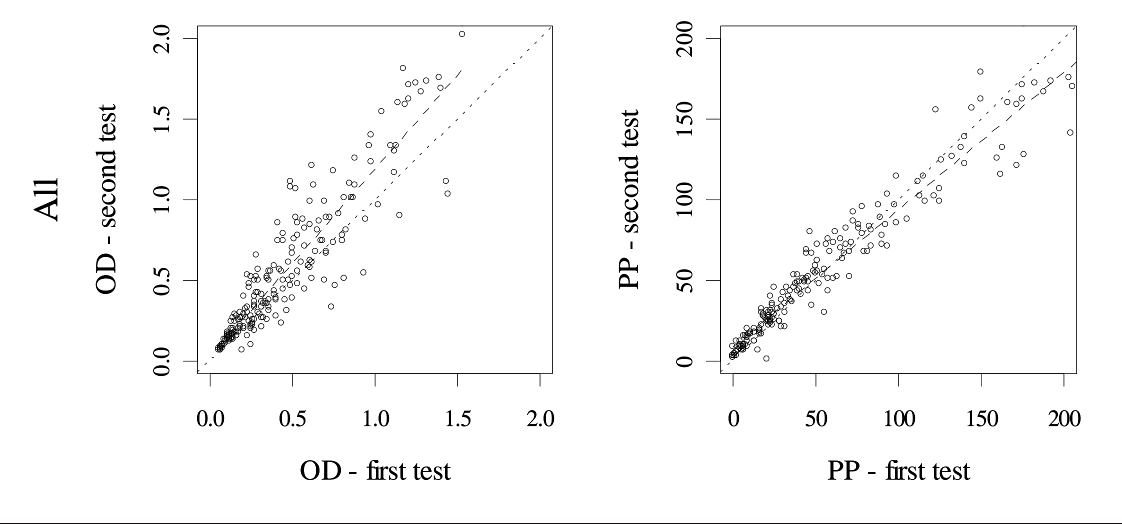
8. Hygiene has also been identified as a significant risk factor; however, it is difficult to make firm recommendations about this, as it is determined to a large degree by the farm-specific design and facilities.

Appendix A

Repeatability of the BDD-associated *Treponema* spp. ELISAs




T3552B
T56
TG187
OD - second test
OD - first test
PP - second test
PP - first test
0.0
0.5
1.0
1.5
2.0
0
50
100
150
200


All
OD - second test
0.0
0.5
1.0
1.5
2.0
OD - first test
PP - second test
0
50
100
150
200
PP - first test

Appendix B

Maps of the study area, showing farm locations

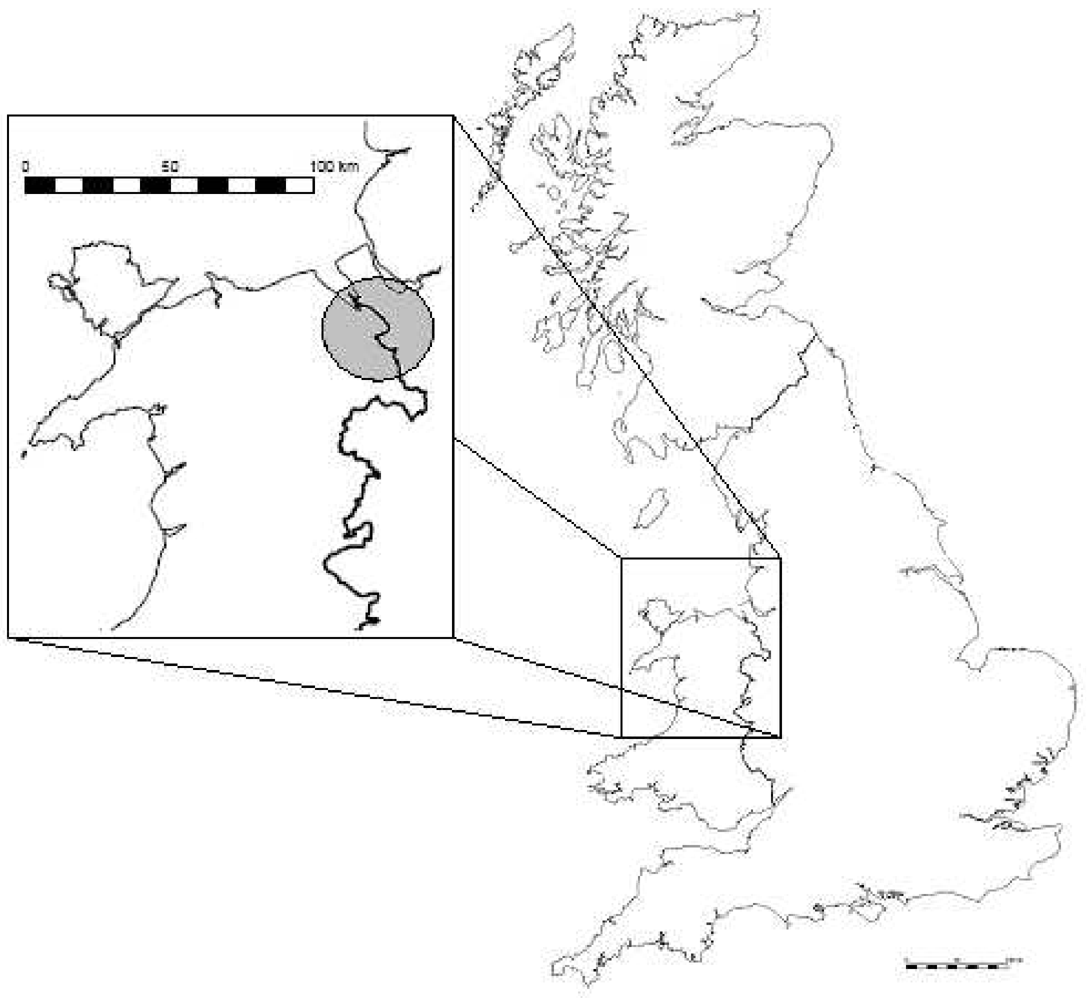


Figure B.1: Map of the UK, showing the study area in North-Western England and North Wales.

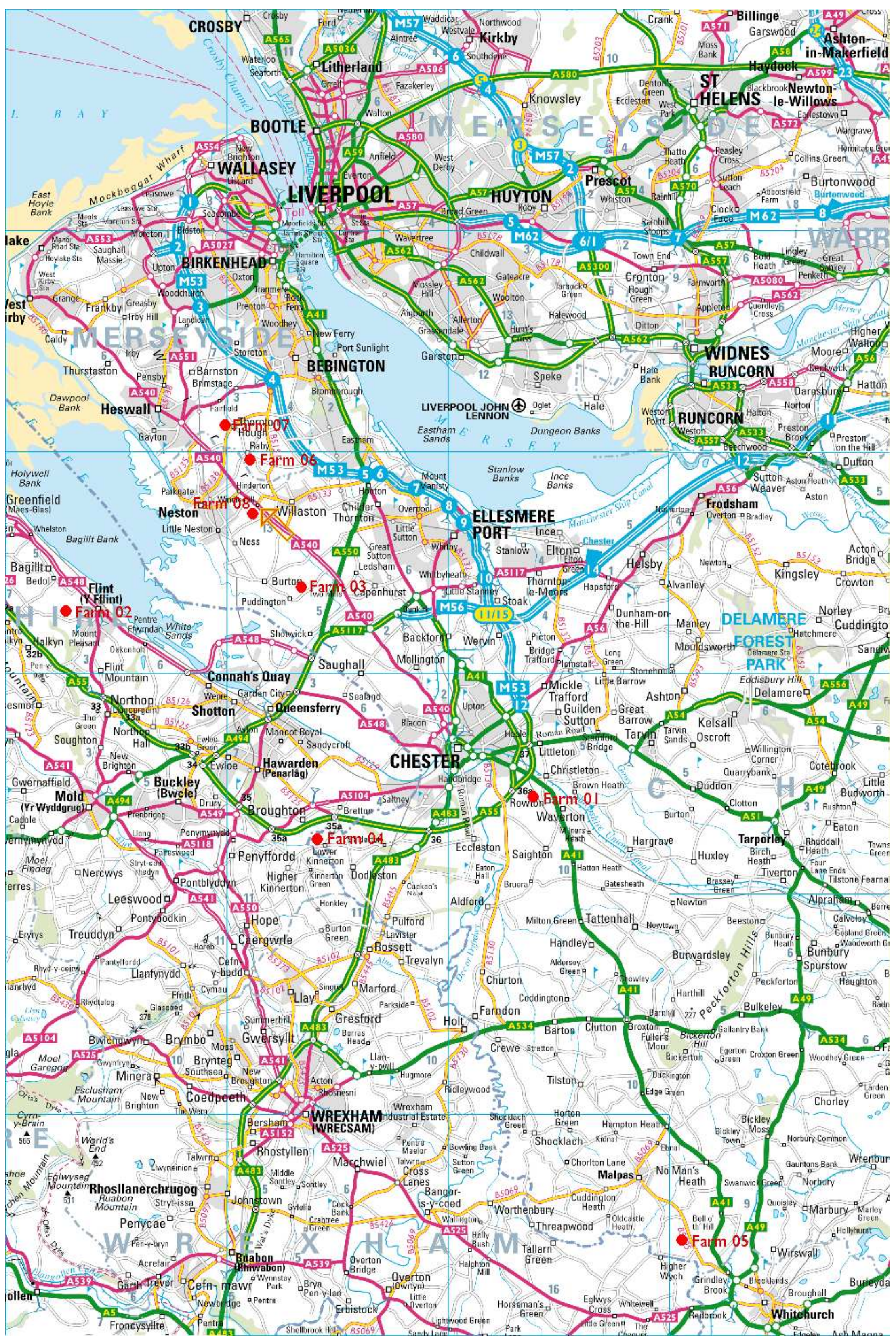


Figure B.2: Detailed map of the study area. Four of the farms (03, 06, 07 and 08) were located on the Wirral peninsula; two farms (01 and 04) were just south of Chester; one farm (02) was nearby Flint in North Wales; and one farm (05) was located near Whitchurch, just over the county border in Shropshire. The arrow marks Leahurst.

Appendix C

Specifications of the rigid borescope

C.1 Description

A rigid borescope is an industrial instrument used to visualize inspection areas where straight-line access is available.

For technical details, see http://www.olympusindustrial.com/.

C.2 Components

The borescope consists of the following components:

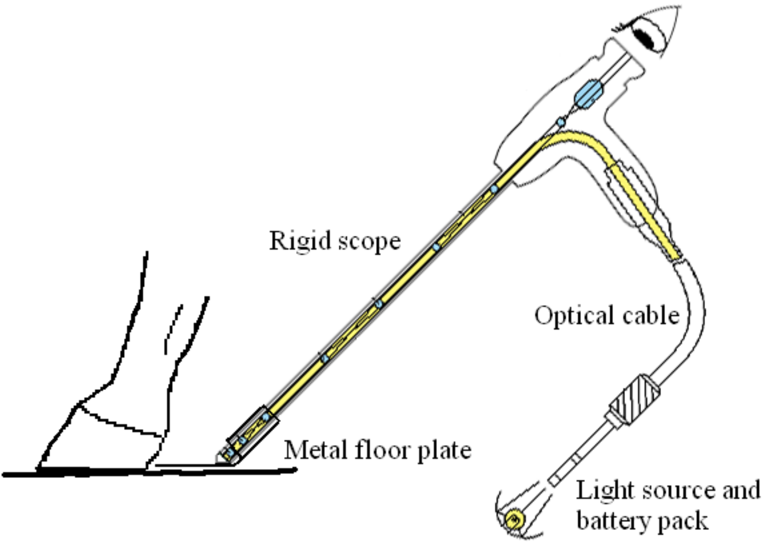


Figure C.1: The modified Olympus industrial borescope

- A durable stainless steel insertion tube of length 64cm and diameter 80mm, incorporating a multiple-element optical lens system. This tube has a direction of view of 45°.

- Illumination is transmitted to the subject area via a detachable optical fibre cable.
- A 75W tungsten-halogen portable light source is used, which has a spring clip for mounting onto a battery belt. Dimensions: 140 x 80 x 60mm.

Figure C.2: Light source

- The light source uses separate, rechargeable 12V nickel-cadmium battery cells, which snap onto a battery mount on the belt.

Figure C.3: ‘Lok On’ belt with power pack

- For resting the end of the borescope on the floor at an appropriate angle, a metal base plate is connected to end of the rigid tube.

C.3 Use for inspecting cows’ feet

Borescopes are used for diverse purposes. This modification was devised by Roger Blowey as a method of visualizing the ‘typical’ BDD lesion area more effectively, without lifting the foot.

In the standing cow, the most basic lesion inspection is performed by hosing down the foot to remove superficial slurry, squatting behind the animal and illuminating the

plantar area of the hind feet with a powerful torch. This is rather uncomfortable at best, and a health and safety hazard at worst! Due to the angle the foot makes with the floor, it is rarely possible to adequately inspect the 'typical' lesion area.

Using the borescope, the floor plate is slid to the back of the foot, which enables direct visualization of the plantar skin between and directly above the heel bulbs. The light source provides ample illumination, and the optical system provides magnification of the area of specific interest. This greatly improves inspection, particularly when the heel height of the animal is low. See below for an illustration of the borescope 'in action'.

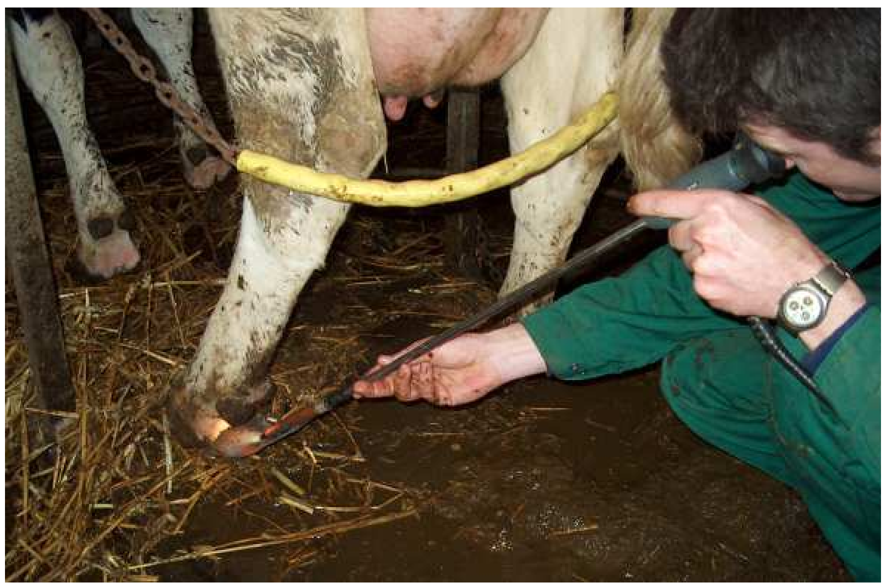


Figure C.4: Foot inspection using the modified Olympus industrial borescope

C.4 Limitations

The field of view is still limited. Magnification can distort the image, and it can be difficult to accurately assess the size of the lesion. The soles of the feet cannot be inspected (e.g. for concurrent lesions); neither can the interdigital cleft.

Some experience and familiarity facilitates use of the scope. Empirically, there is a tendency to assess lesions as being more severe than when confirming by lifting the foot. The implication is that the rate of false positives is relatively high, i.e. lesions classified as active by borescope inspection were classified as 'old' or regressed on lifting.

Nevertheless, routine lifting of all feet would be prohibitive in terms of labour intensity, time limitations and physical constraints! In this context, the borescope is a hugely useful device.

Appendix D

Development of a Bayesian model to determine conditional probability of infection

D.1 WinBUGS model code

```
Model
    {
     for(i in 1:N){
                  s[i] ~ dnorm(mmu[i],ttau[i])
                    mmu[i] <- ((1 - I[i]) * mu[1])  + (I[i] * mu[2])
                    ttau[i] <- ((1 - I[i]) * tau[1])  + (I[i] * tau[2])
                  I[i] ~ dbern(p[i])
                  p[i]<- (q[i] * PVP) + ((1 - q[i]) * (1 - PVN))
                  L[i] ~ dbern(q[i])
                  logit(q[i]) <- b[1] + (b[2] * xc[i]) + (b[3] * zc[i])
                  }
        PVP <- (Prev * Se) / ((Prev * Se) + (1 - Prev) * (1 - Sp))
        PVN <- ((1 - Prev) * Sp) / ((1 - Prev) * Sp + Prev * (1 - Se))
        Se ~ dbeta(10.9,7.6)
        Sp ~ dbeta(42.6,5.6)
        Prev ~ dbeta (1.9,1.9)
     for (j in 1:2){
                   tau[j] ~ dgamma(0.1,0.1)
                   mu[j] ~ dnorm(c[j],d[j])
                   }
        qtilde[1] ~ dbeta(5.4022,14.2067)
        qtilde[2] ~ dbeta(6.4978,7.7195)
        qtilde[3] ~ dbeta(7.3057,12.7106)
        qtilde[4] ~ dbeta(5.0973,10.5603)
        b[1] <- logit(qtilde[1])
        b[2] <- [logit(qtilde[2]) - b[1]] / ((7 - 5.4697) / 1.5539)
        b[3] <- [logit(qtilde[4]) - logit(qtilde[3])] / ((6 - 3.72) / 2.56))
    }
```

D.2 Model approach

Requirements of the model are to:

- incorporate a latent variable for infection status;
- incorporate an appropriate structure in the model which reflects the structure of the dataset;
- determine the conditional probability of infection (CPI) without cut-offs;
- obtain predictive probability of infection (PPI) curves which can be generally applied (which therefore assumes the study population is representative of the larger population).

Assumptions and explanation:

- The serologic outcome `s` for all `N` observations is assumed to be normally distributed (through log transformation) with mean `mmu` and precision `ttau`.
- The means and precision are assumed to be different for infected and uninfected animals (if `I[i]` = 0, `mmu[i]` = `mu[1]` and if `I[i]` = 1, `mmu[i]` = `mu[2]` and likewise for `tau`).
- Infection status `I` is binary, hence Bernoulli distributed with probability `p[i]` of an animal being infected.
- To quantify `I`, we express it as a function of `q[i]`, `PVP` and `PVN`. As this is undesirable, we substitute `Se`, `Sp` and `Prev` into the model and place priors on these (see below).
- Lesion status `L` is binary, hence Bernoulli distributed with probability `q[i]` of an inspected animal having a lesion.
- We model lesion status with a straightforward regression model, i.e. using the logit link function.
- To allow derivation of informed prior distributions (see below) and avoid autocorrelation between `b[1]` and `b[2]`, the covariates `x` (FHS) and `z` (age) have been standardized by centring:

  $$\texttt{xc[i]} = \frac{(x_i - \bar{x})}{\text{sd}(x)}$$

  where $\text{sd}(x)$ is the standard deviation of x (and likewise for `zc[i]`).
- The precision has a gamma distribution, which is an appropriate conjugate prior for precision in the normal distribution.

- The means `mu[1]` and `mmu[2]` are normally distributed; the initial values of `c` and `d` are specified in the data file as c(0,1) and c(1,1), respectively.

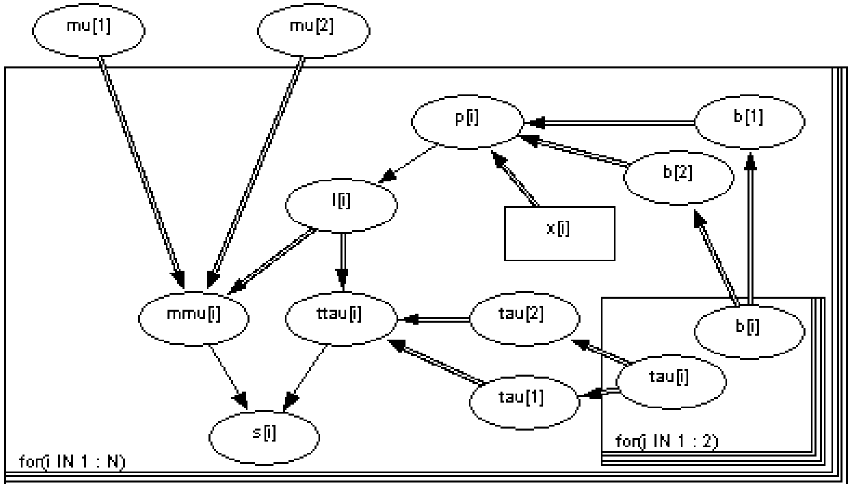


Figure D.1: DoodleBUG of basic model

D.3 Specifying the prior distributions

Relevant information on BDD includes the prevalence of lesions in cow groups; information was derived from the literature (Somers et al., 2005; Holzhauer et al., 2006; Murray et al., 1996), and the author's experience on regional dairy farms. The prevalence of infection in this group can also be perceived as the probability of a cow within this group of having a BDD lesion.

To construct informed beta priors for the covariates `x[i]` and `z[i]`, it is necessary to first standardize them; this has already been performed to obtain `xc[i]` and `zc[i]`.

D.3.1 First covariate: FHS

Let $\widetilde{Q}_1 = P(L \mid X = \overline{x})$, i.e. what is the probability of a cow with an average FHS, on an 'average' farm, having a BDD lesion?

$$\text{logit}(q_i) = b_1 + b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}$$

so

$$\text{logit}(\widetilde{Q}_1) = b_1 + b_2 * \frac{(\bar{x} - \bar{x})}{\text{sd}(x)}$$

Therefore,

$$b_1 = \text{logit}(\widetilde{Q}_1)$$

Now, a 'dirtier' cow with a higher FHS is considered (e.g. x_h); let $\widetilde{Q}_2 = P(L \mid X = x_h)$. This FHS is arbitrary, but the probability of having a BDD lesion should be estimable with a good degree of confidence. By substituting,

$$\text{logit}(\widetilde{Q}_2) = b_1 + b_2 * \frac{(x_h - \bar{x})}{\text{sd}(x)}$$

The average FHS was 5.47, and a higher FHS of 7 was chosen. The standard deviation was 1.55.

$$\text{logit}(\widetilde{Q}_2) = b_1 + b_2 * \frac{(7 - 5.47)}{1.55}$$

$$b_2 = \text{logit}(\widetilde{Q}_2) - b_1 * \frac{1.55}{(7 - 5.47)}$$

A cow of average FHS was considered to have a probability of 25% of showing a BDD lesion, with a 95% certainty that this probability was greater than 12.5%. A cow with FHS 7 was considered to have a probability of 45% of showing a BDD lesion, with a 95% certainty that this probability was greater than 25%. These estimates were used to obtain beta distributions (using BetaBuster).

D.3.2 Second covariate: age

As before, let $\widetilde{Q}_3 = P(L \mid Z = \overline{z})$, and $\widetilde{Q}_4 = P(L \mid Z = z_h)$. Adding the second covariate to the regression equation,

$$\text{logit}(q_i) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right) + \left(b_3 * \frac{(z_i - \bar{z})}{\text{sd}(z)}\right)$$

so

$$\text{logit}(\widetilde{Q}_3) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right) + \left(b_3 * \frac{(\bar{z} - \bar{z})}{\text{sd}(z)}\right)$$

i.e.

$$\text{logit}(\widetilde{Q}_3) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right)$$

and

$$\text{logit}(\widetilde{Q}_4) = b_1 + \left(b_2 * \frac{(x_i - \bar{x})}{\text{sd}(x)}\right) + \left(b_3 * \frac{(z_4 - \bar{z})}{\text{sd}(z)}\right)$$

Therefore,

$$\text{logit}(\widetilde{Q}_4) = \text{logit}(\widetilde{Q}_3) + \left(b_3 * \frac{(z_4 - \bar{z})}{\text{sd}(z)}\right)$$

so rearranging and solving for b_3 we get

$$b_3 = \text{logit}(\widetilde{Q}_4) - \text{logit}(\widetilde{Q}_3) * \left(\frac{\text{sd}(z)}{z_4 - \bar{z}}\right)$$

The average age was 3.72, and a higher age of 6 was chosen. The standard deviation was 2.56. A cow of the average age was considered to have a probability of 35% of showing a BDD lesion, with a 95% certainty that this probability was greater than 20%. A cow of age 6 was considered to have a probability of 30% of showing a BDD lesion, with a 95% certainty that this probability was greater than 15%. These estimates were used to obtain beta distributions (using BetaBuster).

D.3.3 Sensitivity, specificity and prevalence

Let `I[i]` represent the distribution of the true infection status `p[i]` (latent variable). We now need an equation specifying the variable `p[i]` in the model:

```
P[Infected] = (P[Lesions] * P[Infected | Lesions]) +
(P[No lesions] * P[Infected | No lesions])
```

`P[Infected | Lesions]` is predictive value positive (`PVP`) and
`P[Infected | No lesions]` is (1 - predictive value negative (`PVN`))

```
p[i]<-q[i] * PVP + (1-q[i]) * (1-PVN)
```

Unlike the sensitivity and specificity, these predictive values are dependent on the prevalence of infection in the population. A model that is valid for our study population therefore would not be applicable for a larger population. We therefore want to enter `Se` and `Sp` back in the model.

Lesion	Infection		
	Positive	Negative	Total
Positive	a	b	(a + b)
Negative	c	d	(c + d)
Total	(a + c)	(b + d)	(a + b + c + d)

Referring to the 2x2 table above,

```
PVP = a / (a+c)

PVP = {((a+c)/(a+b+c+d)) * a/(a+c)} / {((a+c)/(a+b+c+d) * a/(a+c))
+ ((b+d)/(a+b+c+d) * b/(b*d))}

PVP = (Prev * Se) / (Prev * Se) + ((1 - Prev) * (1 - Sp))
```

Similarly,

```
PVN = d / (c+d)

PVN = {((b+d)/(a+b+c+d)) * d/(b+d)} / {((b+d)/(a+b+c+d) * d/(b+d)) +
((a+c)/(a+b+c+d) * c/(a*c))}

PVN = ((1 - Prev) * Sp) / ((1 - Prev) * Sp) + (Prev * (1 - Se))
```

Prior beta distributions can now be specified. It was assumed specificity is very high with a high level of certainty (if lesions are seen, the probability of *not* being infected are very low, i.e. (1 - `Sp`) is very low, i.e. `Sp` is very high). Sensitivity is lower with a low level of certainty (if no lesions are seen, the animal may still be infected, i.e. (1 - `Se`) is higher, i.e. `Se` is lower). The following prior distributions were specified:

`Se` $\approx$ dbeta(10.9,7.6): `Se` has mode of 0.6; 95% certain that `Se` >0.4

`Sp` $\approx$ dbeta(42.6,5.6): `Sp` has mode of 0.9; 95% certain that `Sp` >0.8

A non-informed prior was assumed for `Prev` (as the true prevalence is determined by latent infection variable):

`Prev` $\approx$ dbeta(1.9,1.9): `Prev` has mode of 0.5; 95% certain that `Prev` >0.13

D.4 Model convergence and performance

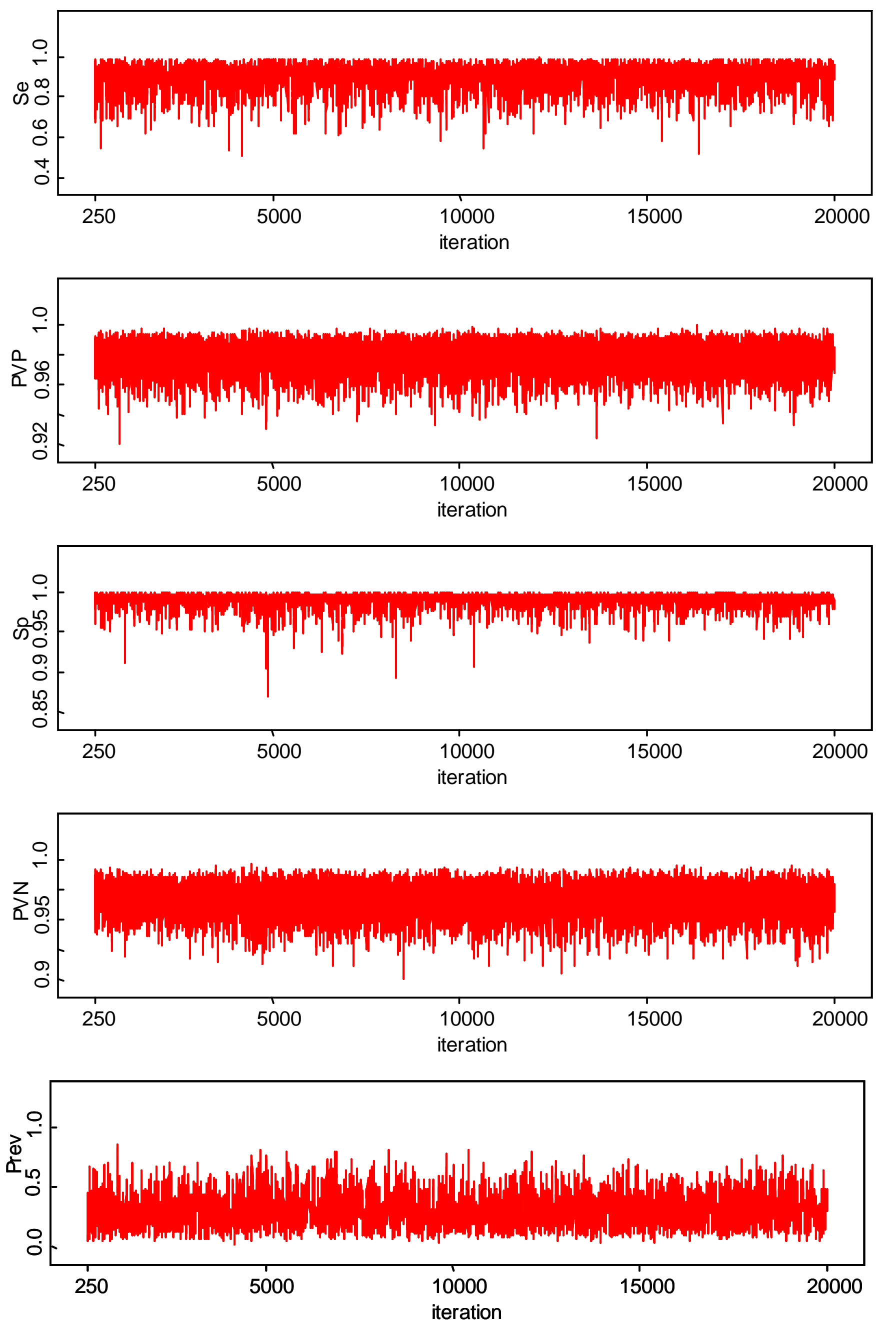


Figure D.2: Traces of model estimation of sensitivity, specificity, predictive value positive, predictive value negative and prevalence. Iterations 0 – 250 were discarded (burn-in)

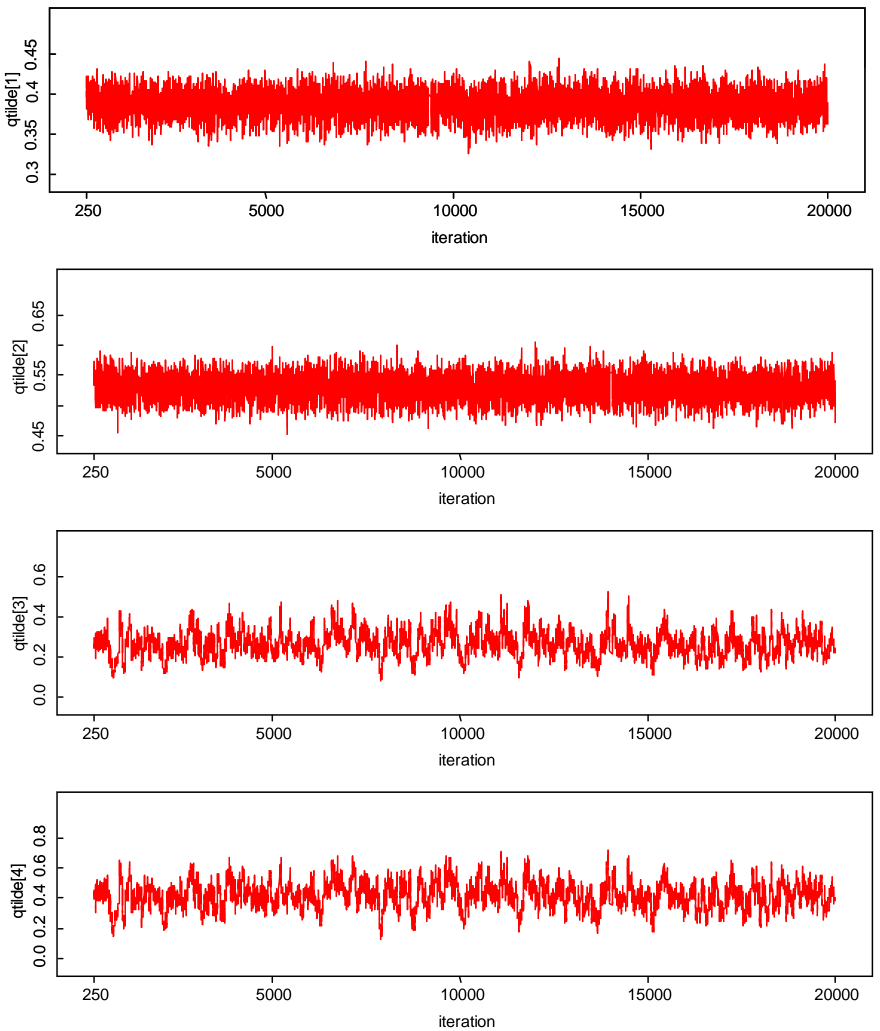


Figure D.3: Traces of model estimation of average FHS, high FHS, average Age and above average Age. Iterations 0 – 250 were discarded (burn-in)

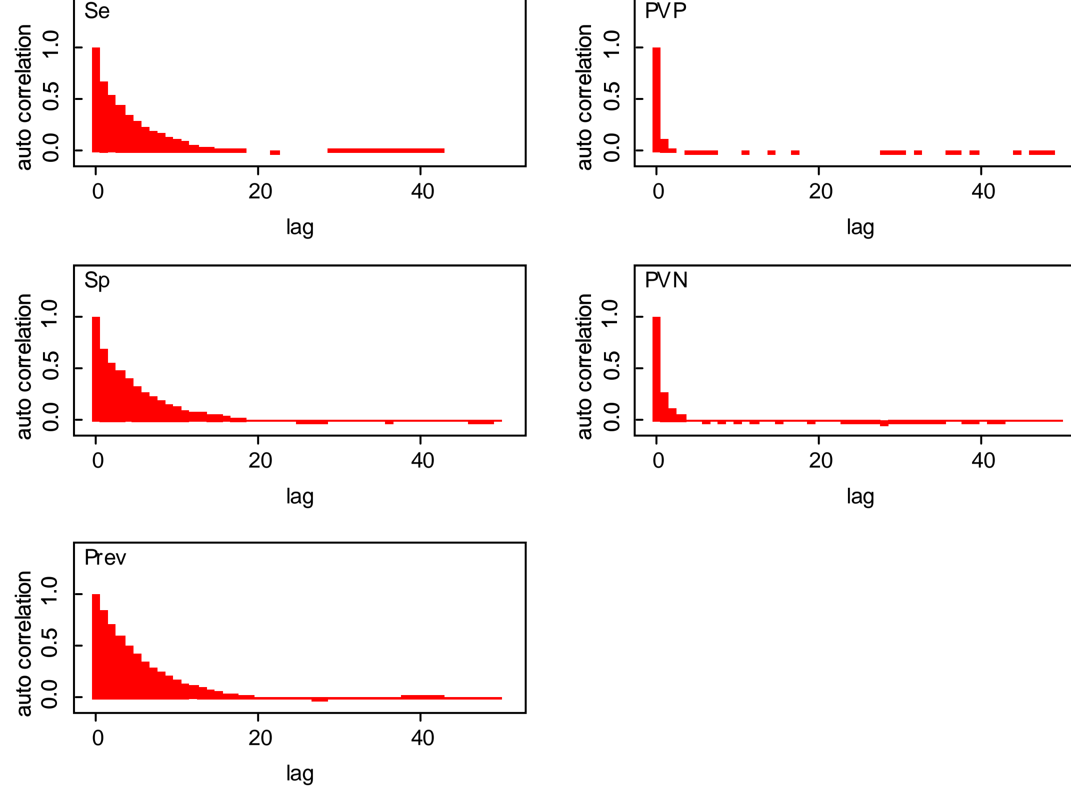


Figure D.4: Plots of autocorrelation of sensitivity, specificity, predictive value positive, predictive value negative and prevalence, up to a lag of 50

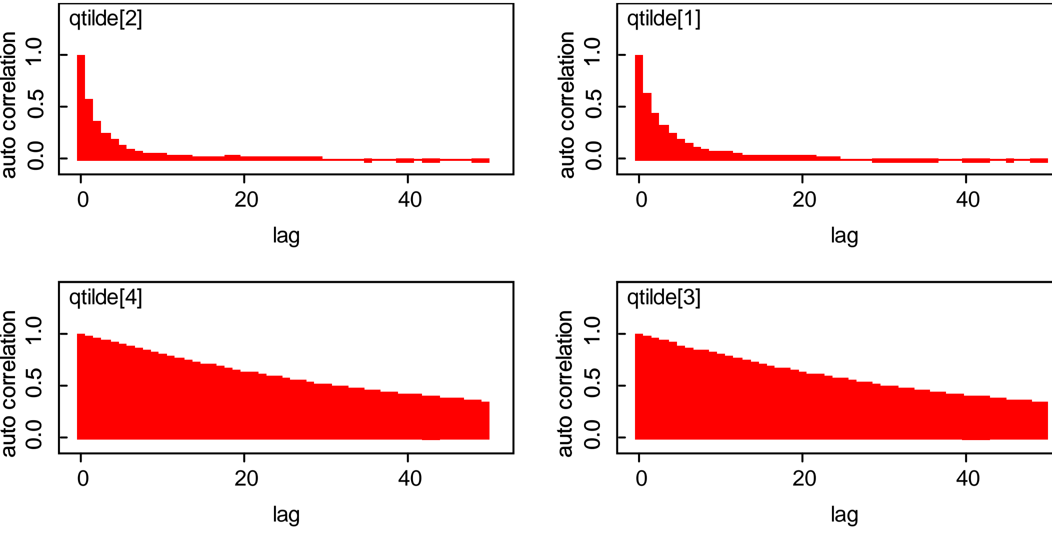


Figure D.5: Plots of autocorrelation of average FHS, high FHS, average Age and above average Age, up to a lag of 50

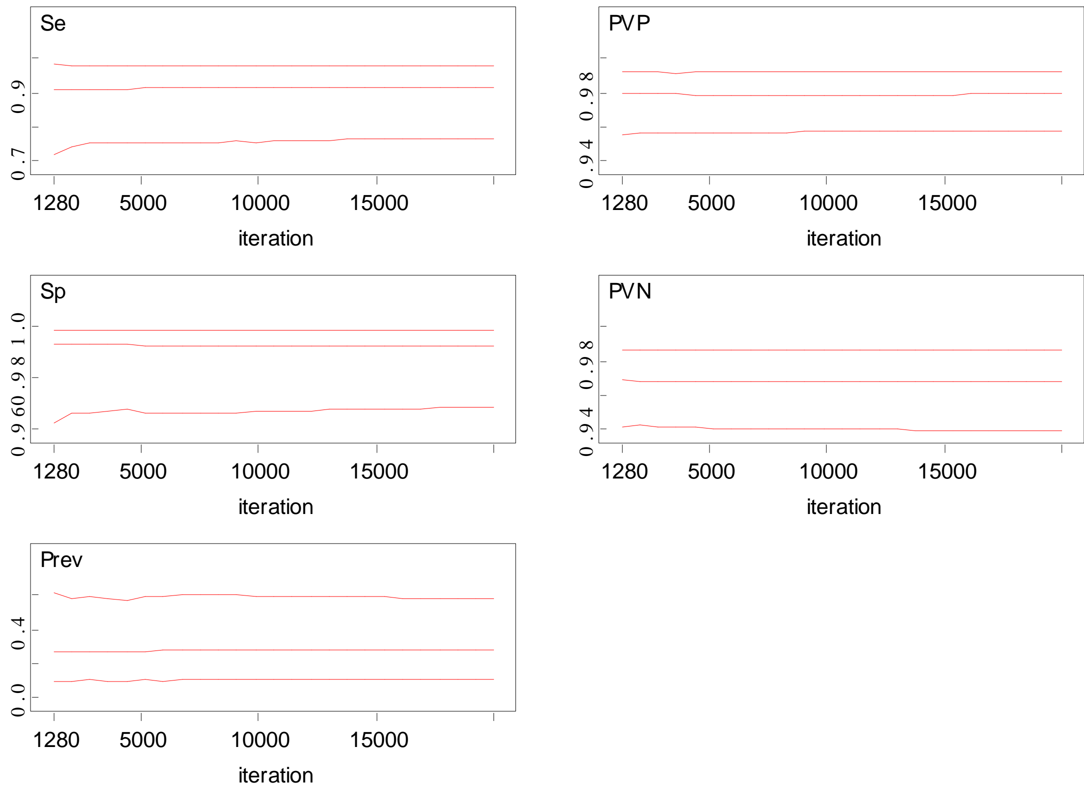


Figure D.6: Plots of quantiles about the estimated median sensitivity, specificity, predictive value positive, predictive value negative and prevalence

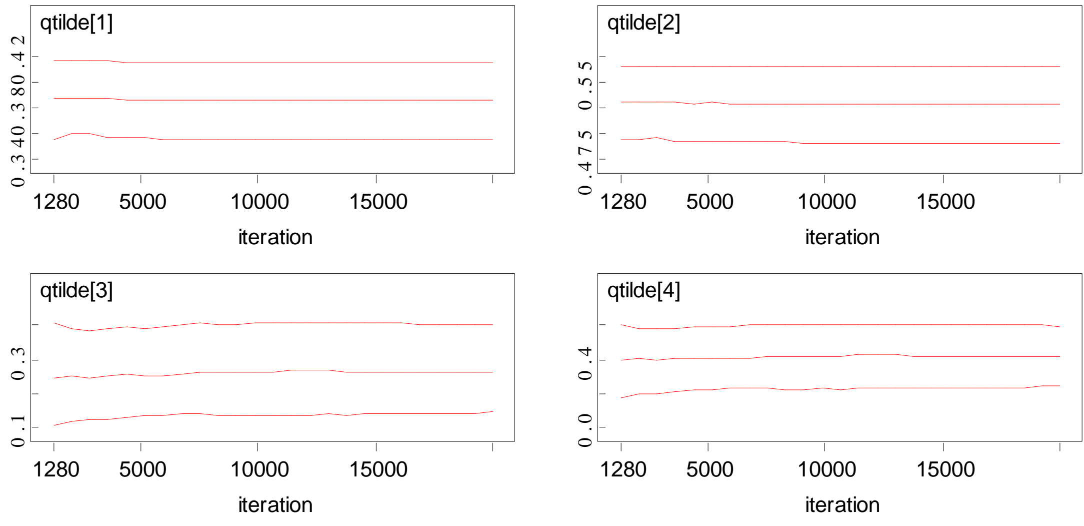


Figure D.7: Plots of quantiles about the estimated medians of average FHS, high FHS, average Age and above average Age

D.5 Model output and results

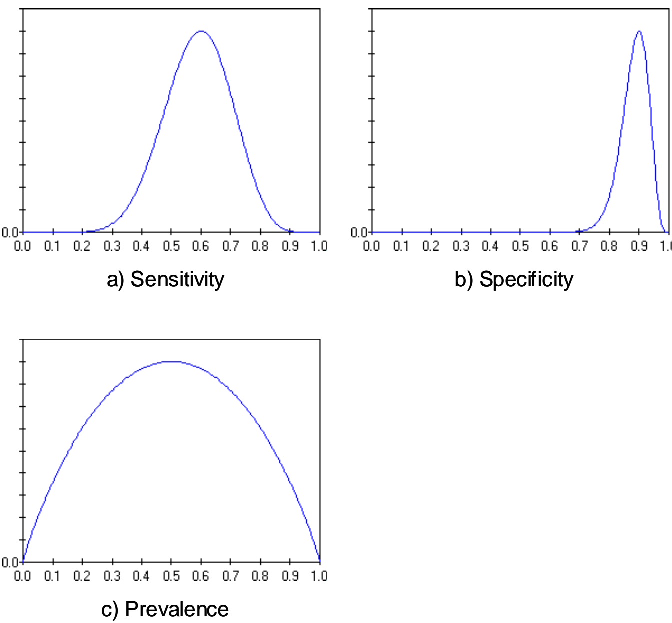


Figure D.8: Plots of the prior distributions of sensitivity ($\sim$ Beta$(10.9, 7.6)$), specificity ($\sim$ Beta$(42.6, 5.6)$) and prevalence ($\sim$ Beta$(1.9, 1.9)$)

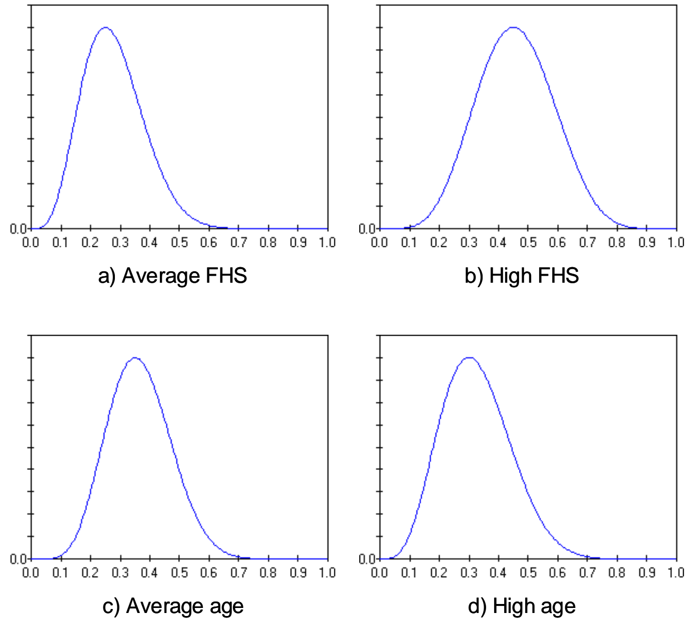


Figure D.9: Plots of the prior distributions of average FHS ($\sim$ Beta$(5.40, 14.21)$), high FHS ($\sim$ Beta$(6.50, 7.72)$), average Age ($\sim$ Beta$(7.31, 12.71)$) and high Age ($\sim$ Beta$(5.10, 10.56)$)

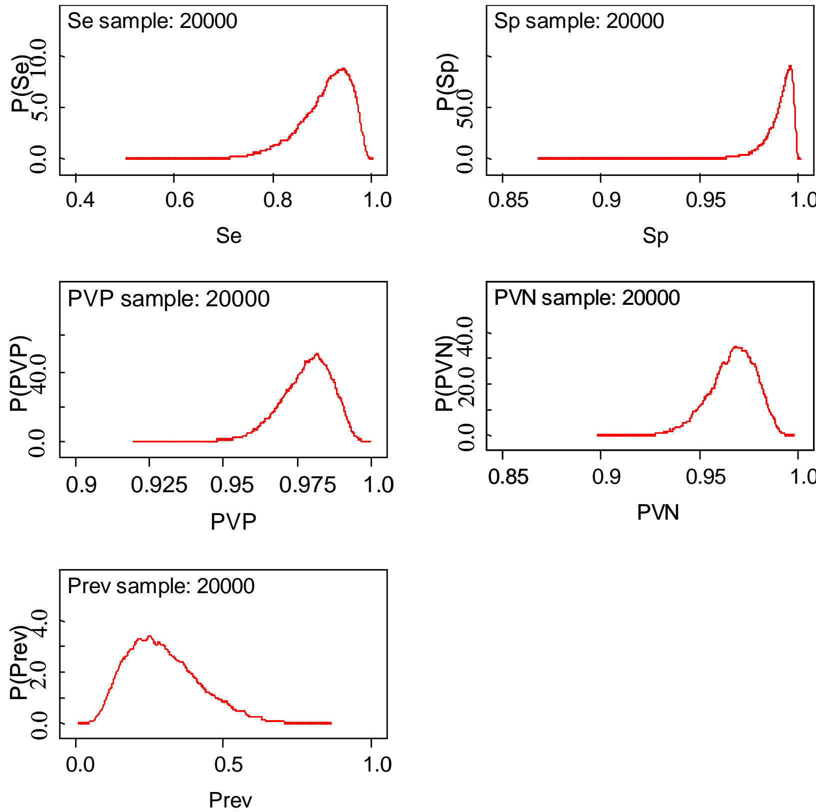


Figure D.10: Kernel density plots of model estimates of sensitivity, specificity, predictive value positive, predictive value negative and prevalence

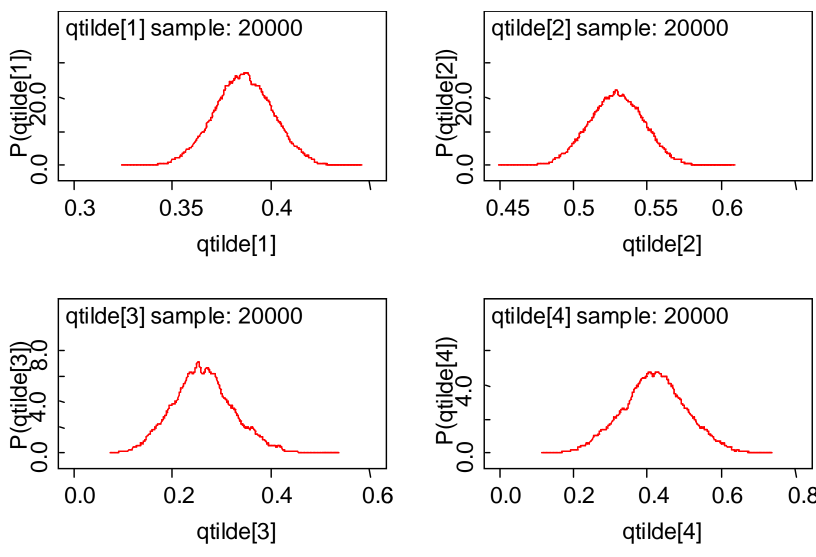


Figure D.11: Kernel density plots of model estimates of average FHS, high FHS, average Age and above average Age

Appendix E

Questionnaire and recording forms

E.1 Cross-sectional study

E.1.1 Questionnaire

Control of digital dermatitis in cattle: understanding transmission and spread of disease

Cross-sectional study

2004

Confidential farm management questionnaire

Epidemiology Group
Department of Veterinary Clinical Science
Leahurst
Neston
South Wirral
CH64 7TE

Farm details

Farm name / address	____
	____
	____
	____
	____
Person(s) interviewed (tick all that apply)	☐ farm owner(s) ☐ farm manager(s) ☐ stockperson(s) ☐ other: ____
Farm telephone / mobile	Tel. ______________________ Mobile____________________
Classification of farm DD status	☐ DD free ☐ low DD prevalence ☐ medium DD prevalence ☐ high DD prevalence
Date of interview	____ / ____ / 2004
Veterinary practice through which farm was recruited	____

Farm level variables

Cohabitation

1. We would like to know the numbers of animals on the farm at this moment. Please could you specify:

Species / type	**Total number on farm at date of interview**
Dairy cattle	
Beef cattle	
Sheep – resident	

Animal replacements and contacts

2. Research has indicated that ***bringing animals onto the farm from outside*** is one of the most important ways of introducing digital dermatitis on previously negative farms. How many of the following animals have been brought onto the farm ***in the last 12 months***?

Category / group	**Number of animals**	**Source(s) of acquired animals (tick all that apply)**	**Frequency of introduction (number of occasions)**
Replacement calves / heifers		☐ other farmer ☐ market ☐ known dealer ☐ other: specify _______________________________	
Breeding cows		☐ other farmer ☐ market ☐ known dealer ☐ other: specify _______________________________	
Fattening cattle		☐ other farmer ☐ market ☐ known dealer ☐ other: specify _______________________________	
Breeding bulls		☐ other farmer ☐ market ☐ known dealer ☐ other: specify _______________________________	
Any other cattle (specify)		☐ other farmer ☐ market ☐ known dealer ☐ other: specify _______________________________	
Sheep		☐ other farmer ☐ market ☐ known dealer ☐ other: specify _______________________________	

Footcare

3. Are *footbaths* used?
   - ☐ yes
   - ☐ no

4. If so, describe the following *details*:

During which months do you use them?	
In this period, what is the frequency of use?	every ______ weeks
What formulations (or combinations thereof) are used? Include approximate concentrations.	
How many times do you use the dipping solution before refeshing it?	
What procedures are followed?	
On which animals is footbathing performed?	

5. What is the *farm strategy regarding foot trimming?*

Which animals?	Which feet?	When?
	☐ all feet ☐ back feet only ☐ affected feet only	☐ routinely: specify ________________ ☐ irregularly: specify _______________ ☐ incidentally, whenever problems present themselves

6. *Who* trims the feet of cattle on the farm? (tick all that apply)
   - ☐ farmer ________ % of total; formally trained? yes / no
   - ☐ stockman/stockmen ________ % of total; formally trained? yes / no
   - ☐ certified claw trimmer ________ % of total
   - ☐ vet ________ % of total

Digital dermatitis on the farm level

7. Which *year* was DD first observed on this farm? __________

8. Since this time, which of the following statements best describes the *farm history* of DD infection:

- ☐ decreased incidence
- ☐ more or less stable
- ☐ gradual increase in incidence
- ☐ sharp increase in incidence: specify year(s) and season(s) if possible

____

____

9. Please describe the *seasonal variation / patterns* in the occurrence of DD:

- ☐ worst during housing period
- ☐ more or less even all year round
- ☐ worst during grazing period

10. In which *management groups* does DD occur and in which is it most common? First, assign a score of N to groups in which it has <u>not</u> been seen, and then rank the other groups in order from most common (1) to less common (2 and upwards):

- — unweaned calves
- — weaned calves
- — bulling heifers
- — in calf heifers
- — first lactation cows
- — second and higher lactation cows
- — dry cows

11. At *what stage of lesion development* is individual treatment for DD carried out? **[treat as OPEN QUESTION]**

- ☐ immediately when a small lesion is observed or possible onset of DD is suspected
- ☐ when the DD lesion is easily visible, bleeds and is painful
- ☐ when the DD lesion has become a large, wart-like growth
- ☐ never
- ☐ other: specify

____

12. *Where and when* are individual treatments for DD carried out?

- ☐ in the parlour, when DD lesions (regardless of size and appearance) are observed during milking
- ☐ in the crush, when DD lesions (regardless of size and appearance) are observed after foot trimming

14. What are the *routine prevention measures* for DD? (tick all that apply) **[treat as OPEN QUESTION]**

- ☐ regular functional trimming and treatment
- ☐ closed farm concept: no purchase of replacement stock or use of hired bulls
- ☐ vigilance: special attention to recognition and prompt treatment (e.g. in parlour)
- ☐ cow comfort in the housing environment (deep bedding, correct cubicle dimensions etc.)
- ☐ balanced / appropriate nutrition, e.g. in early lactation
- ☐ breeding: attention to foot conformation
- ☐ other: specify

____

____

____

____

____

15. What are the *routine control measures* for DD? (tick all that apply) **[treat as OPEN QUESTION]**

- ☐ footbaths (see above for details)
- ☐ vigilance: special attention to recognition and prompt treatment (e.g. in parlour)
- ☐ hygiene in the housing environment
- ☐ avoidance of contact between management groups and extra care to regrouping and rehousing
- ☐ other: specify

____

____

____

____

Management group level variables

For each management group, a ***limited set of similar questions*** will be asked to clarify basic management procedures. This will be supplemented by information gathered during the inspection of the housing and animals.

Unweaned calves and weaned calves

	Unweaned calves	**Weaned calves**
Total number on farm at present		
Housing system(s) used	☐ individual pens ☐ group pens	☐ individual pens ☐ group pens
Frequency of bedding down	____ times per day / week	____ times per day / week
Frequency of completely cleaning out bedding	☐ ____ times per week / month ☐ after each group ☐ never: dissipates spontaneously ☐ other: specify _________________	☐ ____ times per week / month ☐ after each group ☐ never: dissipates spontaneously ☐ other: specify _________________
Frequency of use of bedding additives, e.g. lime	☐ never ☐ ____ times per week / month ☐ between groups	☐ never ☐ ____ times per week / month ☐ between groups
Frequency of steam cleaning / disinfection	☐ after each group ☐ average interval of ______ months ☐ never	☐ after each group ☐ average interval of ______ months ☐ never
Criteria for moving on to the next management group	***weaning:*** ☐ age: ________ weeks ☐ body size / weight ☐ forage intake ☐ concentrate intake ☐ convenience, e.g. to make room in housing	***moving on to heifers group:*** ☐ weight: ________ kg ☐ age: ________ months ☐ other: ____________ ____________
If not by age, at what average age does this take place?	_______ weeks	________ months

Observations:
____
____
____
____
____

Bulling heifers and pregnant heifers

	Bulling heifers	Pregnant heifers
Total number on farm at present		
Housing system(s) used	☐ individual pens ☐ group pens / yards ☐ cubicles	☐ individual pens ☐ group pens / yards ☐ cubicles
Frequency of bedding down	____ times per week	____ times per week
Frequency of completely cleaning out bedding	☐ ____ times per week / month ☐ after each group ☐ never: dissipates spontaneously ☐ other: specify __________________	☐ ____ times per week / month ☐ after each group ☐ never: dissipates spontaneously ☐ other: specify __________________
Frequency of use of bedding additives, e.g. lime	☐ never ☐ ____ times per week / month ☐ between groups	☐ never ☐ ____ times per week / month ☐ between groups
Frequency of steam cleaning / disinfection	☐ after each group ☐ average interval of ______ months ☐ never	☐ after each group ☐ average interval of ______ months ☐ never
Criteria for moving on to the next management group	***moving on to pregnant heifers group:*** ☐ non return / positive pregnancy diagnosis ☐ age: ________ months ☐ other: _________________________	
If not by age, at what average age does this take place?	_______ months	***average age of delivery of first calf:*** ________ months

Observations:
____
____
____
____
____
____
____
____

Lactating cows and dry cows

	Lactating cows	Dry cows
Total number on farm at present		
Housing system(s) used	☐ cubicles ☐ straw yards	☐ cubicles ☐ straw yards
Frequency of bedding down	____ times per week	____ times per week
Frequency of completely cleaning out bedding	☐ ____ times per week / month ☐ after each group ☐ never: dissipates spontaneously ☐ other: specify __________________	☐ ____ times per week / month ☐ after each group ☐ never: dissipates spontaneously ☐ other: specify __________________
Frequency of use of bedding additives, e.g. lime	☐ never ☐ ____ times per week / month ☐ between groups	☐ never ☐ ____ times per week / month ☐ between groups
Frequency of steam cleaning / disinfection	☐ after each group ☐ average interval of ______ months ☐ never	☐ after each group ☐ average interval of ______ months ☐ never
Criteria for moving on to the next management group	***moving on to dry cows group:*** ☐ lactation stage: ______ D.I.M. ☐ expected calving date ☐ other: _________________________ _	
If not by lactation stage, what is the average duration of lactation?	_______ days	

Observations:

____

____

____

____

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E.1.2 Housing inspection form

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Housing inspection

Notes

The different types of housing have been numbered as follows:

1. Individual pens
2. Group pens
3. Yards / barns
4. Cubicle housing
5. Calving area

The housing types on the farm have been further subdivided in sections. Sections are lettered (A, B, ...). A section should be considered to be a collection of units of the types mentioned above, in which the conditions may be assumed to be identical. For instance, a row of individual pens on the same side of a corridor. A row of pens on the other side of the corridor could be given a different section letter.

For each type of housing, the management group inhabiting it and the section should be completed. There should be no duplicates on one farm.

The general housing layout should be sketched out, and the housing types and sections clearly marked.

1. Individual pens

Section		
Management group	☐ unweaned calves ☐ weaned calves	☐ unweaned calves ☐ weaned calves
Number of pens in section		
Dimensions	____ cm x ____ cm x ____ cm	____ cm x ____ cm x ____ cm
Floor type	☐ wooden slats ☐ concrete ☐ other: specify __________________	☐ wooden slats ☐ concrete ☐ other: specify __________________
Floor consistency	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
Bedding material	☐ straw ☐ sawdust ☐ other: _________________________ ☐ none	☐ straw ☐ sawdust ☐ other: _________________________ ☐ none
Hygiene score of housing (tick one from each)	☐ 1: no or very few faecal deposits ☐ 2: moderate amount of soiling ☐ 3: very soiled with faeces / water	☐ 1: no or very few faecal deposits ☐ 2: moderate amount of soiling ☐ 3: very soiled with faeces / water
Faecal consistency (score 4 pats from different areas / pens)	Scoring: 1: very dry / lumpy 2: stiff / semi formed pats 3: circular / moist / symmetrical 4: loose / thinly spread 5: liquid pools with undigested particles	Scoring: 1: very dry / lumpy 2: stiff / semi formed pats 3: circular / moist / symmetrical 4: loose / thinly spread 5: liquid pools with undigested particles
	Pat A ____ Pat C ____ Pat B ____ Pat D ____	Pat A ____ Pat C ____ Pat B ____ Pat D ____
Is there any direct contact between this and other sections?	☐ yes: specify section: ________ ☐ no	☐ yes: specify section: ________ ☐ no
Observations		

3. Yards / barns

Section		
Management group	☐ bulling heifers ☐ pregnant heifers ☐ lactating cows ☐ dry cows	☐ bulling heifers ☐ pregnant heifers ☐ lactating cows ☐ dry cows
Number of yards / barns in section		
Group size per yard / barn		
Dimensions	_____ m x ____ m	_____ m x ____ m
Floor type	☐ solid concrete ☐ concrete slats ☐ other: specify __________________	☐ solid concrete ☐ concrete slats ☐ other: specify __________________
Floor consistency	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
Bedding material	☐ chopped straw ☐ big bale straw ☐ sawdust ☐ sand ☐ paper ☐ mats	☐ chopped straw ☐ big bale straw ☐ sawdust ☐ sand ☐ paper ☐ mats
Hygiene score of housing (tick one from each)	Lying area ☐ 1: no or very few faecal deposits ☐ 2: moderate amount of soiling ☐ 3: very soiled with faeces / water	Lying area ☐ 1: no or very few faecal deposits ☐ 2: moderate amount of soiling ☐ 3: very soiled with faeces / water
	Feeding and watering area ☐ 1: no or very few faecal deposits ☐ 2: moderate amount of soiling ☐ 3: very soiled with faeces / water	Feeding and watering area ☐ 1: no or very few faecal deposits ☐ 2: moderate amount of soiling ☐ 3: very soiled with faeces / water
Faecal consistency (score 4 pats from different areas / yards / barns	Scoring: 1: very dry / lumpy 2: stiff / semi formed pats 3: circular / moist / symmetrical 4: loose / thinly spread 5: liquid pools with undigested particles	Scoring: 1: very dry / lumpy 2: stiff / semi formed pats 3: circular / moist / symmetrical 4: loose / thinly spread 5: liquid pools with undigested particles
	Pat A ____ Pat C ____ Pat B ____ Pat D ____	Pat A ____ Pat C ____ Pat B ____ Pat D ____
Is there any direct contact between this and other sections?	☐ yes: specify section: ________ ☐ no	☐ yes: specify section: ________ ☐ no

4. Cubicle housing

Section		
Management group	☐ pregnant heifers ☐ lactating cows ☐ dry cows	☐ pregnant heifers ☐ lactating cows ☐ dry cows
Total number of cubicles in section		
Herd / group size		
Dimensions of cubicles	_____ cm x ____ cm x ____ cm	_____ cm x ____ cm x ____ cm
Floor type	☐ solid concrete ☐ concrete slats ☐ other: specify __________________	☐ solid concrete ☐ concrete slats ☐ other: specify __________________
Floor consistency	Floor surface in cubicle housing:	Floor surface in cubicle housing:
	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
	Floor surface in passageways:	Floor surface in passageways:
	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
Frequency of removing slurry from the floor	☐ ________ times per day ☐ other: specify __________________	☐ ________ times per day ☐ other: specify __________________
Method of removing slurry from the floor	☐ automatic scraper ☐ tractor scraper ☐ other: specify __________________ ___________________________________	☐ automatic scraper ☐ tractor scraper ☐ other: specify __________________ ___________________________________
Do pools of slurry collect that are not cleared?	☐ yes ☐ no	☐ yes ☐ no
Do cows have to pass through these regularly?	☐ yes ☐ no	☐ yes ☐ no
Is slurry spread between groups via the scrapers / removal?	☐ yes ☐ no	☐ yes ☐ no
Bedding material	☐ straw ☐ sawdust ☐ sand ☐ paper ☐ mats ☐ cow mattresses ☐ none	☐ straw ☐ sawdust ☐ sand ☐ paper ☐ mats ☐ cow mattresses ☐ none

4. Cubicle housing

Section		
Management group	☐ pregnant heifers ☐ lactating cows ☐ dry cows	☐ pregnant heifers ☐ lactating cows ☐ dry cows
Total number of cubicles in section		
Herd / group size		
Dimensions of cubicles	_____ cm x ____ cm x ____ cm	_____ cm x ____ cm x ____ cm
Floor type	☐ solid concrete ☐ concrete slats ☐ other: specify ___________________	☐ solid concrete ☐ concrete slats ☐ other: specify ___________________
Floor consistency	Floor surface in cubicle housing:	Floor surface in cubicle housing:
	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
	Floor surface in passageways:	Floor surface in passageways:
	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
Frequency of removing slurry from the floor	☐ ________ times per day ☐ other: specify ___________________	☐ ________ times per day ☐ other: specify ___________________
Method of removing slurry from the floor	☐ automatic scraper ☐ tractor scraper ☐ other: specify ___________________ ____________________________________	☐ automatic scraper ☐ tractor scraper ☐ other: specify ___________________ ____________________________________
Do pools of slurry collect that are not cleared?	☐ yes ☐ no	☐ yes ☐ no
Do cows have to pass through these regularly?	☐ yes ☐ no	☐ yes ☐ no
Is slurry spread between groups via the scrapers / removal?	☐ yes ☐ no	☐ yes ☐ no
Bedding material	☐ straw ☐ sawdust ☐ sand ☐ paper ☐ mats ☐ cow mattresses ☐ none	☐ straw ☐ sawdust ☐ sand ☐ paper ☐ mats ☐ cow mattresses ☐ none

Section		
Management group	☐ pregnant heifers ☐ lactating cows ☐ dry cows	☐ pregnant heifers ☐ lactating cows ☐ dry cows
Total number of cubicles in section		
Herd / group size		
Dimensions of cubicles	_____ cm x ____ cm x ____ cm	_____ cm x ____ cm x ____ cm
Floor type	☐ solid concrete ☐ concrete slats ☐ other: specify ___________________	☐ solid concrete ☐ concrete slats ☐ other: specify ___________________
Floor consistency	Floor surface in cubicle housing:	Floor surface in cubicle housing:
	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
	Floor surface in passageways:	Floor surface in passageways:
	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined	☐ rough / course ☐ sufficient traction ☐ smooth / slippery ☐ broken up ☐ can't be determined
Frequency of removing slurry from the floor	☐ ________ times per day ☐ other: specify ___________________	☐ ________ times per day ☐ other: specify ___________________
Method of removing slurry from the floor	☐ automatic scraper ☐ tractor scraper ☐ other: specify ___________________ ____________________________________	☐ automatic scraper ☐ tractor scraper ☐ other: specify ___________________ ____________________________________
Do pools of slurry collect that are not cleared?	☐ yes ☐ no	☐ yes ☐ no
Do cows have to pass through these regularly?	☐ yes ☐ no	☐ yes ☐ no
Is slurry spread between groups via the scrapers / removal?	☐ yes ☐ no	☐ yes ☐ no
Bedding material	☐ straw ☐ sawdust ☐ sand ☐ paper ☐ mats ☐ cow mattresses ☐ none	☐ straw ☐ sawdust ☐ sand ☐ paper ☐ mats ☐ cow mattresses ☐ none

E.1.3 Individual animal record form

Animal details and production record Sample code: ____

Sampling date: / / 200	Management group:	
Farm code:	☐ unweaned calves	☐ pregnant heifers
Animal code: ______________	☐ weaned calves	☐ lactating cows
Animal ID number (eartag): ____________________	☐ bulling heifers	☐ dry cows
Date of birth: ____ / ____ / _______	Breed:	
	☑ HF	☐ HF - cross
Parity: ____	☐ Other: specify _____________________________	

Left hindquarter (diagram labels: A, B, C, D)

	Body hygiene score			
	1	2	3	4
A: flank	☐	☐	☐	☐
B: hindleg	☐	☐	☐	☐
C: udder	☐	☐	☐	☐
D: tail	☐	☐	☐	☐

Right hindquatrer (diagram labels: A, B, C, D)

	Body hygiene score			
	1	2	3	4
A: flank	☐	☐	☐	☐
B: hindleg	☐	☐	☐	☐
C: udder	☐	☐	☐	☐
D: tail	☐	☐	☐	☐

	Right hind	Left hind	Right fore	Left fore
Foot hygiene score	☐ 1 ☐ 2 ☐ 3 ☐ 4	☐ 1 ☐ 2 ☐ 3 ☐ 4	☐ 1 ☐ 2 ☐ 3 ☐ 4	☐ 1 ☐ 2 ☐ 3 ☐ 4

E.2 Longitudinal study

E.2.1 Individual animal record form

Animal identifcation	Sample code:	

Sampling date:	____ / ____ / 2005	Management group:	
Farm code:	____	☐ unweaned calves	☐ pregnant heifers
Animal code:	________________	☐ weaned calves	☐ lactating cows
Animal ID number (eartag):	______________________	☐ bulling heifers	☐ dry cows

Left hindquarter — Body hygiene score

	1	2	3	4
A: flank	☐	☐	☐	☐
B: hindleg	☐	☐	☐	☐
C: udder	☐	☐	☐	☐
D: tail	☐	☐	☐	☐

Right hindquatrer — Body hygiene score

	1	2	3	4
A: flank	☐	☐	☐	☐
B: hindleg	☐	☐	☐	☐
C: udder	☐	☐	☐	☐
D: tail	☐	☐	☐	☐

	Left hind	Right hind	Left fore	Right fore
Foot hygiene score	☐ 1: clean ☐ 2: superficial fresh soiling ☐ 3: encrusted soiling ☐ 4: deeply encrusted	☐ 1: clean ☐ 2: superficial fresh soiling ☐ 3: encrusted soiling ☐ 4: deeply encrusted	☐ 1: clean ☐ 2: superficial fresh soiling ☐ 3: encrusted soiling ☐ 4: deeply encrusted	☐ 1: clean ☐ 2: superficial fresh soiling ☐ 3: encrusted soiling ☐ 4: deeply encrusted
Toe length	☐ 1: short ☐ 2: average ☐ 3: long	☐ 1: short ☐ 2: average ☐ 3: long	☐ 1: short ☐ 2: average ☐ 3: long	☐ 1: short ☐ 2: average ☐ 3: long
Toe angle	☐ 1: steep ☐ 2: average ☐ 3: shallow	☐ 1: steep ☐ 2: average ☐ 3: shallow	☐ 1: steep ☐ 2: average ☐ 3: shallow	☐ 1: steep ☐ 2: average ☐ 3: shallow
Claw symmetry	☐ 1: augmented lateral ☐ 2: symmetrical ☐ 3: augmented medial	☐ 1: augmented lateral ☐ 2: symmetrical ☐ 3: augmented medial	☐ 1: augmented lateral ☐ 2: symmetrical ☐ 3: augmented medial	☐ 1: augmented lateral ☐ 2: symmetrical ☐ 3: augmented medial
Heel height	☐ 1: high ☐ 2: average ☐ 3: low	☐ 1: high ☐ 2: average ☐ 3: low	☐ 1: high ☐ 2: average ☐ 3: low	☐ 1: high ☐ 2: average ☐ 3: low
Stance	☐ 1: symmetrical ☐ 2: base-wide			
Observations				

	DD inspection VISUAL			
DD lesion diameter (widest part)	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm
DD lesion aspect	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing
DD lesion location	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft

	Left hind	Right hind	Left fore	Right fore
DD inspection FEET LIFTED	☐ routine ☐ suspect lesion	☐ routine ☐ suspect lesion	☐ routine ☐ suspect lesion	☐ routine ☐ suspect lesion
DD lesion diameter (widest part)	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm	☐ 0: no lesion ☐ 1: < 2 cm ☐ 2: 2 – 3 cm ☐ 3: 3 – 4 cm ☐ 4: > 4 cm
DD lesion aspect	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing	☐ 1: ulcerative / exsudative ☐ 2: mixed ☐ 3: granulomatous ☐ 4: proliferative ☐ 5: scabbed / regressing
DD lesion location	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft ☐ 5: on coronary band ☐ 6: confluent:__________ __________________	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft ☐ 5: on coronary band ☐ 6: confluent:__________ __________________	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft ☐ 5: on coronary band ☐ 6: confluent:__________ __________________	☐ 1: midway between heels ☐ 2: above outer heel bulb ☐ 3: above inner heel bulb ☐ 4: interdigital cleft ☐ 5: on coronary band ☐ 6: confluent:__________ __________________
Concurrent conditions	☐ interdigital dermatitis ☐ heel horn erosion ☐ interdigital phlegmon ☐ tyloma ☐ sole ulcer ☐ solar hemorrhage ☐ white line defect ☐ other ______________ __________________	☐ interdigital dermatitis ☐ heel horn erosion ☐ interdigital phlegmon ☐ tyloma ☐ sole ulcer ☐ solar hemorrhage ☐ white line defect ☐ other ______________ __________________	☐ interdigital dermatitis ☐ heel horn erosion ☐ interdigital phlegmon ☐ tyloma ☐ sole ulcer ☐ solar hemorrhage ☐ white line defect ☐ other ______________ __________________	☐ interdigital dermatitis ☐ heel horn erosion ☐ interdigital phlegmon ☐ tyloma ☐ sole ulcer ☐ solar hemorrhage ☐ white line defect ☐ other ______________ __________________
Observations				

Appendix F

Serological distributions of the cross-sectional study

F.1 Frequency histograms

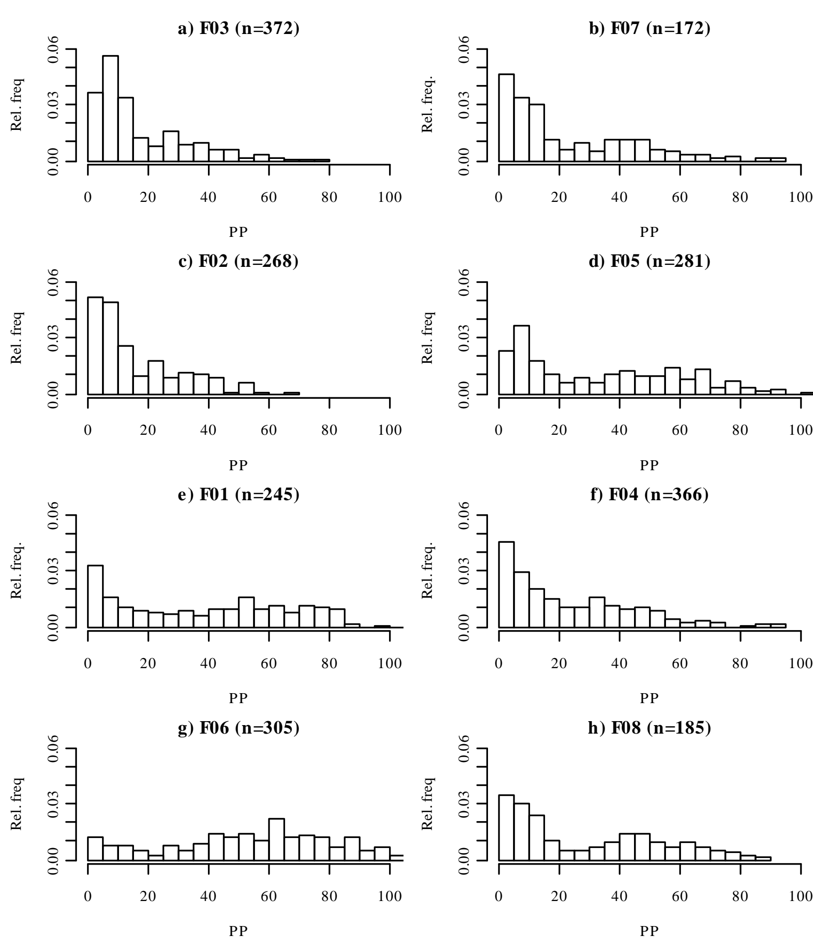


Figure F.1: Relative frequency histograms of the ELISA on eight study farms, grouped by clinical BDD prevalence: a-b) low $< 20\%$; c-d) medium 20 - 40%; e-h) high $>40\%$

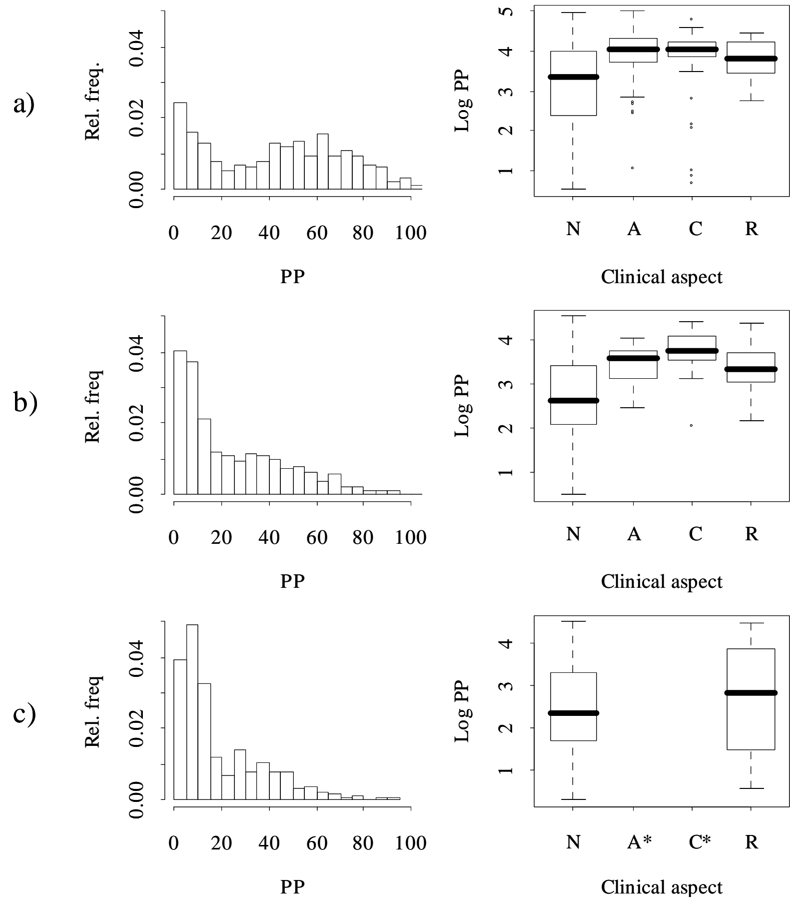


Figure F.2: Relative frequency histograms of the ELISA and boxplots of lesion status, stratified into high (a), medium (b) and low (c) clinical BDD prevalence farms. N: negative; A: acute; C: chronic; R: regressing. * no A and C lesions observed

F.2 Q-Q plots

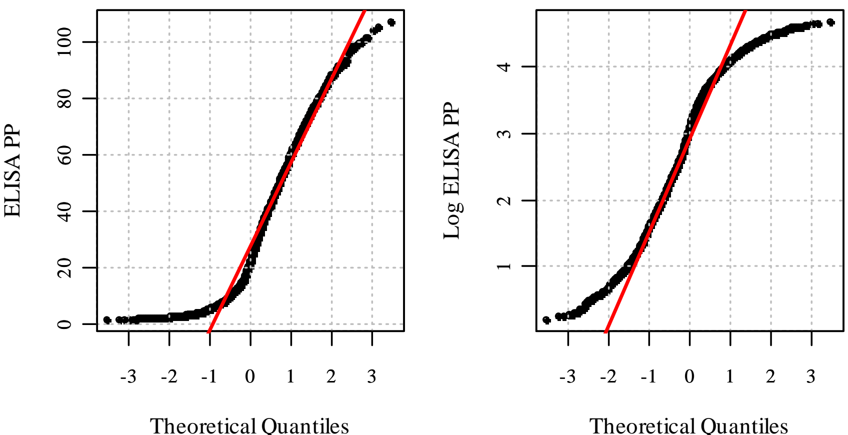


Figure F.3: Whole dataset (n=2204): non-transformed (Shapiro Wilk's statistic W = 0.89, $p < 0.001$) and log transformed (Shapiro Wilk's statistic W = 0.95, $p < 0.001$)

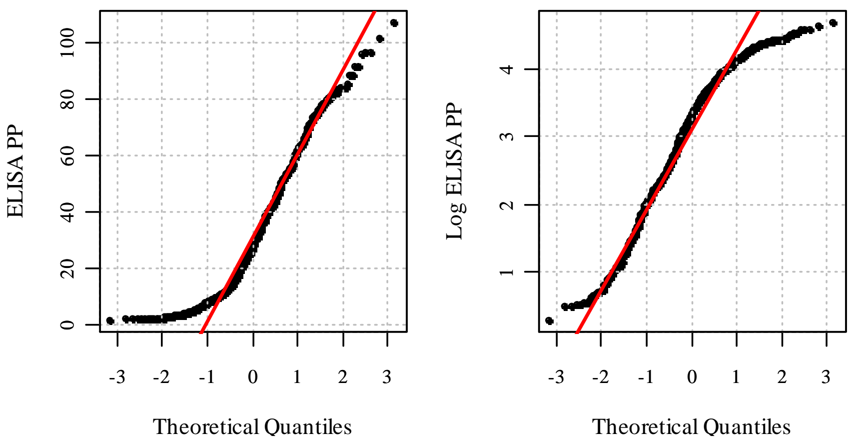


Figure F.4: Clinically inspected subset (n=609): non-transformed (Shapiro Wilk's statistic W = 0.91, $p < 0.001$) and log transformed (Shapiro Wilk's statistic W = 0.95, $p < 0.001$)

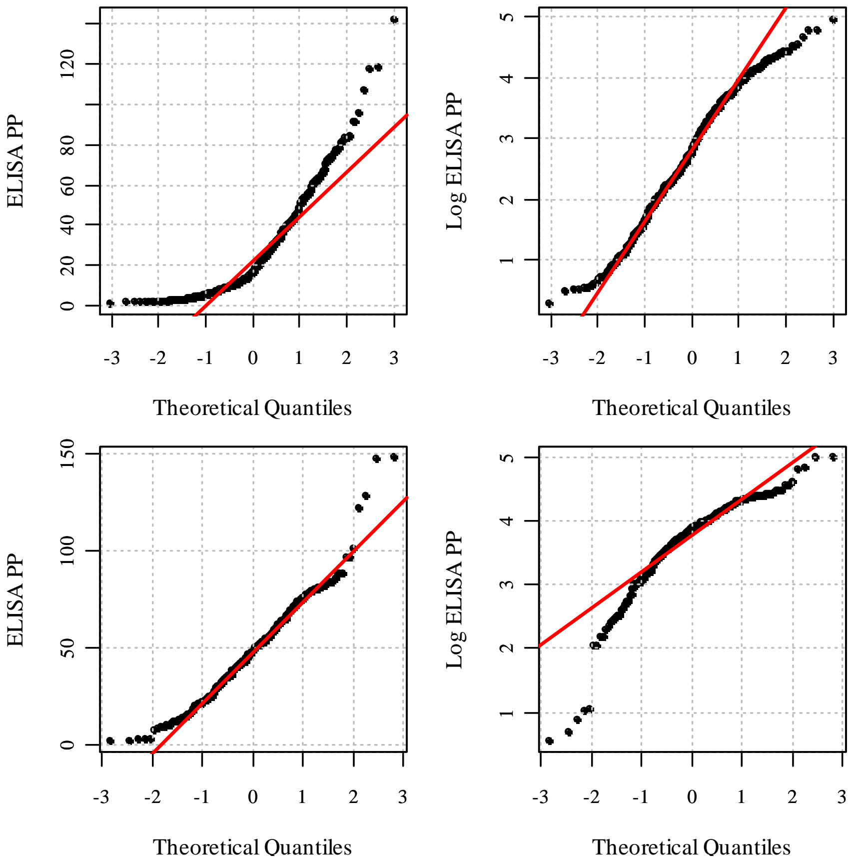


Figure F.5: Clinical negatives (n=398) (top): non-transformed (Shapiro Wilk's statistic W = 0.85, $p < 0.001$) and log transformed (Shapiro Wilk's statistic W = 0.98, $p < 0.001$) Clinical positives (n=211) (bottom): non-transformed (Shapiro Wilk's statistic W = 0.97, $p < 0.001$) and log transformed (Shapiro Wilk's statistic W = 0.87, $p < 0.001$)

	Shapiro – Wilk normality test					
	Non transformed			Log transformed		
Whole dataset	W =	0.89, p =	< 0.001	W =	0.95, p =	< 0.001
All clinical inspected	W =	0.91, p =	< 0.001	W =	0.95, p =	< 0.001
Clinical positive	W =	0.85, p =	< 0.001	W =	0.98, p =	< 0.001
Clinical negative	W =	0.97, p =	0.008	W =	0.87, p =	< 0.001

Table F.1: Shapiro – Wilk normality test of serological distributions of the study population, and subsets thereof

F.3 Lactation data subset

Serological frequency distributions show little difference between all cows and the subgroup for which parity and lactation stage data were obtained.

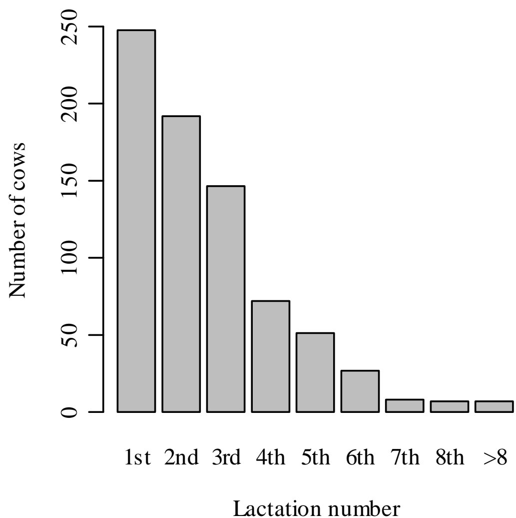


Figure F.6: Lactation numbers of 761 cows included in the study

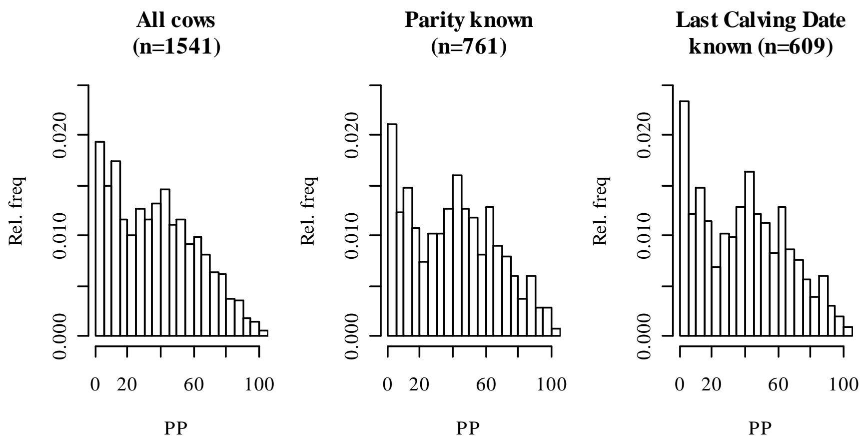


Figure F.7: Frequency distributions of sub-populations of cows

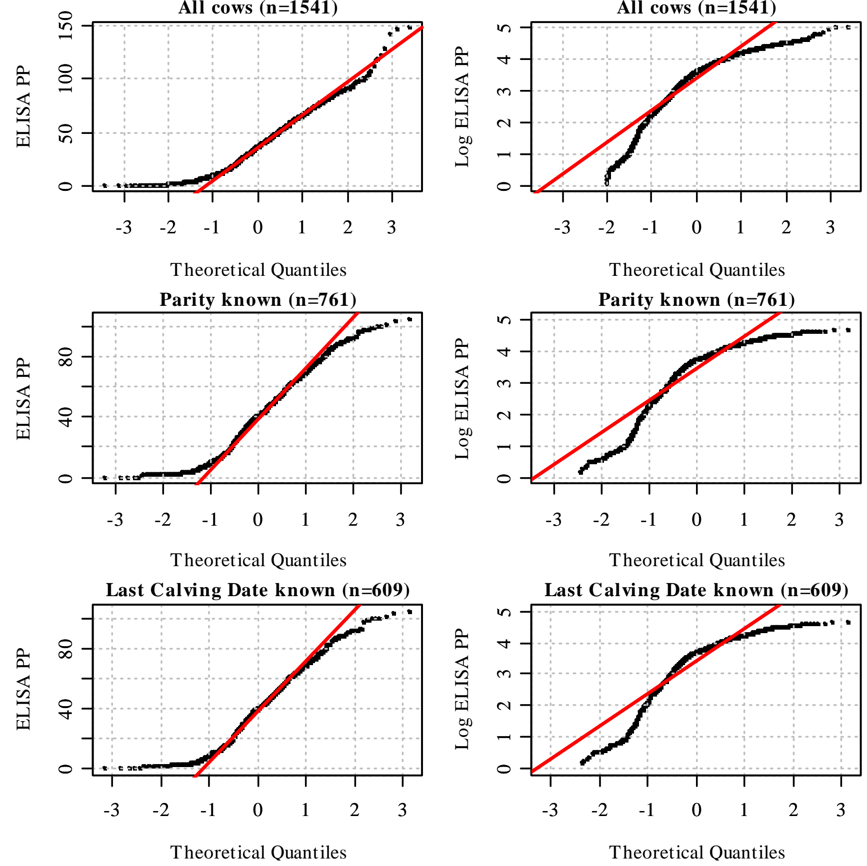


Figure F.8: Q-Q plots of sub-populations of cows

	Shapiro – Wilk normality test					
	Non transformed			Log transformed		
All cows in dataset	W =	0.96, p =	$< 2.2e^{-16}$	W =	0.88, p =	$< 2.2e^{-16}$
Parity	W =	0.96, p =	$< 1.6e^{-13}$	W =	0.87, p =	$< 2.2e^{-16}$
Lactation stage	W =	0.86, p =	$< 3.5e^{-12}$	W =	0.87, p =	$< 2.2e^{-16}$

Table F.2: Shapiro – Wilk normality test of serological distributions of cows in the study population, and stratified by parity and lactation stage

Appendix G

Outputs of univariable statistical models of the cross-sectional study

G.1 Binary outcome: BDD lesion status

Analysis was performed using the R statistical software package (available from http://www.r-project.org/, version 2.3.1.

Several useful references on implementing mixed-effects models in R are available (Verzilli, 2003; Bates and Pinheiro, 2000; Venables and Ripley, 2002). The TINN editor (http://www.sciviews.org/Tinn-R/) was used to interface with R.

The generalized linear models (GLMs) were fitted using the `glm` function from the `stats` package; generalized linear mixed models (GLMMs) were fitted with `lmer` from the `lme4` package (Bates, 2005).

Generalized linear model (GLM)

Each available covariate in sequence was added into a generalized linear model with clinical BDD lesion status as binary outcome variable. Assuming clustering in the data at the farm level, a correction is made by including the farm identifier as a fixed effect; the fixed effects analysis effectively estimates a separate parameter for every herd, and thus separates between-herd variation from the residual variation (Dohoo et al., 2003). The univariable models output is listed in the table below.

Variable		Coefficient	SE	OR	95% CI	*P*-value
Foot hygiene score	1	Referent				
	2	0.93	0.35	2.52	1.27 – 5.02	< 0.01
	3	1.20	0.31	3.31	1.79 – 6.13	< 0.001
	4	1.29	0.32	3.62	1.92 – 6.83	< 0.001
Body hygiene score	1	Referent				
	2	1.90	0.57	6.70	2.21 – 20.28	< 0.001
	3	2.45	0.56	11.61	3.84 – 35.10	< 0.001
	4	2.64	0.61	13.97	4.24 – 45.99	< 0.001
Age (quadratic)	Age.cent	0.37	0.08	1.45	1.25 – 1.68	< 0.01
	Age.centsq	-0.09	0.02	0.91	0.89 – 0.94	< 0.01
Age (categorical)	0-2y	Referent				
	>2-3y	2.23	0.77	9.29	2.07 – 41.70	< 0.01
	>3-4y	3.29	0.76	26.93	6.02 – 120.41	< 0.001
	>4-5y	3.16	0.77	23.50	5.24 – 105.37	< 0.001
	>5-6y	2.96	0.78	19.26	4.22 – 87.92	< 0.001
	> 6y	2.34	0.76	10.34	2.33 – 45.98	< 0.01
Management group	YS	Referent				
	LC	2.84	0.49	17.09	6.53 – 44.70	< 0.001
	DC	1.86	0.58	6.43	2.07 – 19.91	< 0.01
Housing hygiene	1	Referent				
	2	1.92	0.48	6.84	2.65 – 17.67	< 0.001
	3	2.79	0.7	16.21	4.12 – 63.71	< 0.001
	4	1.45	0.48	4.26	1.67 – 10.87	< 0.01
Housing comfort	1	Referent				
	2	0.8	0.35	0.45	0.22 – 0.90	0.02
Footbaths	0	Referent				
	1	2.99	0.79	19.94	4.28 – 92.83	< 0.001
	2	2.61	0.76	13.59	3.09 – 59.83	< 0.001
BDD treatment	0	Referent				
	1	2.41	0.37	11.16	4.63 – 26.92	< 0.001
	2	1.35	0.54	3.84	1.88 – 7.86	< 0.001
Parity	1	Referent				

	2	1.41	0.54	4.10	1.42 – 11.79	< 0.01
	3	0.27	0.61	1.31	0.40 – 4.32	0.65
	$\geq$4	0.58	0.57	1.78	0.59 – 5.40	0.31
Lactation stage (quadratic)	DIM.cent	0.00	0.00	1.00	1.00 – 1.01	0.06
	DIM.centsq	0.00	0.00	1.00	1.00 – 1.00	0.27
Lactation stage (categorical)	0-50d	Referent				
	51-100d	0.63	0.86	0.53	0.10 – 2.88	0.47
	101-150d	0.42	0.82	0.66	0.13 – 3.24	0.61
	151-225d	0.73	0.77	2.08	0.46 – 9.43	0.34
	226-300d	-1.24	0.91	0.29	0.05 – 1.70	0.17
	$\geq$ 301d	1.70	0.83	5.45	1.08 – 27.61	0.04
	dry	0.85	0.81	0.43	0.09 – 2.11	0.30

Table G.1: Univariable relationships between explanatory variables and clinical BDD as binary outcome variable, determined by inclusion of each variable in a generalized linear model (GLM). YS: young stock; LC: lactating cows; DC: dry cows

Generalized linear mixed model (GLMM) with farm as random effect

The GLM was extended to a mixed effects model, specifying farm as the random effect. Each available covariate in sequence was added as the fixed effect into this generalized linear mixed model (GLMM). For estimation of the coefficient, preference was given to the Laplace (ML) approximation, rather than a penalized quasi likelihood (PQL) based method, as it is considered more accurate. The univariable models output is listed in the table below.

Variable		Coefficient	SE	OR	95% CI	*P*-value
Foot hygiene score	1	Referent				
	2	0.96	0.35	2.61	1.32 – 5.17	< 0.01
	3	1.22	0.31	3.39	1.84 – 6.24	< 0.001
	4	1.35	0.32	3.85	2.05 – 7.23	< 0.001
Body hygiene score	1	Referent				
	2	1.93	0.57	6.88	2.27 – 20.83	< 0.001
	3	2.48	0.56	11.92	3.94 – 36.05	< 0.001
	4	2.64	0.61	13.98	4.27 – 45.83	< 0.001
Age (quadratic)	Age.cent	0.37	0.09	1.45	1.22 – 1.72	< 0.01
	Age.centsq	-0.09	0.02	0.91	0.87 – 0.96	< 0.01
Age (categorical)	0-2y	Referent				
	>2-3y	2.25	0.77	9.47	2.08 – 43.18	< 0.01
	>3-4y	3.30	0.77	27.05	5.97 – 122.69	< 0.01
	>4-5y	3.18	0.77	23.94	5.26 – 108.95	< 0.01
	>5-6y	2.98	0.78	19.68	4.25 – 91.17	< 0.01
	>6y	2.36	0.77	10.58	2.35 – 47.76	< 0.01
Management group	YS	Referent				
	LC	2.82	0.49	16.81	6.42 – 43.98	< 0.01
	DC	1.86	0.58	6.39	2.07 – 19.77	< 0.01
Housing hygiene	1	Referent				
	2	1.85	0.46	6.33	2.58 – 15.56	< 0.01
	3	2.47	0.57	11.84	3.89 – 36.05	< 0.01
	4	1.40	0.46	4.06	1.65 – 10.00	< 0.01
Housing comfort	1	Referent				
	2	-0.94	0.34	0.39	0.20 – 0.76	< 0.01
Footbaths	0	Referent				
	1	3.11	0.65	22.36	6.31 – 79.25	< 0.01
	2	2.89	0.64	17.96	5.16 – 62.56	< 0.01
BDD treatment	0	Referent				
	1	2.30	0.40	9.94	4.51 – 21.89	< 0.01
	2	1.53	0.36	4.61	2.27 – 9.36	< 0.01
Parity	1	Referent				

	2	1.42	0.52	4.14	1.49 – 11.48	< 0.01
	3	0.34	0.60	1.40	0.43 – 4.55	0.58
	$\geq$ 4	0.59	0.55	1.80	0.61 – 5.29	0.28
LS (quadratic)	DIM.cent	0.00	0.00	1.00	1.00 – 1.01	0.05
	DIM.centsq	0.00	0.00	1.00	1.00 – 1.00	0.27
LS (categorical)	0-50d	Referent				
	51-100d	-0.60	0.84	0.55	0.11 – 2.85	0.47
	101-150d	-0.41	0.80	0.66	0.14 – 3.17	0.61
	151-225d	0.75	0.75	2.12	0.49 – 9.22	0.32
	226-300d	-1.20	0.89	0.30	0.05 – 1.73	0.18
	$\geq$ 301d	1.70	0.80	5.48	1.15 – 26.15	0.03
	dry	-0.79	0.77	0.46	0.10 – 2.07	0.31

Table G.2: Univariable relationships between explanatory variables and clinical BDD as binary outcome variable, determined by inclusion of each variable in a generalized linear mixed model (GLMM) specifying farm as random effect, using the LaPlace ML algorithm. YS: young stock; LC: lactating cows; DC: dry cows

Generalized linear mixed model (GLMM) with management group as random effect

For further comparison between models, the random effects term was changed to management group, and the univariable analyses were repeated. Note that the management group level variables cannot now be included as fixed effects, as these are already accounted for by the random effects.

Variable		Coefficient	SE	OR	95% CI	P-value
Foot hygiene score	1	Referent				
	2	0.81	0.35	2.24	1.13 – 4.44	0.02
	3	0.65	0.31	1.92	1.04 – 3.53	0.04
	4	0.63	0.32	1.87	1.00 – 3.50	0.05
Body hygiene score	1	Referent				
	2	1.49	0.58	4.43	1.43 – 13.69	< 0.01
	3	1.59	0.57	4.90	1.59 – 15.11	< 0.01
	4	1.34	0.60	3.81	1.17 – 12.40	0.03
Age (quadratic)	Age.cent	0.24	0.08	1.27	1.09 – 1.49	< 0.01
	Age.centsq	-0.06	0.02	0.94	0.91 – 0.97	< 0.01
Age (categorical)	0-2y	Referent				
	>2-3y	2.16	0.83	8.63	1.71 – 43.65	< 0.01
	>3-4y	2.92	0.84	18.49	3.56 – 96.14	< 0.01
	>4-5y	2.87	0.84	17.63	3.39 – 91.72	< 0.01
	>5-6y	2.75	0.85	15.59	2.95 – 82.50	< 0.01
	> 6y	2.14	0.84	8.49	1.64 – 43.83	0.01
Parity	1	Referent				
	2	1.34	0.47	3.80	1.51 – 9.55	< 0.01
	3	0.59	0.57	1.81	0.59 – 5.53	0.30
	≥ 4	0.51	0.50	1.67	0.63 – 4.43	0.30
LS (quadratic)	DIM.cent	0.00	0.00	1.00	1.00 – 1.01	0.02
	DIM.centsq	0.00	0.00	1.00	1.00 – 1.00	0.30
LS (categorical)	0-50d	Referent				
	51-100d	-0.41	0.76	0.67	0.15 – 2.94	0.59
	101-150d	-0.29	0.72	0.75	0.18 – 3.07	0.69
	151-225d	0.84	0.67	2.31	0.63 – 8.51	0.21
	225-300d	-0.69	0.81	0.50	0.10 – 2.44	0.39
	$\geq$ 301d	1.72	0.70	5.57	1.42 – 22.86	0.01
	dry	-0.13	0.59	0.88	0.28 – 2.83	0.83

Table G.3: Univariable relationships between explanatory variables and clinical BDD as binary outcome variable, determined by inclusion of each variable in a generalized linear mixed model (GLMM) specifying management group as random effect, using the LaPlace ML algorithm. YS: young stock; LC: lactating cows; DC: dry cows

Generalized linear mixed model (GLMM) with management group nested within farm as random effect

Finally, the model was specified with management group as a nested random effect within farm; a more explicit multilevel structure was thus defined.

Variable		Coefficient	SE	OR	95% CI	P-value
Foot hygiene score	1	Referent				
	2	0.33	0.40	1.38	0.63 – 3.03	0.42
	3	0.59	0.37	1.80	0.87 – 3.74	0.11
	4	0.31	0.39	1.36	0.63 – 2.94	0.43
Body hygiene score	1	Referent				
	2	1.51	0.64	4.54	1.30 – 15.90	0.02
	3	1.71	0.64	5.55	1.59 – 19.37	< 0.01
	4	1.68	0.68	5.35	1.42 – 20.07	0.01
Age (quadratic)	Age.cent	0.24	0.11	1.28	1.03 – 1.58	0.03
	Age.centsq	-0.07	0.03	0.93	0.89 – 0.99	0.01
Age (categorical)	0-2y	Referent				
	>2-3y	1.76	0.99	5.79	0.84 – 40.09	0.08
	>3-4y	2.71	1.00	15.03	2.13 – 105.99	< 0.01
	>4-5y	2.62	1.00	13.74	1.95 – 97.02	< 0.01
	>5-6y	2.47	1.01	11.84	1.65 – 85.11	0.01
	> 6	1.79	0.99	5.97	0.85 – 41.90	0.07
Parity	1	Referent				
	2	1.35	0.52	3.85	1.39 – 10.67	< 0.01
	3	0.32	0.61	1.38	0.42 – 4.53	0.60
	≥ 4	0.63	0.54	1.88	0.65 – 5.48	0.25
LS (quadratic)	DIM.cent	0.00	0.00	1.00	1.00 – 1.01	0.05
	DIM.centsq	0.00	0.00	1.00	1.00 – 1.00	0.27
LS (categorical)	0-50d	Referent				
	51-100d	-0.62	0.84	0.54	0.10 – 2.78	0.46
	101-150d	-0.43	0.79	0.65	0.14 – 3.08	0.59
	151-225d	0.74	0.74	2.09	0.49 – 8.98	0.32
	226-300d	-1.20	0.89	0.30	0.05 – 1.74	0.18
	$\geq$ 301d	1.67	0.79	5.32	1.14 – 24.89	0.03
	dry	-0.71	1.07	0.49	0.06 – 3.97	0.51

Table G.4: Univariable relationships between explanatory variables and clinical BDD as binary outcome, determined by inclusion of each variable in a generalized linear mixed model (GLMM) specifying management group nested within farm as random effects term, using the LaPlace ML algorithm. YS: young stock; LC: lactating cows; DC: dry cows

G.2 Continuous outcome: serology

The linear models (LMs) were fitted with `lm` from the `stats` package; and linear mixed effects models (LMEs) were fitted with `lme` from the `nlme` package (Pinheiro and Bates, 1995).

Linear regression model (LM)

Each available covariate in sequence was added into a linear model with serology as continuous outcome variable. As for the GLM, a correction for clustering is made by including the farm identifier as a fixed effect; the fixed effects analysis effectively estimates a separate parameter for every herd, and thus separates between-herd variation from the residual variation (Dohoo et al., 2003). The univariable models output is listed in the table below.

Variable		Estimate	SE	t	95% CI	P-value
Foot hygiene score	1	Referent				
	2	15.12	1.64	9.23	11.90 – 18.33	< 0.001
	3	20.61	1.15	17.95	18.36 – 22.87	< 0.001
	4	24.78	1.44	17.24	21.97 – 27.60	< 0.001
	All					< 0.001
Body hygiene score	1	Referent				
	2	11.23	1.54	7.30	8.21 – 14.24	< 0.001
	3	18.60	1.58	11.75	15.50 – 21.70	< 0.001
	4	22.86	2.10	10.88	18.74 – 26.98	< 0.001
	All					< 0.001
Age (quadratic)	Age.cent	5.38	0.20	27.24	5.00 – 5.77	< 0.001
	Age.centsq	-0.51	0.04	-11.71	-0.59 – -0.42	< 0.001
Age (categorical)	0-1y	Referent				
	>1-2y	6.04	1.59	3.81	2.93 – 9.14	< 0.001
	>2-3y	12.53	1.50	8.38	9.60 – 15.47	< 0.001
	>3-4y	28.30	1.52	18.68	25.33 – 31.27	< 0.001
	>4-5y	30.28	1.51	20.05	27.32 – 33.24	< 0.001
	>5-6y	32.46	1.67	19.39	29.18 – 35.73	< 0.001
	> 6y	32.65	1.47	22.19	29.76 – 35.53	< 0.001
	All					< 0.001
Management group	C	Referent				
	H	5.18	1.60	3.24	2.05 – 8.31	< 0.01
	LC	26.85	1.15	23.32	24.60 – 29.11	< 0.001
	DC	25.52	1.61	15.88	22.37 – 28.67	< 0.001
	All					< 0.001
Housing hygiene	1	Referent				
	2	3.91	1.98	1.98	0.03 – 7.79	0.05
	3	12.63	1.52	8.30	9.65 – 15.61	< 0.001
	4	18.55	1.69	10.97	15.23 – 21.87	< 0.001

	All					< 0.001
Housing comfort	1	Referent				
	2	-1.82	2.60	-0.70	-6.92 – 3.28	0.48
	All					0.48
Footbaths	0	Referent				
	1	26.50	1.74	15.26	23.09 – 29.90	< 0.001
	2	28.19	1.72	16.38	24.82 – 31.56	< 0.001
	All					< 0.001
BDD treatment	0	Referent				
	1	17.95	1.26	14.25	15.48 – 20.42	< 0.001
	2	18.65	1.88	9.91	14.96 – 22.34	< 0.001
	All					< 0.001
Parity	1	Referent				
	2	7.18	2.05	3.50	3.16 – 11.21	< 0.001
	3	7.54	2.19	3.45	3.26 – 11.82	< 0.001
	≥ 4	9.39	2.03	4.62	5.41 – 13.38	< 0.001
	All					< 0.001
LS (quadratic)	DIM.cent	0.02	0.01	3.01	0.01 – 0.03	< 0.01
	DIM.centsq	0.00	0.00	-0.80	0.00 – 0.00	0.43
LS (categorical)	0-50d	Referent				
	51-100d	6.42	2.87	2.24	0.79 – 12.05	0.03
	101-150d	2.84	2.91	0.98	-2.86 – 8.53	0.33
	151-225d	7.12	2.76	2.58	1.71 – 12.52	0.01
	225-300d	8.50	2.91	2.92	2.79 – 14.21	< 0.01
	$\geq$ 301d	9.14	2.79	3.28	3.68 – 14.60	< 0.01
	All					< 0.01

Table G.5: Univariable relationships between explanatory variables and serology as continuous outcome variable, determined by inclusion of each variable in a linear regression model (LM). C: calves; H: heifers; LC: lactating cows; DC: dry cows

Linear mixed effects model (LME) with farm as random effect

The LM was extended to a mixed effects model, specifying farm as the random effect. Each available covariate in sequence was added as the fixed effect into this linear mixed effects model (LME). The univariable models output is listed in the table below.

Variable		Estimate	SE	t	95% CI	*P*-value
Foot hygiene score	1	Referent				
	2	15.22	1.64	9.29	12.01 – 18.43	< 0.001
	3	20.66	1.15	18.01	18.41 – 22.91	< 0.001
	4	24.83	1.44	17.29	22.01 – 27.64	< 0.001
	All					< 0.001
Body hygiene score	1	Referent				
	2	11.30	1.54	7.36	8.29 – 14.31	< 0.001
	3	18.65	1.58	11.80	15.56 – 21.75	< 0.001
	4	22.87	2.10	10.90	18.76 – 26.99	< 0.001
	All					< 0.001
Age (quadratic)	Age.cent	5.40	0.20	27.33	5.01 – 5.78	< 0.001
	Age.centsq	-0.51	0.04	-11.73	-0.59 – -0.42	< 0.001
Age (categorical)	0-1y	Referent				
	>1-2y	6.04	1.59	3.81	2.93 – 9.15	< 0.001
	>2-3y	12.57	1.50	8.41	9.64 – 15.50	< 0.001
	>3-4y	28.34	1.51	18.71	25.37 – 31.31	< 0.001
	>4-5y	30.34	1.51	20.09	27.38 – 33.30	< 0.001
	>5-6y	32.52	1.67	19.44	29.24 – 35.80	< 0.001
	> 6y	32.70	1.47	22.24	29.82 – 35.58	< 0.001
	All					< 0.001
Management group	C	Referent				
	H	5.20	1.60	3.25	2.07 – 8.33	< 0.01
	LC	26.91	1.15	23.39	24.66 – 29.17	< 0.001
	DC	25.57	1.61	15.92	22.42 – 28.72	< 0.001
	All					< 0.001
Housing hygiene	1	Referent				
	2	3.99	1.96	2.03	0.14 – 7.83	0.04
	3	12.45	1.52	8.20	9.47 – 15.43	< 0.001
	4	18.55	1.69	11.00	15.24 – 21.85	< 0.001
	All					< 0.001
Housing comfort	1	Referent				
	2	-2.65	2.56	-1.04	-7.68 – 2.37	0.30
	All					0.30
Footbaths	0	Referent				
	1	26.44	1.71	15.50	23.10 – 29.78	< 0.001
	2	28.23	1.69	16.69	24.91-31.55	< 0.001
	All					< 0.001

BDD treatment	0	Referent				
	1	17.97	1.25	14.33	15.51 – 20.42	< 0.001
	2	18.82	1.86	10.11	15.17 – 22.48	< 0.001
	All					< 0.001
Parity	1	Referent				
	2	7.19	2.05	3.50	3.16 – 11.22	< 0.001
	3	7.55	2.19	3.45	3.27 – 11.83	< 0.001
	≥ 4	9.43	2.03	4.64	5.44 – 13.42	< 0.001
	All					< 0.001
LS (quadratic)	DIM.cent	0.02	0.01	3.05	0.01 – 0.03	< 0.01
	DIM.centsq	0.00	0.00	-0.80	0.00 – 0.00	0.42
LS (categorical)	0-50d	Referent				
	51-100d	6.42	2.87	2.23	0.78 – 12.05	0.03
	101-150d	2.82	2.91	0.97	-2.88 – 8.52	0.33
	151-225d	7.17	2.76	2.60	1.77 – 12.57	< 0.01
	226-300d	8.57	2.91	2.94	2.86 – 14.28	< 0.01
	≥ 301d	9.20	2.78	3.30	3.74 – 14.65	< 0.01
	All					< 0.001

Table G.6: Univariable relationships between explanatory variables and serology as continuous outcome variable, determined by inclusion of each variable in a linear mixed effects (LME) regression model, specifying farm as random effect. C: calves; H: heifers; LC: lactating cows; DC: dry cows

Linear mixed effects model (LME) with management group as random effect

For further comparison between models, the random effects term was changed to management group, and the univariable analyses were repeated. Note that the management group level variables cannot now be included as fixed effects, as these are already accounted for by the random effects.

Variable		Estimate	SE	t	95% CI	P-value
Foot hygiene score	1	Referent				
	2	6.67	1.90	3.50	2.93 – 10.40	< 0.001
	3	11.48	1.35	8.52	8.84 – 14.12	< 0.001
	4	12.94	1.65	7.83	9.70 – 16.18	< 0.001
	All					< 0.001
Body hygiene score	1	Referent				
	2	6.27	1.69	3.70	2.95 – 9.58	< 0.001
	3	6.95	1.81	3.83	3.39 – 10.50	< 0.001
	4	6.21	2.27	2.74	1.76 – 10.66	< 0.01
	All					0.02
Age (quadratic)	Age.cent	5.22	0.37	14.10	4.49 – 5.95	< 0.001
	Age.centsq	-0.45	0.06	-6.98	-0.57 – 0.32	< 0.001
Age (categorical)	0-1y	Referent				
	>1-2y	7.17	2.18	3.28	2.89 – 11.45	< 0.01
	>2-3y	13.40	2.24	6.00	9.02 – 17.78	< 0.001
	>3-4y	29.03	2.30	12.61	24.51 – 33.54	< 0.001
	>4-5y	32.32	2.30	14.03	27.81 – 36.83	< 0.001
	>5-6y	35.60	2.43	14.65	30.84 – 40.36	< 0.001
	> 6y	35.09	2.25	15.60	30.68 – 39.50	< 0.001
	All					< 0.001
Parity	1	Referent				
	2	6.46	2.36	2.74	1.84 – 11.08	< 0.01
	3	6.53	2.50	2.61	1.63 – 11.42	< 0.01
	≥ 4	10.66	2.32	4.59	6.11 – 15.21	< 0.001
	All					< 0.001
LS (quadratic)	DIM.cent	0.04	0.01	4.86	0.02 – 0.05	< 0.001
	DIM.centsq	0.00	0.00	-1.21	0.00 – 0.00	0.23
LS (categorical)	0-50d	Referent				
	51-100d	7.74	3.24	2.39	1.38 – 14.09	0.02
	101-150d	4.43	3.24	1.37	-1.92 – 10.79	0.17
	151-225d	11.51	3.06	3.76	5.50 – 17.51	< 0.001
	226-300d	13.21	3.24	4.07	6.85 – 19.56	< 0.001
	≥ 301d	14.41	3.03	4.76	8.47 – 20.34	< 0.001
	All					< 0.001

Table G.7: Univariable relationships between explanatory variables and serology as continuous outcome variable, determined by inclusion of each variable in a linear mixed effects (LME) regression model, specifying management group as random effect. C: calves; H: heifers; LC: lactating cows; DC: dry cows

Linear mixed effects model (LME) with management group nested within farm as random effect

Finally, the model was specified with management group as a nested random effect within farm; a more explicit multilevel structure was thus defined.

Variable		Estimate	SE	t	95% CI	*P*-value
Foot hygiene score	1	Referent				
	2	0.45	1.78	0.25	-3.04 – 3.94	0.80
	3	7.34	1.42	5.15	4.55 – 10.13	< 0.001
	4	9.08	1.84	4.93	5.47 – 12.69	< 0.001
	All					< 0.001
Body hygiene score	1	Referent				
	2	4.39	2.11	2.08	0.25 – 8.52	0.04
	3	5.26	2.20	2.39	0.95 – 9.57	0.02
	4	7.09	2.57	2.76	2.06 – 12.13	< 0.01
	All					0.02
Age (quadratic)	Age.cent	4.42	0.35	12.66	3.74 – 5.10	< 0.001
	Age.centsq	-0.41	0.06	-7.00	-0.53 – -0.30	< 0.001
Age (categorical)	0-1y	Referent				
	>1-2y	7.51	2.47	3.04	2.67 – 12.35	< 0.01
	>2-3y	12.06	2.73	4.42	6.71 – 17.40	< 0.001
	>3-4y	26.85	2.83	9.48	21.30 – 32.40	< 0.001
	>4-5y	28.56	2.83	10.10	23.02 – 34.10	< 0.001
	>5-6y	30.46	2.90	10.49	24.77 – 36.15	< 0.001
	> 6y	30.75	2.81	10.95	25.25 – 36.25	< 0.001
	All					< 0.001
Parity	1	Referent				
	2	7.19	2.05	3.50	3.16 – 11.22	< 0.001
	3	7.55	2.19	3.45	3.27 – 11.83	< 0.001
	≥4	9.43	2.03	4.64	5.44 – 13.42	< 0.001
	All					< 0.001
LS (quadratic)	DIM.cent	0.02	0.01	3.05	0.01 – 0.03	< 0.01
	DIM.centsq	0.00	0.00	-0.80	0.00 – 0.00	0.42
LS (categorical)	0-50d	Referent				
	51-100d	6.42	2.87	2.23	0.78 – 12.05	0.03
	101-150d	2.82	2.91	0.97	-2.88 – 8.52	0.33
	151-225d	7.17	2.76	2.60	1.77 – 12.57	< 0.01
	226-300d	8.57	2.91	2.94	2.86 – 14.28	< 0.01
	≥ 301d	9.20	2.78	3.30	3.74 – 14.65	< 0.01
	All					< 0.001

Table G.8: Univariable relationships between explanatory variables and serology as continuous outcome variable, determined by inclusion of each variable in a linear mixed effects (LME) regression model, specifying management group nested within farm as random effects term. C: calves; H: heifers; LC: lactating cows; DC: dry cows

Appendix H

Outputs of statistical models of the longitudinal study

H.1 Binary outcome: BDD lesion status

Analysis was performed using the R statistical software package, version 2.3.1. The TINN editor was used to interface with R. Several useful references on implementing mixed-effects models in R are available (Verzilli, 2003; Bates and Pinheiro, 2000; Venables and Ripley, 2002).

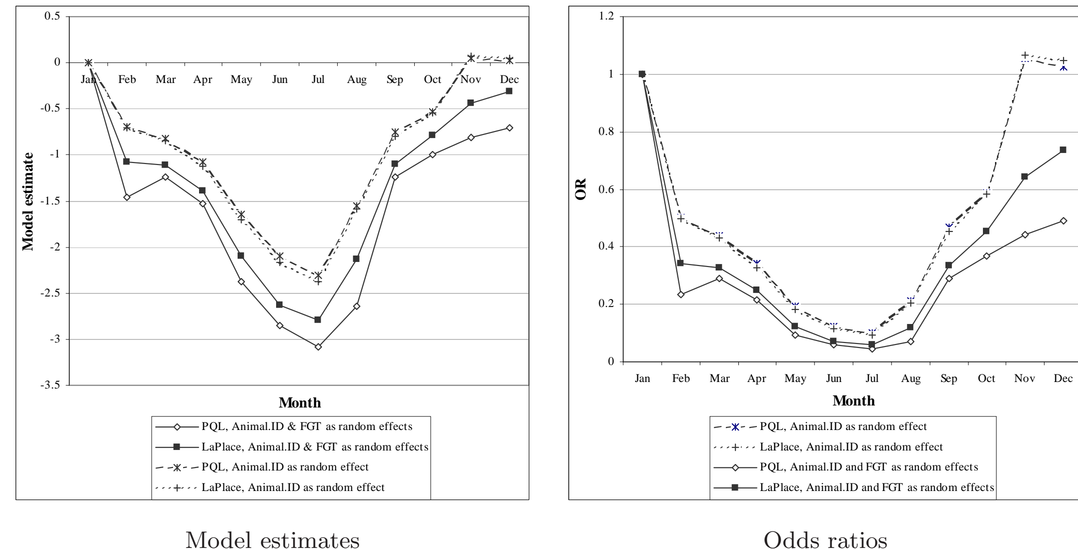
Generalized linear mixed effects model were fitted with lesion as binary outcome. We elected to use `lmer` from the lme4 package (Bates, 2005); this allows the likelihood algorithm to be specified (PQL, Laplace or AGQ). Laplace and AGQ are both maximum likelihood algorithms; Laplace was used.

H.1.1 Covariance structure

Likelihood algorithm and random effects

To decide which random effects structure was best, GLMMs were fitted with month (categorical) as a fixed effect. Animal ID was first included as a single random effect, followed by Animal ID and FGT as crossed random effects. This was performed using the PQL algorithm as well as the LaPlace algorithm. The outputs of the four models were compared by ANOVA and graphed (Figure H.1). Using `Animal.ID` and `FGT` as crossed random effects, the LaPlace algorithm gives a smoother plot, and better log likelihood values. Another advantage of using `FGT` is 'biological plausibility': the model now implicitly incorporates farm, management group and time in the random effects. We therefore decided to use this structure in subsequent models.

Investigating temporal aspects, i.e. seasonality of infection: Figure H.1 shows a very strong seasonal component with a peak in November to January, and a trough in July. This appears to approach a sinusoidal pattern, so we specified a sine function in the model instead of month as a categorical variable. Using the estimated model coefficients to plot the resulting curves, Figure H.2 was obtained. The likelihood ratio



Model estimates Odds ratios

Figure H.1: Longitudinal study model estimates and corresponding odds ratios per calendar month, as estimated using different random effects and likelihood estimation algorithms; January is the referent

test (LRT) of this model was much better than the GLMM using month as categorical explanatory variable; also, use of this function reduces the number of model parameters by 9. By de-trending the seasonal effects in this way, this source of covariance in the data is effectively removed.

H.1.2 Fixed effects

Univariable analyses were performed using the individual-level variables; the results will not be reproduced here. Multivariable models were fitted using explanatory variables significant at the $p = 0.1$ level; the following variables were included in the final model.

Age

Age could not be fitted as a continuous variable as this would imply a linear effect (which from the EDA is clearly not the case); it could not be approximated with a polynomial either. It was therefore fitted as a categorical variable with five levels (0-2y, 2-3y, 3-4y, 4-5y and >5y); the categories 0-1y and 1-2y were merged as there were no clinical positives in these categories.

BHS

BHS is scored on four regions (flank, hindleg, udder, tail) on the left and right side; it is scored on a four-point scale. A summed score ranging from 8 to 32 can thus be obtained. This was recategorised to a four-point scale by dividing by 8 and rounding.

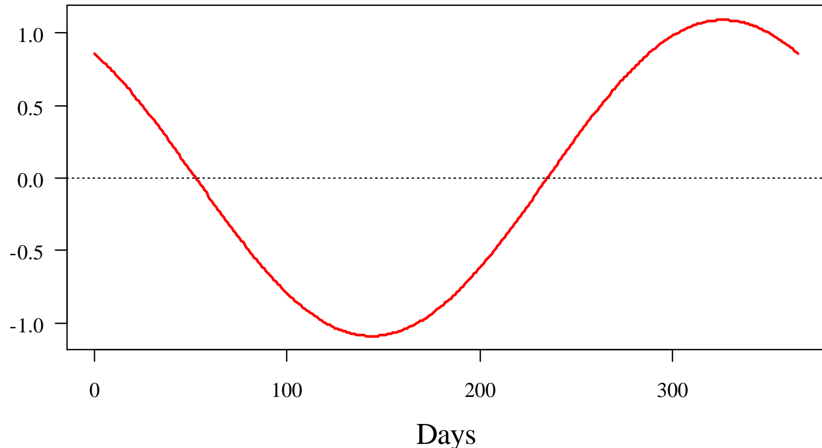


Figure H.2: Seasonal variability of clinical BDD prevalence as predicted by a GLMM incorporating a sine function to simulate the temporal effect. Day 0 represents 1 January; day 365 represents 31 December. Minimum prevalence occurs towards the end of May; maximum prevalence occurs in late November

Final multivariable model

```
GLMM_FINAL<-lmer(Les.bin ~ sin(days/365*2*pi) + cos(days/365*2*pi)
              + as.factor(Age.cat2a) + as.factor(FHS)
              + (1|Animal.ID) + (1|FGT),
              data=LSdata,
              family=binomial,
              method="Laplace")
summary(GLMM_FINAL)
```

H.1.3 Autocorrelation structure: transition model

To fit a first-order transition model, the observation at time (t-1) is specified as an additional explanatory variable (Pinheiro and Bates, 2000), coded as `Les.bin1`.

```
modelTRANS <- update(GLMM_FINAL, . ~ . + Les.bin1)
summary(modelTRANS)
anova(GLMM_FINAL,modelTRANS)
```

H.2 Continuous outcome: BDD serology

The continuous outcome of serology is simpler to model; serial autocorrelation can be investigated in more detail. Linear mixed effects models (LMEs) were fitted with serology as continuous outcome, using the `lme` function from the `nlme` package.

A limitation of this package was encountered when specifying two separate random effects with `lme`. Nesting one within the other is straightforward, but it was not possible to specify `Animal.ID` and `FGT` as separate (crossed) random effects. Therefore, only `Animal.ID` was included as random effect.

H.2.1 Covariance structure

Likelihood algorithm and random effects

For this class of models, a choice between maximum likelihood (`ML`) or restricted maximum likelihood (`REML`) could be made. According to Dohoo et al. (2003), both are valid; `ML` was specified.

As with the BDD lesion modelling, the effects of seasonality were investigated. Neither the model (LME with ELISA PP as outcome and month of year as categorical fixed effect), nor the EDA (scatterplots and boxplots – refer to text in Chapter 5) showed any evidence of seasonality for serology, although clinically BDD positive animals had significantly higher titres.

H.2.2 Fixed effects

Age

Age could in this instance be fitted as a categorical variable with six levels (0-1y, 1-2y, 2-3y, 3-4y, 4-5y and >5y).

BHS

The same categories for BHS were used as for the transition model (see above).

Management group

Five categories were initially created: unweaned calves, weaned calves, heifers, lactating cows and dry cows. The lactating cow category was further subdivided by lactation stage: pre-preak (up to 100 days in milk), peak (101 to 200 days in milk) and post-peak (over 201 days in milk). This way, lactation stage effects were also incorporated into the model.

Final multivariable model

The multivariable model was then specified thus:

```
LME_FINAL<-lme(ELISA.PP ~ as.factor(Age.cat) + as.factor(Man.groupLS)
              + as.factor(BHS),
              random = ~ 1 | Animal.ID,
              method = "ML",
              data=LSdata)
summary(LME_FINAL)
```

H.2.3 Autocorrelation structure: CAR(1) model

The CAR(1) model was then simply specified:

```
modelCAR1<-update(LME_FINAL,
       corr = corCAR1(0.95, ~ days | Animal.ID),
       method = "ML")
summary(modelCAR1)
anova(LME_FINAL,modelCAR1)
```

Appendix I

Outputs of a mathematical models for BDD

I.1 Model code

The model was written using Berkeley Madonna (http://www.berkeleymadonna.com/), a specialised software package for solving differential equations. The model code is included below. Semi-colons and curly braces 'protect' comments.

```
METHOD RK4

STARTTIME = 0
STOPTIME=52
DT = 0.02


{PREAMBLE}

; As clinical disease is almost exclusively in the cow groups, only consider
; these, i.e. exclude the young stock.

; As proportions of "true" and "false" positives with clinical inspection &
; serology are comparable for lactating cows and dry cows, further reduce the
; model by considering one cow group.

; The initial model was SEIR. We don't have the microbiological tools to
; differentiate between E and R. EDA of CSS and LS datasets make an SEIS
; structure plausible, where S can pass to E, form which it can return to S or
; become I. I can return to E or directly to S (see thesis text for flow
; diagram).

; There is an age effect, with older animals having less S and more E; the
; proportion of I is the same. Therefore split the cows into two age groups:
; less than 5 (young) and over 5 (old).

; Seasonal component of infection is very prominent. Define two periods:
; housing and grazing. The dynamics will be different in these periods. Specify
; the model for a one-year period, i.e. 12 months. Let time t=0 represent
; beginning of housing.
```

```
{1. DIFFERENTIAL EQUATIONS}

{YOUNG COWS GROUP}

    d/dt(X_y) = Ix + gamma_y*Y_y - mu_y*X_y - beta_y*(X_y*E_y/N_y)*Env +
                sigma_y*E_y - rho*X_y
    d/dt(E_y) = Ie + beta_y*(X_y*E_y/N_y)*Env + delta2_y*Y_y -
                (mu_y + sigma_y)*E_y - delta_y*E_y*Env - rho*E_y
    d/dt(Y_y) = Iy + delta_y*E_y*Env - (mu_y + gamma_y + delta2_y)*Y_y - rho*Y_y

{OLD COWS GROUP}

    d/dt(X_o) = rho*X_y + gamma_o*Y_o - mu_o*X_o - beta_o*(X_o*E_o/N_o)*Env +
                sigma_o*E_o
    d/dt(E_o) = rho*E_y + beta_o*(X_o*E_o/N_o)*Env + delta2_o*Y_o -
                (mu_o + sigma_o)*E_o - delta_o*E_o*Env
    d/dt(Y_o) = rho*Y_y + delta_o*E_o*Env - (mu_o + gamma_o + delta2_o)*Y_o

{ENVIRONMENT}

    d/dt(Env) = alpha*(Y_y + Y_o) - epsilon*Env


{2. POPULATION PARAMETERS}

{YOUNG COWS GROUP}

    N_y=X_y+E_y+Y_y     ; From LS data: see Figure 6.3
    N_y0=90
    E_y0=(0.3*N_y0)
    Y_y0=(0.4*N_y0)

    INIT X_y = N_y0-(E_y0+Y_y0)
    INIT E_y = E_y0
    INIT Y_y = Y_y0

    {Calving of animals: assume steady state:}

    It=(mu_y*N_y)+(mu_o*N_o)
    Ix=0.65*It
    Ie=0.1*It
    Iy=0.25*It          ; From LS data: see Figure 6.3

    {Age-determined transition from y to o:}

    rho=1/36            ; Calving at 2 years => 3 years in this group => 36 months

{OLD COWS GROUP}

    N_o=X_o+E_o+Y_o     ; From LS data: see Figure 6.3
    N_o0=60
    E_o0=(0.5*N_o0)
    Y_o0=(0.4*N_o0)

    INIT X_o = N_o0-(E_o0+Y_o0)
    INIT E_o = E_o0
    INIT Y_o = Y_o0
```

```
{3. RATES OF TRANSITIONS}

{YOUNG COWS GROUP}

{Periodic functions housing <-> grazing}

    gamma_y =  IF TIME <= 6 THEN gamma_yh ELSE gamma_yg
    sigma_y = IF TIME <= 6 THEN sigma_yh ELSE sigma_yg
    delta_y =  IF TIME <= 6 THEN delta_yh ELSE delta_yg
    delta2_y =  IF TIME <= 6 THEN delta2_yh ELSE delta2_yg
    I =  IF TIME <= 6 THEN I_yh ELSE I_yg
    R =  IF TIME <= 6 THEN R_yh ELSE R_yg
    L =  IF TIME <= 6 THEN L_yh ELSE L_yg
    L2 =  IF TIME <= 6 THEN L2_yh ELSE L2_yg

{Housing season}

    sigma_yh = 1/I_yh   ; rate of E -> S
    gamma_yh = 1/R_yh   ; rate of I -> S
    delta_yh = 1/L_yh   ; rate of E -> I
    delta2_yh = 1/L2_yh ; rate of I -> E
    I_yh = (25/2)*6     ; time from E -> S
    R_yh = 10E+6        ; time from I -> S
    L_yh = (25/8)*6     ; time from E -> I
    L2_yh = (47/7)*6    ; time from I -> E

{Grazing season}

    sigma_yg = 1/I_yg   ; rate of E -> S
    gamma_yg = 1/R_yg   ; rate of I -> S
    delta_yg = 1/L_yg   ; rate of E -> I
    delta2_yg = 1/L2_yg ; rate of I -> E
    I_yg = (25/3)*6     ; time from E -> S
    R_yg = (40/3)*6     ; time from I -> S
    L_yg = (25/7)*6     ; time from E -> I
    L2_yg = (40/10)*6   ; time from I -> E

{OLD COWS GROUP}

{Periodic functions housing <-> grazing}

    gamma_o =  IF TIME <= 6 THEN gamma_oh ELSE gamma_og
    sigma_o = IF TIME <= 6 THEN sigma_oh ELSE sigma_og
    delta_o =  IF TIME <= 6 THEN delta_oh ELSE delta_og
    delta2_o =  IF TIME <= 6 THEN delta2_oh ELSE delta2_og
    I =  IF TIME <= 6 THEN I_oh ELSE I_og
    R =  IF TIME <= 6 THEN R_oh ELSE R_og
    L =  IF TIME <= 6 THEN L_oh ELSE L_og
    L2 =  IF TIME <= 6 THEN L2_oh ELSE L2_og

{Housing season}

    sigma_oh = 1/I_oh   ; rate of E -> S
    gamma_oh = 1/R_oh   ; rate of I -> S
    delta_oh = 1/L_oh   ; rate of E -> I
    delta2_oh = 1/L2_oh ; rate of I -> E
    I_oh = (22/2)*6     ; time from E -> S
    R_oh = (32/2)*6     ; time from I -> S
    L_oh = (22/2)*6     ; time from E -> I
```

```
    L2_oh = (32/5)*6    ; time from I -> E

{Grazing season}

    sigma_og = 1/I_og   ; rate of E -> S
    gamma_og = 1/R_og   ; rate of I -> S
    delta_og = 1/L_og   ; rate of E -> I
    delta2_og = 1/L2_og ; rate of I -> E
    I_og = (32/7)*6     ; time from E -> S
    R_og = 10E+6        ; time from I -> S
    L_og = (32/13)*6    ; time from E -> I
    L2_og = (33/8)*6    ; time from I -> E


{4. TRANSMISSION PARAMETERS}

{YOUNG COWS GROUP}

{Periodic functions housing <-> grazing}

    beta_y = IF TIME <= 6 THEN beta_yh ELSE beta_yg
    beta_yh = 0.550     ; From LS data: see Table 6.3
    beta_yg = 0.735     ; From LS data: see Table 6.3

{OLD COWS GROUP}

{Periodic functions housing <-> grazing}

    beta_o = IF TIME <= 6 THEN beta_oh ELSE beta_og
    beta_oh = 1.052     ; From LS data: see Table 6.3
    beta_og = 0.548     ; From LS data: see Table 6.3


{5. DEATH RATES}

; Combine culling and natural death rate into one parameter to reduce by one
; term

    mu_y =  5/(90*12)  ; natural death rate + culling rate young cows:
                       ; 5 cows per 90 in group over 12-month period
    mu_o = 15/(60*12)  ; natural death rate + culling rate old cows
                       ; 15 cows per 60 in group over 12-month period

{6. ENVIRONMENTAL COMPARTMENT}

; These values were assumed - no information exists to base figures on

{Periodic functions housing <-> grazing}

    INIT Env = IF TIME <=6 THEN Env_h ELSE Env_g
    alpha = IF TIME <= 6 THEN alpha_h ELSE alpha_g
    epsilon = IF TIME <= 6 THEN epsilon_h ELSE epsilon_g

{Housing season}

    Env_h = 1.5E+1
    alpha_h = 1/A_h    ; rate of shedding in housing
    epsilon_h = 1/S_h  ; rate of environmental death in housing
    S_h = 1            ; time to environmental death in housing
    A_h = 4
```

```
{Grazing season}

    Env_g = 1.5E-0
    alpha_g = 1/A_g    ; rate of shedding at pasture
    epsilon_g = 1/S_g  ; rate of environmental death at pasture
    S_g = 0.5          ; time to environmental death at pasture
    A_g = 4
```

I.2 Estimation of the transmission coefficient, β

I.2.1 Cows ≤5 years of age

Time point (t)	Housing type	S_{tY}	E_{tY}	I_{tY}	N_{tY}	Z_{tY}	$S_{tY}E_{tY}/N_{tY}$	$\log(S_{tY}E_{tY}/N_{tY})$
1	H	51	17	23	91	–	–	–
2	H	45	12	32	89	2	7.80	0.89
3	G	57	10	23	90	4	6.20	0.79
4	G	53	10	27	90	2	6.11	0.79
5	G	53	16	21	90	1	7.66	0.88
6	G	26	34	29	89	8	9.68	0.99
7	G	29	22	39	90	0	8.51	0.93
8	G	30	14	37	90	4	6.58	0.82
9	H	35	17	38	90	0	6.34	0.80
10	H	26	17	48	91	0	5.73	0.76
11	H	15	15	60	90	0	3.68	0.57
12	H	25	15	50	90	2	3.33	0.52
13	H	31	14	45	90	2	4.49	0.65
14	H	31	15	44	90	0	4.99	0.70
15	G	36	19	34	89	2	6.43	0.81
16	G	31	27	33	91	0	8.44	0.93
17	G	27	31	31	89	0	9.30	0.97
18	G	27	27	35	89	0	8.80	0.94
19	G	36	14	41	91	0	6.86	0.84
20	G	4	11	45	90	0	4.85	0.69
21	H	32	21	37	90	5	5.81	0.76
22	H	37	7	47	91	0	5.16	0.71
23	H	39	8	43	90	0	3.16	0.50

Table I.1: Number of susceptible (S_{tY}), exposed (E_{tY}) and infected (I_{tY}) animals of age ≤5 years at time t. The time points are months in study; the corresponding housing type is cubicle housing (H) or grazing (G). Z_{tY} represents new infections since the previous month. These data were extracted from the longitudinal cohort data and scaled to a total number (N_{tY}) of 90 in a 150 cow milking herd. The value of $S_{tY}E_{tY}/N_{tY}$ is calculated as the mean for time $(t-1)$ and time t

I.2.2 Cows ≥5 years of age

Time point (t)	Housing type	S_{tY}	E_{tO}	I_{tO}	N_{tO}	Z_{tO}	$S_{tO}E_{tO}/N_{tO}$	$\log(S_{tO}E_{tO}/N_{tO})$
1	H	5	26	29	60	–	–	–
2	H	2	27	31	60	1	1.53	0.19
3	G	3	29	28	60	0	1.18	0.07
4	G	2	34	24	60	2	1.29	0.11
5	G	6	33	21	60	0	2.22	0.35
6	G	3	43	14	60	0	2.72	0.44
7	G	0	35	25	60	0	1.08	0.03
8	G	5	30	25	60	0	1.25	0.10
9	H	5	12	43	60	0	1.75	0.24
10	H	4	14	41	59	2	0.97	-0.01
11	H	0	12	48	60	0	0.47	-0.32
12	H	5	10	46	61	0	0.41	-0.39
13	H	9	16	35	60	2	1.61	0.21
14	H	8	18	34	60	2	2.40	0.38
15	G	5	18	37	60	0	1.95	0.29
16	G	11	19	30	60	3	2.49	0.40
17	G	5	16	38	59	0	2.42	0.38
18	G	12	12	36	60	0	1.88	0.27
19	G	11	26	23	60	0	3.58	0.55
20	G	8	23	30	61	4	3.89	0.59
21	H	15	11	34	60	0	2.88	0.46
22	H	9	20	31	60	6	2.88	0.46
23	H	9	21	30	60	0	3.08	0.49

Table I.2: Number of susceptible (S_{tO}), exposed (E_{tO}) and infected (I_{tO}) animals of age >5 years at time t. The time points are months in study; the corresponding housing type is cubicle housing (H) or grazing (G). Z_{tO} represents new infections since the previous month. These data were extracted from the longitudinal cohort data and scaled to a total number (N_{tO}) of 60 in a 150 cow milking herd. The value of $S_{tO}E_{tO}/N_{tO}$ is calculated as the mean for time $(t-1)$ and time t

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