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Aetiology and consequences of reproductive tract diseases in dairy cows

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Abstract

Reproductive tract diseases of dairy cows are common world-wide and results in a decrease in reproductive performance. The research presented in this thesis evaluates the available diagnostic methods for reproductive tract diseases, including the quality of published reports describing these methods in dairy cows. To improve the accuracy of cow-side diagnostic tests for reproductive tract diseases more research is needed, specifically to establish optimal cut-points, timing of examination and test variability (i.e. intra- and interobserver agreement). Moreover, future manuscripts reporting on diagnostic methods for reproductive tract diseases could be improved by using checklists for quality of design and reporting as a guideline.

Research was also done to assess the presence of intrauterine bacteria in early postpartum New Zealand dairy cows and their association with the subsequent reproductive tract infection, inflammation and reproductive performance. The isolation of intrauterine bacteria, irrespective of type, at 23 days postpartum was associated with a decrease in pregnancy within three weeks for the start of the seasonal breeding programme (planned start of mating; PSM; $P = 0.05$). *Escherichia coli* isolated at 23 days postpartum tended to increase the time to pregnancy ($P = 0.09$). However, the presence of *E. coli* within the first week postpartum was not significantly associated with isolation of *Trueperella pyogenes* three weeks later ($P = 0.53$). An interesting finding was the positive association between the elevated recruitment of polymorphonuclear cells in the early postpartum period and a decreased time to pregnancy ($P = 0.05$).

Susceptibility data, based on minimum inhibitory concentration (MIC), was generated for a range of antimicrobials against *E. coli* and *T. pyogenes* from intrauterine origin. Between-herd and between age-

group differences in MIC were detected ($P \leq 0.05$). Cows diagnosed with intrauterine *E. coli* with an MIC of $\geq 8 \mu\text{g/mL}$ at 23 days postpartum tended to be at lower risk of pregnancy within six weeks of PSM relative to an MIC of $< 8 \mu\text{g/mL}$ ($P = 0.09$). No interpretative criteria are available for MIC data of antimicrobials against uterine isolates. Hence, more research is required on pharmacokinetic and pharmacodynamic profiles for veterinary antimicrobials.

This thesis describes the first isolation of apparent antibodies to bovine herpesvirus type 4 and the DNA of bovine lymphotropic herpesvirus in New Zealand dairy cattle, both of which may play an important role in the pathogenesis of reproductive tract diseases. Further studies are required to investigate the true impact of these viruses.

The research presented in this thesis provided data useful for further improvement of diagnosis and treatment of reproductive tract diseases in dairy cows.

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As I'm sitting here surrounded by piles of paper collected over the last number of years, notes, draft versions of manuscripts, and multiple printouts of peer-reviewed manuscripts I'm reflecting on the last few years that have been entirely dedicated towards the creation of this thesis. Returning to New Zealand to start this PhD project was life-changing in many ways. I am pleased to have this opportunity to thank a large number of people. Without them this demanding journey would have been an ordeal.

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Abbreviations

AI	Artificial insemination
BCS	Body condition score
BHBA	β -hydroxybutyric acid
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
BLHV	Bovine lymphotropic herpesvirus
BoHV-4	Bovine herpesvirus type 4
bp	Base pair
BUN	Blood urea nitrogen
CCFA	Ceftiofur crystalline free acid
CFU	Colony forming unit
CL	Corpus luteum
CLSI	Clinical and Laboratory Standards Institute
DIM	Days in milk
ELISA	Enzyme-linked immunosorbent assay
EnPEC	Endometrial pathogenic <i>Escherichia coli</i>
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MAC	Macrophages
MIC	Minimum inhibitory concentration
MIC ₅₀	The antimicrobial concentration that inhibits 50% of the bacterial isolates
MIC ₉₀	The antimicrobial concentration that inhibits 90% of the bacterial isolates

NEFA	Non-esterified fatty acid
OD	Optical densities
ONPG	An enzymatic test for Ortho-nitrophenyl- β -galactosidase
OUMI	ONPG, urase, motility, indol agar tests
PBMC	Peripheral blood mononuclear cells
PGE ₂	Prostaglandin E ₂
PGF _{2α}	Prostaglandin F _{2α}
PMN	Polymorphonuclear cells
PSM	Planned start of mating (the seasonal start of the breeding season)
PVD	Purulent vaginal discharge
RFM	Retained foetal membranes
ROC	Receiver-operating characteristic analysis
Se	Sensitivity (the proportion of diseased animals that test positive)
Sp	Specificity (the proportion of non-diseased animals that test negative)
TAGS	Tests in absence of a gold standard
TSI	Triple sugar iron agar test
VDS	Vaginal discharge score

List of publications

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MW de Boer, SJ LeBlanc, J Dubuc, S Meier, W Heuwieser, S Arlt, RO Gilbert, S McDougall. 2014. *Invited review: Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows.* Journal of Dairy Science 97:3983-3999.

Melvin de Boer, Bryce M. Buddle, Cord Heuer, Tao Zheng, Stephen LeBlanc, Hassan Hussein, Scott McDougall. 2014. Risk factors for metritis and endometritis in NZ dairy cows: Preliminary results of associations between intrauterine bacterial infection and reproductive tract inflammation. Proceedings of the Food Safety, Animal Welfare & Biosecurity, Epidemiology & Animal Health Management, and Industry branches of the NZVA (FEI), 5.1.

Melvin de Boer, Bryce Buddle, Cord Heuer, Hassan Hussein, Tao Zheng, Stephen LeBlanc, Scott McDougall. 2014. Associations between intrauterine bacterial infection, reproductive tract inflammation, and reproductive performance in New Zealand dairy cows. Proceedings of the XXVIII World Buiatrics Congress 2014 Cairns, Australia, in press.

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Chapter 1

General introduction

1.1. INTRODUCTION

For a 365-day inter-calving interval to be maintained in seasonal breeding herds, it is crucial that cows are bred and conceive within a limited breeding season (Macmillan, 2002a; b). Reproductive performance of dairy cattle appears to be declining both internationally (Royal et al., 2000; Lucy, 2001) and in New Zealand (Macmillan, 2002a; b), with reported increased inter-calving intervals, increased culling rates and decreased pregnancy rates (Esslemont, 2003). However, in the last decade, the overall decline in reproductive performance plateaued in New Zealand with breeding genetics improving over time (Brownlie, 2012; Anonymous, 2013). International studies have found that peripartum diseases, including dystocia, retained foetal membranes (RFM), metritis and endometritis, have a negative effect on reproductive performance (Curtis et al., 1985; Erb et al., 1985; Heuer et al., 1999). Under New Zealand conditions, around 20% of cows have been estimated to have a peripartum disease, with diseased animals having reduced 4 and 8 week in-calf rates and increased non-pregnant rates (McDougall, 2001b). McDougall et al. (2007) found that cows with grossly evident purulent material in the vagina had lower 56-day and final pregnancy rates compared with herd-mates without this condition. New Zealand and international studies have found noticeable production losses due to reproductive tract diseases (Esslemont, 2003; Gilbert et al., 2005; McDougall et al., 2011).

The overall objective of this thesis was to address the decreasing reproductive performance as a result of reproductive tract disease, focusing on pasture-based New Zealand dairy cows. This was achieved through the:

a) evaluation of available diagnostic methods for reproductive tract diseases in dairy cows world-wide by systematic literature review;

b) elucidation of potential reasons for decreasing reproductive performance as a result of intrauterine bacterial infections in New Zealand dairy cattle;

c) improvement in the knowledge of antimicrobial susceptibility of isolates from postpartum uterine bacterial infections; and

d) identification of novel intrauterine pathogens in New Zealand dairy cattle that may potentially cause reproductive tract diseases.

A number of reviews have recently been published on bovine reproductive tract diseases (Földi et al., 2006; Sheldon et al., 2006; Azawi, 2008; LeBlanc, 2008; Sheldon et al., 2009; LeBlanc et al., 2011; Haimerl et al., 2012; LeBlanc, 2012; Lefebvre and Stock, 2012; Sannmann et al., 2012). Therefore, this Chapter, specifically describes the New Zealand pasture-based and seasonal dairy system, the available definitions for reproductive tract diseases, immunology and risk factors for reproductive tract diseases, an objective evaluation of diagnostic methods, intrauterine bacterial pathogens, treatments against these pathogens, and bovine herpesvirus type 4 (BoHV-4).

1.2. NEW ZEALAND PASTURE-BASED AND SEASONAL DAIRY SYSTEM

In the 2012/2013 season, the New Zealand dairy industry consisted of approximately five million dairy cows within approximately 12,000 herds, located predominately in the North Island. The Waikato region contains New Zealand's largest dairy population with 24% of all dairy cows (Anonymous, 2013). The

majority of New Zealand dairy herds are managed using a seasonal, spring-calving system and cows are on pasture year-round. A typical pasture ration consists predominately of rye grass and clover with the majority of farms providing some supplementary feed, such as maize silage or palm kernel extract, when pasture growth is insufficient. The seasonal system evolves so that the peak milk production occurs coincident with the peak spring grass growth. Hence, the date of the herd's planned start of calving is set accordingly (Macmillan, 2002a; Verkerk, 2003). In the Waikato region, the start of the seasonal breeding programme (planned start of mating; PSM) commonly occurs in the first part of October on an annual basis. In general, the breeding programme consists of artificial insemination (AI) for approximately three to six weeks from the PSM followed by a period (eight to ten weeks) where bulls are grazed with the herd (Macmillan, 2002b). In total, approximately 75% of cows were artificially inseminated in the 2012/2013 breeding season (Anonymous, 2013). The current industry-set target for completion of the entire breeding programme within a single season is less than 12 weeks (Anonymous, 2007).

A short calving to conception interval is required to maintain the seasonal calving pattern (Macmillan, 2002a). Since the 1970s, induction of parturition of late-conceiving cows has been used in New Zealand to retain a compact calving period, but is now regulated by strict guidelines and actively discouraged at the national level by the dairy industry in response to concerns regarding potentially reduced animal welfare (Macmillan, 2002a; Verkerk, 2003; Fisher and Webster, 2013). Consequently, adaption of alternative strategies, such as hormonal treatment of anoestrus cows at PSM, has been required (McDougall, 2010).

The New Zealand dairy industry sets reproductive targets based on data derived from the top quartile of herds (Xu and Burton, 2003). For example, the target for cows submitted for AI by Day 21 of the breeding period ('3-week submission risk'), pregnant by Day 42 of the seasonal breeding period ('6 week

in-calf risk') and first service to conception were set at 90%, 78% and 60%, respectively (Anonymous, 2007). These targets are currently used in the InCalf™ program which, through a series of facilitated training meetings for herd managers, is designed to improve herd reproduction (Anonymous, 2007). Although participants in this farmer extension program have been found to be more likely to have herds with improved reproductive performance (as measured using 6 week in-calf risk; Brownlie et al., 2012), further research is still required to determine methods by which reproductive health and performance of pasture-based cows can be improved and to reach the targets set by the New Zealand dairy industry.

1.3. DEFINITIONS OF REPRODUCTIVE TRACT DISEASES

Sheldon et al. (2006) have proposed definitions for bovine uterine disease. "Puerperal metritis" was defined as the presence of an abnormally enlarged uterus with a fetid, watery, red-brown uterine discharge, signs of systemic illness (e.g. decreased milk yield, dullness or other signs of toxæmia) and a fever of $>39.5^{\circ}\text{C}$ within 21 days postpartum. "Metritis" can be diagnosed in cows that are not ill or pyrexia, but have an abnormally enlarged uterus and a purulent uterine discharge detectable in the vagina within 21 days after parturition. "Clinical endometritis" was defined by the presence of purulent or mucopurulent uterine exudate in the vagina 21 days or more postpartum, not accompanied by systemic signs. Others have defined "subclinical endometritis" as an increase in proportion of inflammatory cells (i.e. polymorphonuclear cells; PMN) in the uterus (Kasimanickam et al., 2004; Gilbert et al., 2005). To clarify the difference between disease as diagnosed by a pathologist and that diagnosed using clinical tests, Runciman et al. (2009) introduced the term "bovine reproductive tract inflammatory disease" for reproductive disease in cows diagnosed following visualization of gross vaginal discharge. In comparison, Dubuc et al. (2010a) proposed the use of purulent vaginal discharge (PVD) as an alternative for "clinical endometritis" and "cytological" endometritis when an increase in inflammatory cells in the endometrium is diagnosed. The pathological definitions of reproductive tract diseases, as described in detail in Chapter 2, are used in this thesis; e.g. PVD is used for cows detected with grossly evident

purulent material in the vagina and endometritis on the influx of inflammatory cells to the endometrium.

1.4. REPRODUCTIVE TRACT IMMUNOLOGY AND RISK FACTORS

Cows with peripartum disease have decreased immunity (Kimura et al., 2002), spend less time eating, and have a lower dry matter intake than cows without peripartum disease (Urton et al., 2005; Huzzey et al., 2007). Following pathogen recognition, the immune response involves the release of pro-inflammatory cytokines, e.g. tumour necrosis factor- α (TNF- α) and interleukins (IL-1, IL-6, IL-8, IL-12). IL-8 chemotactically attracts neutrophils, induces phagocytosis and oxidative burst of these cells. Cytokines instigate the production of acute phase proteins, e.g. haptoglobin in the liver, which reduce tissue damage and stimulate tissue repair (LeBlanc, 2012). The cytokine concentrations in uterine tissue and plasma (Ishikawa et al., 2004; Kim et al., 2005; Ghasemi et al., 2012) and plasma concentrations of acute phase proteins (Williams et al., 2005; Dubuc et al., 2010b; Li et al., 2010) are higher in cows affected by reproductive tract diseases compared with those unaffected. Cows diagnosed with PVD have decreased calcium serum concentrations (Heidarpour et al., 2012). Neutrophil functionality (i.e. phagocytosis and oxidative burst) is impaired in cows with subclinical hypocalcaemia (Martinez et al., 2012). Furthermore, neutrophil functionality may be reduced by lower intracellular glycogen concentrations in cows with reproductive tract diseases (Galvão et al., 2010). An additional factor in the reduced functionality of polymorphonuclear cells may be negative energy balance (Hammon et al., 2006; Moyes et al., 2009).

Negative energy balance as measured by concentrations of non-esterified fatty acid (NEFA; Hammon et al., 2006; Galvão et al., 2010), serum β -hydroxy butyric acid (BHBA; Duffield et al., 2009; Galvão et al., 2010; Heidarpour et al., 2012), acetone (Reist et al., 2003), blood urea nitrogen (BUN) or blood glucose (Senosy et al., 2012a), appears to be associated with reproductive tract diseases. In contrast, other studies have found similar profiles for NEFA, BHBA, BUN and glucose between cows with PVD or

endometritis compared with healthy cows (Burke et al., 2010; Senosy et al., 2012b; Giuliadori et al., 2013).

Risk factors for PVD in pasture based cows are low BCS, herd, primiparity, periparturient disease, dystocia, stillbirth, twin-calving, atonic uterus, large uterus and prolonged anovulation period (McDougall et al., 2007; Runciman et al., 2008a; Plöntzke et al., 2010). In addition, bearing a bull calf, steep vulval angle, lower daily dry matter intakes, low dietary protein over the dry period, left displaced abomasum, milk fever, longer lactation length and short dry periods were found to be risk factors in housed or partially housed cows (Kaneene and Miller, 1995; Bruun et al., 2002; Bell and Roberts, 2007; Gautam et al., 2009; Potter et al., 2010). Recently, studies have described similar risk factors for endometritis, e.g. hyperketonemia, retained placenta, acute puerperal metritis and low BCS (Rutigliano et al., 2008; Dubuc et al., 2010b; Cheong et al., 2011). It is clear that reproductive tract diseases are multifactorial and some can be eliminated by changes in management. The detection of those diseases is reliant on the available diagnostic methods.

1.5. DIAGNOSTIC METHODS

Diagnostic methods are used at multiple levels in veterinary medicine, from individual animal to group and herd level by the practitioner, and at regional, national or international level for surveillance and epidemiological studies (Greiner and Gardner, 2000a). For example, it is common practice in New Zealand to diagnose PVD using an intravaginal sampling device ('Metricheck'). Using this method, within-herd prevalences ranged from 10 to 20% and cows diagnosed with PVD had poorer reproductive outcomes than those without PVD (McDougall et al., 2007). Kelton *et al.* (1998) assessed 43 publications and found prevalences of PVD between 2.2% and 37.3% worldwide. It can be assumed that this large variation is due, at least in part, to different diagnostic tools used with different definitions and individual studies using different designs.

The performance of diagnostic tests should ideally be validated against a test producing only correct results, i.e. a gold standard (Greiner and Gardner, 2000b). For many methods, no gold standard is available (Gardner and Greiner, 2006). There are evidence-based methods available to objectively evaluate the literature describing diagnostic methods of reproductive tract diseases in dairy cows. The Cochrane Collaboration is one of the organisations promoting the use of systematic reviews; an objective literature review. This initiative is an independent non-profit organization named after Archie Cochrane (1909-1988), an epidemiologist who promoted the use of randomized controlled trials to generate reliable data for human health practitioners. According to Cochrane's definition a systematic review involves "a clearly formulated question that uses systematic and explicit methods to identify, select, and critically appraise relevant research, and to collect and analyse data from studies that are included in the review" (Higgins and Green, 2011). Currently, a limited number of veterinary systematic reviews have been published, and asides from metritis (Sannmann et al., 2012), no such reviews are available on diagnostic methods of reproductive tract diseases in dairy cows, its diagnostic criteria and definitions, repeatability or agreement among methods for diagnosis. A study using the systematic review procedure is described in Chapter 2.

1.6. INTRAUTERINE BACTERIA

Bacteria can be isolated from the uterus of over 90% of cows in the first two weeks postpartum with the prevalence declining with time (Griffin et al., 1974). The bacterial flora are commonly mixed cultures of anaerobic and aerobic, either Gram-positive or Gram-negative bacteria (Steffan et al., 1984). *Escherichia coli*, *Trueperella pyogenes*, *Prevotella melaninogenicus*, *Fusobacterium necrophorum* have been associated with significant uterine pathology (Williams et al., 2005). Recently, *Arcanobacterium pyogenes* (formerly *Corynebacterium pyogenes* and *Actinomyces pyogenes*) has been renamed to *Trueperella pyogenes* (Yassin et al., 2011). The growth density of these primary uterine pathogens at Day 7 postpartum, but not Days 14, 21 or 28, is associated with reduced dominant follicle growth rate,

reduced oestradiol concentrations and a smaller corpus luteum following ovulation (Williams et al., 2007). Only *T. pyogenes* has been consistently associated with reduced reproductive performance (Bonnett et al., 1993; Huszenicza et al., 1999). It has been suggested that intrauterine presence of *E. coli* within the first week postpartum may predispose to *T. pyogenes* infection later (Dohmen et al., 2000; Sheldon et al., 2006; Williams et al., 2007). Recently Sheldon *et al.* (2010) described the presence of a uterine adapted *E. coli* (endometrial pathogenic *E. coli*; EnPEC) whose presence may increase the risk of isolation of *T. pyogenes* and of subsequent reduced reproductive performance in the UK. If this hypothesis is true, then management strategies that reduce the risk of uterine infection, such as vaccination against *E. coli* may reduce incidence or severity of uterine disease. However, the causal effect of the presence of *E. coli* predisposing intrauterine infections with *T. pyogenes* has not been directly tested, nor has the presence of uterine adapted *E. coli* been confirmed in any other dairy farming systems such as the New Zealand pasture-based and seasonal system. Chapter 3 reports, among other associations, particularly on the suggested causal effect of presence of *E. coli* on the isolation of *T. pyogenes* later in lactation.

1.7. THERAPY

The treatments for PVD include intrauterine antimicrobial infusion (McDougall, 2001a; Runciman et al., 2008b) or intramuscular injection with prostaglandin $F_{2\alpha}$ (LeBlanc et al., 2002; McDougall et al., 2013). Treatment efficacy depends on time of treatment, severity of uterine infection, presence of a corpus luteum, and the minimum inhibitory concentration of the antimicrobial used. Kaufmann *et al.* (2010) did not find a difference in clinical or reproductive outcomes between parenteral antibiotics (1 mg/kg ceftiofur daily for three days) or two prostaglandin $F_{2\alpha}$ injections 14 days apart. The therapy of metritis involves local or parental antimicrobials, non-steroidal anti-inflammatory drugs and fluids (Drillich et al., 2001; Sheldon et al., 2004; Drillich et al., 2007). Oxytocin, prostaglandin $F_{2\alpha}$, and oestrogens remain controversial and generally unproven in treating metritis (Frazer, 2005). Given the range of bacterial

species isolated from the uterus postpartum, broad spectrum (i.e. Gram-positive and Gram-negative) antimicrobials appear to be indicated. Currently ceftiofur, cephapirin, tylosin, oxytetracycline, trimethoprim/sulphadiazine, and amoxicillin/clavulanic acid are registered for bovine reproductive tract diseases in New Zealand. Interestingly, even though antimicrobial therapy is most commonly used for reproductive tract diseases (Azawi, 2008), limited data are available internationally on the pharmacodynamics (i.e. the prediction of *in vitro* efficacy) and the pharmacokinetics (i.e. the prediction of *in vivo* efficacy) of antimicrobials against bacteria from bovine uterine origin. No such data are available for intrauterine pathogens isolated from pasture-based dairy cows. Chapter 4 addresses this knowledge gap on susceptibility of a range of antimicrobials.

1.8. BOVINE HERPESVIRUS TYPE 4 (BOHV-4)

Most pathogens causing postpartum uterine infections in cows are bacteria. However, BoHV-4 has been one of the few viruses consistently associated with uterine pathology. However, direct causal *in vivo* associations have not yet been established (Sheldon et al., 2009). BoHV-4 has been found in a variety of tissues and commonly macrophages are persistently infected (Osorio and Reed, 1983; Dubuisson et al., 1989; Donofrio et al., 2008). *In vitro*, the virus has been shown to be trophic for bovine endometrial and stromal cells, and to induce cytopathic effects (Donofrio et al., 2007). *Escherichia coli* and its endotoxin lipopolysaccharide promoted the reactivation of BoHV-4 (Donofrio et al., 2008). In addition to *E. coli*, it is proposed that other bacteria, such as *T. pyogenes*, may also be associated with this virus (Sheldon et al., 2009). Like many herpesviruses, BoHV-4 is present in a latent stage after infection, thus detectable in healthy animals. Hence, difficulties exist finding direct associations between healthy and diseased animals (Donofrio et al., 2008). This virus has been reported in the USA (Parks and Kendrick, 1973) and Europe (Castrucci et al., 1986; Czaplicki and Thiry, 1998). However, it is unknown if BoHV-4 is present in New Zealand and whether it is associated with increased severity of uterine infection under pasture-

based New Zealand systems. The presence of this virus in New Zealand is discussed in more detail in Chapter 5.

1.9. STRUCTURE AND CONTENT OF THE SUBSTANTIVE CHAPTERS

The substantive chapters of this thesis are Chapters 2 to 5. Each of those Chapters was prepared for manuscript submission to various peer-reviewed journals. Consequently, some differences in presentation may exist, because the formatting style of the journal of submission was used.

The study reported in Chapter 2 of this thesis evaluates the current gaps in literature of the current available diagnostic methods for reproductive tract disease in dairy cows using the evidence-based method of a systematic review. Diagnostic methods, diagnostic criteria and definitions, repeatability, and agreement among methods for diagnosis of reproductive tract diseases in dairy cows (i.e. vaginitis, cervicitis, endometritis, salpingitis, and oophoritis) are addressed. Additionally, a critical appraisal of the quality of design and reporting of the publications describing those diagnostic methods is reported in Chapter 2.

Chapter 3 describes a field trial that generated data that integrated emerging pieces of information about the role of, and relationships amongst, uterine infections with *E. coli* and *T. pyogenes* and their associations with reproductive inflammation, i.e. PVD and endometritis diagnosed by cytology, and reproductive performance. However, the primary objective was to assess the association between isolation of *E. coli* from intrauterine samples taken from cows within the first few days postpartum and the risk of *T. pyogenes* isolation 21 days later. Additional aims were to assess the associations between early reproductive tract inflammation and subsequent inflammation, and reproductive performance.

The study described in Chapter 4 of this thesis generated novel sensitivity data for a range of antimicrobials against *E. coli* and *T. pyogenes* isolated from pastured-based dairy cows. Further,

associations between minimum inhibitory concentrations of the evaluated antimicrobials with demographic factors and with reproductive performance were assessed.

Chapter 5 reports on the presence of BoHV-4 in samples taken from New Zealand dairy cows diagnosed with clinical metritis. A general discussion of the thesis and overall outcomes is described in Chapter 6.

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Chapter 2

Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows

According to:

Invited review: Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows. *Journal of Dairy Science* 97, 3983-99, 2014

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2.1. ABSTRACT

The objective of this study was to conduct a systematic and critical appraisal of the quality of previous publications, and describe diagnostic methods, diagnostic criteria and definitions, repeatability, and agreement among methods for diagnosis of vaginitis, cervicitis, endometritis, salpingitis, and oophoritis in dairy cows. Publications (n = 1,600) which included the words dairy, cows, and at least one disease of interest were located with online search engines. In total, 51 papers were selected for comprehensive review by pairs of the authors. Only 61% (n = 31) of the 51 reviewed papers provided a definition or citation for the disease or diagnostic method(s) studied, and only 49% (n = 25) of the papers provided the data or a citation to support the test cut-point used for diagnosing disease. Furthermore, a large proportion of the papers did not provide sufficient detail to allow critical assessment of the quality of design or reporting. Of 11 described diagnostic methods, only one complete methodology, i.e. vaginoscopy was assessed for both within- and between-operator repeatability ($\kappa = 0.55 - 0.60$, and 0.44 , respectively). In the absence of a gold standard, comparisons between different tests have been undertaken. There is a low level of agreement between the various diagnostic methods. These discrepancies may indicate that these diagnostic methods assess different aspects of reproductive health and underline the importance of tying diagnostic criteria to objective measures of reproductive performance. Those studies that have used a reproductive outcome to select cut-points and tests have the greatest clinical utility. This approach has demonstrated, for example, that presence of (muco)purulent discharge in the vagina and an increased proportion of leucocytes in cytological preparations following uterine lavage or cytobrush sampling are associated with poorer reproductive outcomes. The lack of validated, consistent definitions and outcome variables makes comparisons of the different tests difficult. The quality of design and reporting in future publications could be improved by using checklists as a guideline. Further high quality research based on published standards to improve study design and reporting, should improve cow-side diagnostic tests. Specifically, more data on intra-

and interobserver agreement are needed to evaluate test variability. Also, more studies are necessary to determine optimal cut-points and time postpartum of examination.

Keywords: vaginitis, purulent vaginal discharge, cervicitis, endometritis

2.2. INTRODUCTION

Systematic reviews use a pre-defined methodology for the selection of studies, and then evaluate those studies based on a series of criteria designed to assess the experimental design, the sample size, the sampling approach, the statistical approach, and the strength of the inferences (Tranfield et al., 2003). Systematic reviews, together with meta-analyses, are regarded as the highest source of scientific evidence (Arlt et al., 2010). This methodology has been more commonly used in human medicine than in veterinary medicine and animal science, but are relevant in the latter as well (Sargeant et al., 2006; Grindlay et al., 2012).

The prevalence of endometritis in dairy cows is reported to be between 5 and 68% (Barlund et al., 2008; Gautam et al., 2009; Cheong et al., 2011). These large variations are at least partially due to inconsistencies of timing of examination relative to calving, diagnostic method, and definition of endometritis as well as true differences in prevalence between populations. Anaerobic and aerobic, Gram-positive and Gram-negative bacteria can be isolated from the uterus of more than 90% of cows in the first 2 wk postpartum, with the prevalence of infection declining with time (Földi et al., 2006). The time required for normal uterine and cervical involution varies among cows from 25 to 47 days after calving (LeBlanc, 2008). To generate more consistency, definitions have been proposed recently to define purulent vaginal discharge ('clinical endometritis'; PVD) and (cytological or 'subclinical') endometritis (Sheldon et al., 2006; Runciman et al., 2009; Dubuc et al., 2010a). Reporting the definition of disease and other critical information in papers on diagnosis of acute postpartum metritis in dairy cows is inconsistent (Sannmann et al., 2012).

High intra- and interobserver agreement are required for good quality tests (Greiner and Gardner, 2000a). Agreement can be statistically analyzed by 2 different methods: kappa-statistics (value between -1 and 1; κ) which calculates agreement beyond chance (Dohoo et al., 2009) and the correlation between tests (value between -1 and 1; r ; Greiner and Gardner, 2000a). The performance of diagnostic tests should ideally be validated against a test producing only correct results, i.e. a gold standard (Greiner and Gardner, 2000b). Some diagnostic tests produce a dichotomous test result (diseased or not diseased). Other tests will produce an ordinal or a continuous outcome (Greiner and Gardner, 2000b), like a gross vaginal discharge score from 0 to 5 (McDougall et al., 2007) or the proportion of polymorphonuclear leucocytes (PMN) in a uterine cytology smear (Gilbert et al., 2005). For tests with ordinal or continuous outcomes, cut-points need to be established to determine if a test result is categorized as positive or negative (Greiner and Gardner, 2000b). Cut-points can be established using receiver-operating characteristic (ROC) analysis which provides an assessment of sensitivity (Se) and specificity (Sp) over the range of test scores (Gardner and Greiner, 2006). Tests are described (“test characteristics”) using Se and Sp which are the probability of a positive test result in a disease-positive animal and the probability of a negative test result in a non-diseased animal, respectively (Greiner and Gardner, 2000b). Used in conjunction with the prevalence of the condition, predictive values for test results can then be calculated to provide interpretive guidance.

Often a gold standard is not available (Gardner and Greiner, 2006). In these circumstances, tests are validated against a non-perfect test or a biological outcome, e.g. calving to pregnancy interval or pregnancy by a given interval postpartum (LeBlanc et al., 2002; Barlund et al., 2008). Statistical methods have also been developed for tests in absence of a gold standard (TAGS); these assume that neither test is perfect and adjust the estimates of Se and Sp accordingly (Pouillot et al., 2002). Finally, Bayesian methods can be used to develop ROC curves to determine cut-points when a gold standard is not available (Choi et al., 2006).

Traditional literature reviews may be biased if authors use criteria for inclusion or exclusion of specific papers that are not robust. For this reason, a more evidence-based approach, such as a systematic review, is required to reduce the potential lack of critical assessment (Tranfield et al., 2003). A systematic review uses a transparent and repeatable process to firstly select the papers to be included in a review, and then secondly uses a consistent approach to assess the quality of the study design, case inclusion, clinical or laboratory procedures, analysis and reporting. Instead of a traditional literature review, the aim of this study was to conduct a systematic review on diagnostic methods for reproductive-tract diseases in cows. No data are currently available on the quality of design and reporting of papers describing diagnostic methods for these diseases other than for metritis (Sannmann et al., 2012). The first objective was to critically appraise the quality of design and reporting of papers selected using an evidence-based method. A systematic review has not been performed on these diagnostic methods, therefore other objectives were to assess diagnostic methods, diagnostic criteria and definitions, repeatability, and agreement among methods for diagnosis of reproductive-tract diseases in dairy cows (i.e. vaginitis, cervicitis, endometritis, salpingitis, and oophoritis). This appraisal was conducted using selection criteria, a data extraction template and a quality checklist, which were developed a priori with the involvement of each of the authors of this manuscript.

2.3. METHODS

A protocol was developed a priori, which included a detailed description of the review process, the inclusion criteria and the reporting process using guidelines from the Cochrane Collaboration (Higgins and Green, 2011) and the Centre for Reviews and Dissemination, University of York (Centre for Reviews and Dissemination, 2009). The populations of interest were postpartum dairy cows tested for vaginitis, cervicitis, endometritis, salpingitis or oophoritis, irrespective of breed, type of housing, geographic location or calving distribution. For this review, pathological definitions of the reproductive-tract diseases were used, that is, including both clinical ('grossly evident') and subclinical (i.e. absence of

clinically evident disease, hence relying on ancillary laboratory tests for diagnosis) disease. Vaginitis, cervicitis, endometritis, salpingitis and oophoritis were defined as inflammation (measured as an increase in inflammatory cells, generally associated with an undesirable outcome or impaired reproductive performance) within the vagina, cervix, uterus, oviduct (uterine tube), or ovaries, respectively.

Studies conducted on dairy cattle that included these conditions, and where comparisons were made between diagnostic tests of any type or between a single test and a reproductive outcome, were selected for critical appraisal. Studies with interventions (treatments) for the reproductive-tract disease were included. These were only included when the study design (i.e. a negative control group was used in which cows received no interventions, or an assessment of diagnostic criteria before treatment) or analytical processes (i.e. stratification to consider the negative control group, or covariate adjustment of the effect of the intervention) dealt appropriately with confounding such that the test characteristics of the test itself could be assessed. Studies that did not control for these interventions, but were included for other reasons, were excluded from test validation assessed using reproductive outcomes.

Data from controlled trials, cohort studies, and quantitative study designs evaluating diagnostic tests were included. Primary papers reporting original data were included; reviews and meta-analyses were excluded. Studies reporting on in vitro and post-mortem effects were excluded, as well as case reports and case series, or if studies were described as preliminary results, personal experiences, and unpublished data other than conference proceedings. Only papers in English and published in peer reviewed journals and conference proceedings that were available online were considered. No date limitations were applied. After the selection process, only reported data were used - authors were not contacted to provide any additional information.

The literature search was performed by the first author on 6 February 2013 using CAB Abstracts, MEDLINE and Web of Science simultaneously within the search engine Web of Knowledge using the search terms “dairy AND (cow* OR cattle OR bovine) AND (vaginitis OR purulent vaginal discharge OR cervicitis OR endometritis OR subclinical endometritis OR clinical endometritis OR cytological endometritis OR salpingitis OR oophoritis)”. The selected search terms were kept broad to increase the search result. For example, search terms around diagnosis, such as “diagnostic tests” were not included to minimize the risk of failing to detect papers.

Selection and assessment of diagnostic papers were performed in 2 stages (Figure 2.1). In Stage 1, all titles and abstracts of the identified studies were assessed by the first author using the eligibility criteria above. Only papers available at the libraries of Massey University, Palmerston North, New Zealand; Freie Universität Berlin, Germany; University of Guelph, Canada; or available on the Internet were included for Stage 2. This stage involved screening of full manuscripts. Each was comprehensively evaluated for inclusion by 2 assessors. The first author evaluated all manuscripts for inclusion. A second evaluation for inclusion was provided by one of the co-authors (22 to 24 papers per second assessor). To prevent bias, none of the authors evaluated manuscripts (co-)authored by themselves. A manuscript was included when both assessors concluded that the inclusion criteria were met and a median of 7 papers per second assessor were included. Following agreement that a paper would be included in the review during the full evaluation at Stage 2, 2 structured assessments were performed. Data on the contents and methods of each paper were entered into a spreadsheet (Excel; Microsoft) developed a priori, modified from the Cochrane Handbook (Appendix 1; Higgins and Green, 2011). Also, the checklist for diagnostic methods for paratuberculosis in ruminants, STRADAS-paraTB (Standards for Reporting of Animal Diagnostic Accuracy Studies for paratuberculosis; Gardner et al., 2011), was modified to include criteria relevant to diagnostic methods for reproductive-tract diseases (Appendix 2). The scoring of the individual items of the checklist was done on a six-point scale (strongly agree to strongly disagree, or not

determined; Arlt et al., 2010). Spreadsheets were collated in a purpose-built SQL database, and data were analyzed using Stata 12.1 (StataCorp, College Station, TX, USA).

2.4. RESULTS AND DISCUSSION

2.4.1. Search Results and Paper Selection

In this review, 51 papers were critically appraised to assess the currently available diagnostic methods for vaginitis, cervicitis, endometritis, salpingitis, and oophoritis in dairy cows (Appendix 3). Initially 689, 40 and 871 publications were identified by the databases CAB Abstracts[®], MEDLINE[®] and Web of Science[®], respectively. The combination of these databases cover the vast majority of veterinary and animal science journals (Grindlay et al., 2012). Therefore, no other methods (e.g. manual searches) beyond the initial database search were used to retrieve additional papers, and manuscripts published after the search date (6 February 2013) were not included.

Included Papers. Selection for inclusion was made on the basis of criteria based on relevance developed a priori and approved by all authors, similar to other systematic reviews (Sannmann et al., 2012). To reduce bias, each full evaluation of the papers was undertaken by 2 assessors. There was fair agreement ($\kappa = 0.33$) between pairs of assessors for including papers. The majority of the discrepancies ($n = 27$) between the assessors were on studies not accounting for treatments of reproductive-tract disease by study design or statistical analyses. Prior to the inclusion process, the inclusion criteria were not tested on papers by the assessors. This may be a possible reason for these disagreements. Therefore, training sessions undertaken prior to start of a systematic review may decrease heterogeneity of interpretation, even when all are involved preparing the inclusion criteria.

Excluded Papers. Of the excluded manuscripts ($n = 1,549$; Figure 2.1), 55 were the same data set published twice, 458 were written in a language other than English, 30 were in vitro or post-mortem

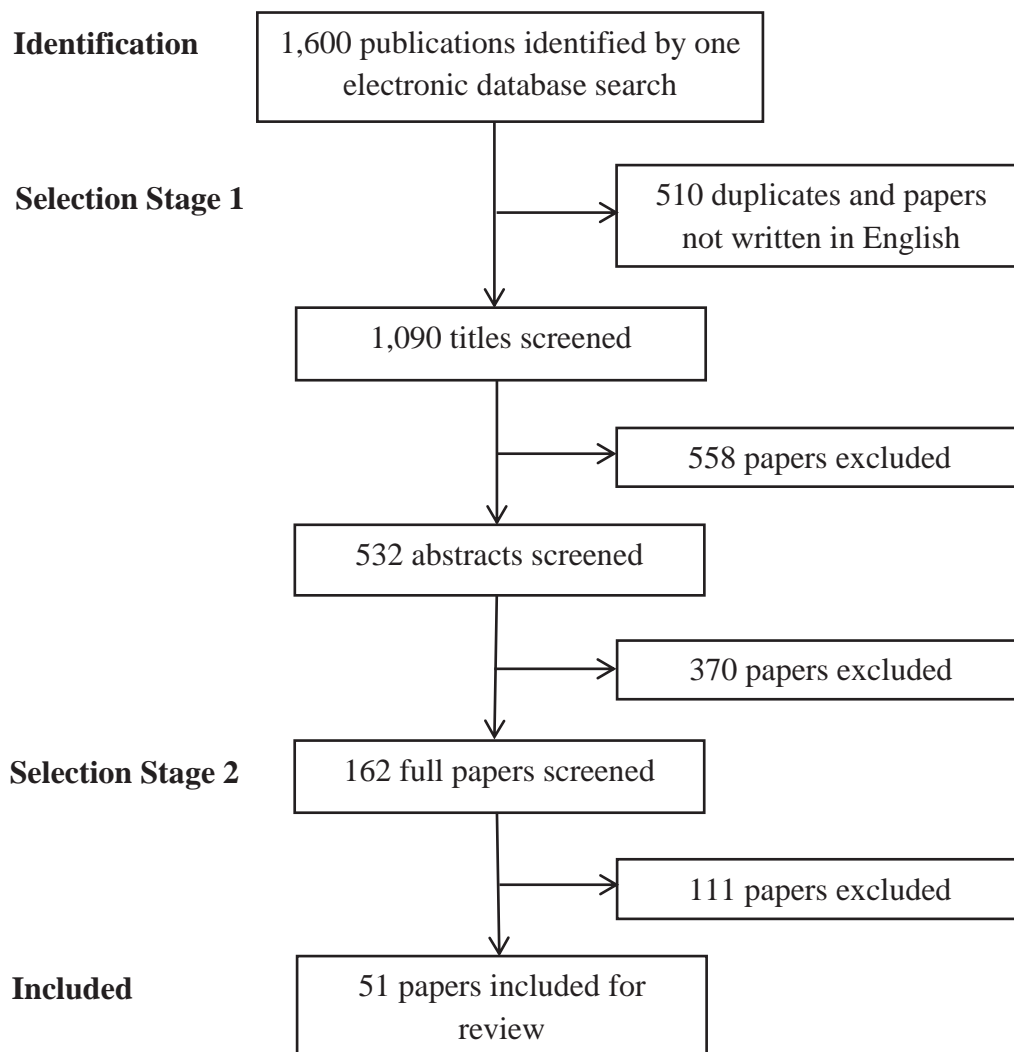


Figure 2.1. Flowchart of selection process of papers identified on 6 February 2013 by using the search terms “dairy AND (cow* OR cattle OR bovine) AND (vaginitis OR purulent vaginal discharge OR cervicitis OR endometritis OR subclinical endometritis OR clinical endometritis OR cytological endometritis OR salpingitis OR oophoritis)” in the 3 databases CAB Abstracts, MEDLINE, and Web of Science simultaneously within the search engine Web of Knowledge for quality appraisal and data synthesis.

studies, 45 were studies on animals other than dairy cows, 78 were not evaluating 2 or more diagnostic tests or one test with reproductive outcomes, 614 did not report on a disease relevant to this review or the disease of interest was an outcome variable for other conditions, 71 did not report original data, 59 were not published in journals or issues of conference proceedings (e.g. books and theses), and 90

publications were not full manuscripts (e.g. short-communications, letters, or abstract only). In 39 cases treatment was not accounted for in the study design or statistical analyses, and 10 were classified as case-control studies or case reports.

Although broad search terms were used, this resulted in exclusion of an unknown number of potential relevant papers, e.g. a publication by Bonnett et al. (1993). Besides “dairy cows”, that publication did not include any of the other search terms used in the title or abstract, and no keywords were described. It is unclear how many publications were missed because of this, and this emphasizes the importance of the use of appropriate key words in papers. Similar to other systematic reviews, papers written in a language other than English and those not published in peer-reviewed journals were excluded (Roy and Keefe, 2012; Sannmann et al., 2012). Papers that appear relevant may not have been included due to the selection of search terms, or may have been selected but were subsequently excluded as they failed to meet the specific inclusion criteria.

2.4.2. Quality Assessment of Included Papers

Disease Definition, Test Characteristics, and Cut Points. A full description of the disease, the diagnostic method and the rationale of the diagnostic cut-point is important information that needs to be provided to make an informed judgement on the validity of the diagnostic test, the test performance, and application of the results. In total, 31 (61%) papers referenced the disease of interest or the diagnostic method for disease detection, whereas the remaining 20 (39%) papers described the disease or diagnostic method without citation. Sannmann et al. (2012) reported an even higher proportion of papers on diagnostic methods for acute postpartum metritis (64%) that did not state or cite the disease definition. In total, 25 (49%) papers included in this review referenced or derived from the study data the described diagnostic cut-points. In 15 (60%) of these papers, cut-points were justified by citing

references, eight (16%) used statistical techniques to analyze cut-points, and 2 (4%) provided both for different tests used (Table 2.1).

The test characteristics of diagnostic methods were discussed in 29 (57%) of the included papers. In 21 (41%) and 23 (45%) of the papers diagnostic cut-points and possible sources of error were discussed, respectively. Only 20% of the reviewed papers discussed all 3 criteria. Test characteristics, cut-points and sources of error are important criteria that can influence selection and application of tests and interpretation of test results. To be able to assess and compare the diagnostic methods or results, this information should be discussed (Sannmann et al., 2012), so that the reader is aware of these factors influencing the study outcome.

Checklist for Quality of Design and Reporting. A large proportion of the papers did not provide sufficient detail to allow critical assessment of the quality of design and reporting (Appendix 2). For only one criterion (item 2: stating the research question or study aims) a large majority of the papers (82%) scored “strongly agree” or “agree”. For 5 criteria (items 1, 5, 16, 17, and 22) including the description of diagnostic test, sampling protocol, or both and cross tabulation of results, scores were “agree” or “strongly agree” for approximately half of the papers, whereas for 8 criteria (items 4, 11, 12, 13, 15, 23, 25, and 26) including a description of the selection methods of animals and herds, and the use of blinding methods, 51 to 100% of the papers were marked “disagree” or “strongly disagree”. Agreements between assessors for each criterion are described in Appendix 2. In total, 19 (66%) criteria had a fair or higher agreement ($\kappa > 0.20$). As would be expected, agreement between both assessors was slightly higher, when responses “agree” and “strongly agree”, and “disagree” and “strongly disagree” were combined (Appendix 2). Reduced agreement may be due to the review design, where the second assessor was any 1 of 7 individuals. Ideally, all observers would have rated all included papers, but because of time limitations, this design was not practical. In common with many published

Table 2.1. Summary of cut points for the proportion of polymorphonuclear leucocytes (PMN) in uterine cytology, leucocyte esterase reagent test strips, and the optical density (OD) of fluid retrieved following uterine lavage for diagnosis of endometritis in dairy cows. Only analyses analyzed using different statistical methods on original data are provided; described or referenced cut-points are not included

Diagnostic technique	DIM	Sample size	Excluded diseases	Statistical method	Reference outcome	Cut-point
Endo cytobrush ¹	21-47	285	RFM ² , metritis, PVD ³	ROC analysis ⁴	Pregnant by 90 DIM	6.7% PMN ⁵
Endo cytobrush	35	1044	None	Survival analysis	Pregnant by 120 DIM	6% PMN
Endo cytobrush	56	1044	None	Survival analysis	Pregnant by 120 DIM	4% PMN
Endo cytobrush	28-41	221	None	Survival analysis	Pregnant by 150 DIM	8% PMN
Endo cytobrush	20-33	228	PVD	ROC analysis	Pregnant by 132 DIM	18% PMN
Endo cytobrush	34-47	228	PVD	ROC analysis	Pregnant by 132 DIM	10% PMN
Endo cytobrush	21-34	168	None	Survival analysis	Pregnant by 300 DIM	6.5% PMN
Endo cytobrush	28	303	None	Descriptive ⁶	-	9% PMN
Endo cytobrush	42	303	None	Descriptive	-	7% PMN
Uterine lavage	21	445	Pyometra, adhesions, abscesses	ROC analysis	Pregnant by 150 DIM	8.5% PMN
Uterine lavage	35	445	Pyometra, adhesions, abscesses	ROC analysis	Pregnant by 150 DIM	6.5% PMN
Uterine lavage	49	445	Pyometra, adhesions, abscesses	ROC analysis	Pregnant by 150 DIM	4% PMN
Cerv cytobrush ⁷	21-34	168	None	Survival analysis	Pregnant by 300 DIM	5% PMN
Uterine lavage	40-60	563	PVD	ROC analysis	Cytology (> 10% PMN)	2 + (LE ⁸)
Uterine lavage	40-60	563	PVD	ROC analysis	Cytology (>10% PMN)	3 + (Protein ⁹)
Uterine lavage	40-60	563	PVD	ROC analysis	Cytology (>10% PMN)	7 (pH ¹⁰)
Uterine lavage	34-36	1742	Pyometra	ROC analysis	Gross uterine discharge ¹²	0.058 (OD ¹¹)
Uterine lavage	34-36	1742	Pyometra	ROC analysis	Cytology (>18% PMN)	0.059 (OD)

¹Endo cytobrush: Endometrial cytobrush.

²RFM: Retained fetal membranes.

³PVD: Purulent vaginal discharge.

⁴ROC analysis: Receiver-operating characteristic analysis.

⁵PMN: Polymorphonuclear leucocytes.

⁶Descriptive: Upper quartile of the distribution.

⁷Cerv cytobrush: Cervical cytobrush.

⁸LE: Leucocyte esterase reagent test strip (0 (no leukocytes), trace, + (small), 2+ (moderate), and 3+ (large)).

⁹Protein: Protein reagent test strip (0 (no proteins), trace, + (30 mg/dL), 2+ (100 mg/dL), 3+ (300 mg/dL), and 4+ (>2000 mg/dL)).

¹⁰pH: pH reagent test strip (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5).

¹¹OD: Optical density (wavelength = 620nm).

¹²Visual gross uterine discharge in uterine lavage fluid.

systematic reviews, the current used 2 assessors during the selection and assessment process (Arlt et al., 2010; Haimerl et al., 2012; Roy and Keefe, 2012). It is recommended to use a minimum of 2 assessors for the evaluation of quality to improve objectivity (Higgins and Green, 2011). Additionally, it is unclear if inclusion of more assessors would improve agreement. Simoneit et al. (2012) found slightly higher interobserver agreements following use of a checklist on bovine reproduction papers when assessed by 14 observers. However, observers only reviewed 3 pre-selected papers, and received additional information on use of the checklist, whereas for this review no additional information beyond the checklist was provided. In the absence of additional data about the effect of number of assessors on repeatability of quality, it is unclear if using more reviewers would have improved the paper selection and assessment process (Arlt et al., 2010). Also, no formal training was done to improve agreements among assessors in the current study. Thus the assessment of quality and statistical validity was reliant on the training and experience of those involved. On the other hand, all assessors were involved in the development and modification of the checklist. Additionally, some of the authors have previously published papers on literature assessments using different checklists (Arlt et al., 2010; Simoneit et al., 2012). In contrast, lower agreement among assessors may be attributable to unclear or incomplete reporting leading to difficulty in making an assessment more than to flaws in the assessment tool (Smidt et al., 2006b). It should also be noted that while kappa statistics are commonly calculated with 2 observers and dichotomous outcomes (e.g. diseased or not diseased) in the current study there were 6 possible scores (or 4 when combined). Hence, a score one unit apart is a relatively small difference, and does not necessarily reflect substantial variation in assessment of the level of quality of reporting in a paper. Although it is common not to publish assessment of agreement between assessors of quality (Siddiqui et al., 2005; Zafar et al., 2008; Fontela et al., 2009), the current study describes fair to

moderate agreement. Therefore, the level of agreement between assessors during the quality criteria assessment in the current study needs to be interpreted with some caution, although it is not clear if our assessments are any more divergent than is typical.

The majority of papers included in the current study did not provide data on all items of the checklist. The quality assessment of papers in our manuscript was modelled on the STRADAS-paraTB statement (Standards for Reporting of Animal Diagnostic Accuracy Studies for paratuberculosis; Gardner et al., 2011). That statement was in turn modified by independent experts from the STARD statement (Standards for Reporting of Diagnostic Accuracy) which aims to improve reporting of test accuracy studies in human medicine (www.stard-statement.org; Bossuyt et al., 2003). The impetus for the development of the STRADAS-paraTB was the review by Nielsen and Toft (2008) on diagnostic tests for paratuberculosis (Gardner et al., 2011). The conclusion of that review was that “the quality of design, implementation, and reporting of evaluations of tests for paratuberculosis was poor” (Nielsen and Toft, 2008). Others report similar concerns in other areas of veterinary and animal science (Arlt et al., 2010; Sannmann et al., 2012), as well as in human medical literature (Siddiqui et al., 2005; Zafar et al., 2008; Fontela et al., 2009).

Although the peer-review system is a good tool that enhances the quality of published manuscripts (Goodman et al., 1994; Purcell et al., 1998), this process has its limitations. The quality of papers varies even in peer-reviewed journals and acceptance for publication does not guarantee the completeness, clarity, or credibility of papers, even in journals with a high impact factor (Kastelic, 2006; Benos et al., 2007; Arlt et al., 2010). Hence, even published in peer-reviewed high-impact journals papers on reproductive diagnostic may lack sufficient information to allow critical appraisal. Besides the STARD and STRADAS-paraTB statements, other statements have been developed to improve publication standards, such as CONSORT (Schulz et al., 2010), STROBE (von Elm et al., 2007) and REFLECT (Sargeant et al., 2010). Despite discrepancies between assessors and the limitations of checklists when applied to

diverse study types (Smidt et al., 2006b), quality of reporting and design has improved in journals adapting these guidelines (Moher et al., 2001; Smidt et al., 2006a). Therefore, authors of future papers on diagnostic methods of reproductive-tract disease are advised to use a guideline to improve the clarity and consistency of study design and reporting.

2.4.3. Distribution of Diseases

In this review, the diseases were described on a pathological basis (i.e. inflammation of some part of the reproductive-tract). This approach was taken to ensure that diagnostic methods that encompassed clinical diseases (e.g. detection of grossly evident purulent material in the vagina) as well as 'subclinical' disease (e.g. definition of endometritis based on PMN% in endometrial cytology) would be included. Purulent vaginal discharge is a symptom or condition of an inflammatory process, as defined by a variety of diagnostic methods, rather than being a specific etiological or pathological diagnosis. Therefore, papers on diagnostic methods for PVD were included in papers describing vaginitis as this is where material is collected for assessment. Thus, even though PVD is often used as proxy for uterine inflammation, it is an assumption that the purulent material originates only from within the uterine lumen. Regardless of origin, it is identified clinically in the vagina. It is clear, from recent studies, that PVD is not always coincident with endometritis and may occur independently (Dubuc et al., 2010a). This suggests that the inflammation originates from the cervix or vagina. Also, even if the primary source of (muco)purulent material is the uterus, presence of such material in the vagina might induce vaginitis. Recent studies have clearly demonstrated that endometritis, cervicitis, and vaginitis (including PVD) are related, but not synonymous conditions (Dubuc et al., 2010a; b; Deguillaume et al., 2012).

The most common diagnosis described in the reviewed papers was endometritis (n = 45; 88%), followed by vaginitis (n = 29; 57%) and cervicitis (n = 4; 8%). It is only recently that a research interest has developed in the effects of inflammation of the cervix, which likely explains the relatively low number of

studies of this condition. No diagnostic studies were included on salpingitis and oophoritis. It is unlikely that these 2 diseases totally coincide with other reproductive-tract diseases; therefore, specific diagnostic methods may be required for their diagnosis. The prevalence of these conditions is assumed to be low, but diagnosis is difficult practically and methods for use in live animals have not been validated.

2.4.4. Distribution of Diagnostic Methods

Cervical diameter, uterine horn size, thickness of the uterine wall, and volume of intrauterine fluids can be assessed by transrectal palpation or ultrasonography (LeBlanc et al., 2002; Barlund et al., 2008), and were reported in 18 (35%) and 10 (20%) papers, respectively. Purulent material in the vagina may be visualized by the use of a speculum (LeBlanc et al., 2002), detected by the introduction and retraction of a clean gloved hand (gloved hand; Plöntzke et al., 2011; n = 5; 10%), or a stainless steel rod with a rubber hemisphere attached at the end (Metricheck, Simcrotech, Hamilton, New Zealand; McDougall et al., 2007; n = 11; 22%). Cervical and uterine samples to evaluate inflammatory cells or bacterial growth can be obtained by a cytobrush or swab (Yavari et al., 2009; Deguillaume et al., 2012; n = 21; 41%), cervical fluid aspiration or uterine lavage (Gilbert et al., 2005; Yavari et al., 2009; n = 13; 25%), and biopsy (Bonnnett et al., 1991; n = 6; 12%).

2.4.5. Cut-Points and Reported Cut-Point Analyses

Similar to many other diagnostic methods in medicine, there is no gold standard test available for reproductive-tract diseases in cows (Sheldon et al., 2006). This complicates the determination of cut-points as well as the evaluation of diagnostic tests (Gardner and Greiner, 2006). The recommended ROC analysis for the determination of cut-points (Gardner and Greiner, 2006) was used in half of the papers. However, without the availability of a gold standard, these papers instead used pregnancy by 90 to 150 DIM or > 10% PMN by uterine lavage as reference outcomes (Table 2.1). Reproductive performance as a

reference outcome has the advantage of being tangible and of economic importance, but it has the disadvantage of being influenced by a multitude of factors other than the disease condition of interest and it does not directly measure pathological processes. Muco-purulent vaginal discharge or a cervical diameter > 7.5 cm at > 20 DIM or PVD at > 26 DIM was predictive for non-pregnancy by 120 DIM (LeBlanc et al., 2002). Four other papers calculated cut-points using similar time to event (pregnancy) techniques and one divided the continuous scoring scale into quartiles (Table 2.1). Bayesian methods can also be used to determine cut-points (Choi et al., 2006). It is interesting to note that none of the studies used these methods, although McDougall et al. (2007) applied this approach for the assessment of the Metrichheck device. Prior assessment of likely test performance is used in Bayesian methodology (Gardner and Greiner, 2006). The Bayesian approach may be a more robust approach for using a distribution of possible values instead of a fixed (unknown) parameter used in classical methods (Enøe et al., 2000). Potential reasons for the limited use of Bayesian analysis is the difficulty to understand and implement these methods, and analytical convergence issues when the range of selected priors is not wide enough. However, it may be valuable to pursue these methods in future research.

2.4.6. Intra- and Interobserver Agreements

Of the 51 included papers, only one reported intra- and interobserver agreements of a diagnostic test (vaginocopy; Table 2.2). Moderate agreement was calculated between 3 operators, of which 'one' operator was a group of inexperienced veterinary students (Leutert et al., 2012). Within and between reader agreements and correlations for the evaluation of cytological microscope slides generated by samples from the cytobrush or uterine lavage techniques were determined by 6 studies (Table 2.2). These studies generally assessed the repeatability of multiple readings of a single slide created by a single cow-side operator at one time point. Hence, these assessments are limited to laboratory variability of the test and not complete methodology (e.g. the on-farm, between-cow, between-operator variability). Low repeatability is associated with lower Se and Sp of the diagnostic method. The

repeatability study of vaginocopy was performed in one herd and cows were examined at one time point (Leutert et al., 2012). Unfortunately, therefore no data are available for tests performed at different time points and between different populations.

Table 2.2. Reported intra- and interobserver agreements of diagnostic methods for reproductive-tract disease in papers (n = 7) included in a systematic review

Diagnostic method	DIM	Intraobserver		Interobserver			Reference
		Statistic ¹	Value	Number observers	Statistic	Value	
Vaginocopy	21 - 27	κ	0.55 - 0.60	3	κ	0.44	Leutert et al., 2012
Cytology slide (cytobrush) ²	35	κ	0.82	2	κ	0.77	Dubuc et al., 2010b
Cytology slide (cytobrush)	20 - 47	r	0.84	2	r	0.84	Kasimanickam et al., 2004
Cytology slide (cytobrush)	28 - 41	r	0.85				Barlund et al., 2008
Cytology slide (cytobrush)	29 & 43			2	r	0.82	McDougall et al., 2011
Cytology slide (uterine lavage) ²	40 - 60	κ	0.86				Gilbert et al., 2005
Cytology slide (uterine lavage)	28 - 41	r	0.76				Barlund et al., 2008
Cytology slide (both) ²	20 - 47			2	r	0.90	Kasimanickam et al., 2005

¹The Kappa statistic gives a value between -1 and 1, where ≤ 0 no agreement and 1 is perfect agreement beyond chance. The correlation coefficient (r) gives a value between -1 and 1, where -1 is perfect negative association and 1 is perfect positive association. Complete independence has a value of 0.

²Cytology slide: These studies assessed the repeatability of multiple readings of a single cytology slide created by a single cow-side operator at one time point using the cytobrush, uterine lavage, or both techniques. Hence these assessments are limited to laboratory variability of the test, and not the complete methodology (e.g. on-farm, between-cow, between-operator variability).

2.4.7. Comparison Among Tests

Agreement between various diagnostic methods was reported in 12 of the 51 included papers. In 11 of these papers, more than one agreement was assessed. Inter-test agreements between vaginocopy and other tests were reported in 5 papers, whereas agreements between Metricheck, ultrasonography, cytobrush, swab, uterine lavage, biopsy and other tests (e.g. leucocyte esterase test) were reported in 6,

Table 2.3. Reported agreements between diagnostic methods of reproductive-tract disease in papers included in a systematic review

Diagnostic method	Outcome	Comparison method	Outcome	DIM	Statistic ¹	Value	Reference
Metricheck	PVD ²	Vaginoscopy	PVD	33 ± 16 ³	κ	0.45	McDougall et al., 2010
Metricheck	VDS (0 – 5) ⁴	Vaginoscopy	VDS (0 – 5)	33 ± 16 ³	κ	0.27	McDougall et al., 2010
Metricheck	PVD	Vaginoscopy	PVD	7 to 28	κ	0.73	Runciman et al., 2009
Metricheck	VDS (0 – 3) ⁵	Vaginoscopy	VDS (0 – 3)	7 to 28	κ	0.59	Runciman et al., 2009
Metricheck	VDS (0 – 3)	Ultrasound	Uterine horn diameter	18 to 46	r	0.52 ⁶	Senosy et al., 2009
Metricheck	VDS (0 – 3)	Ultrasound	IUF ⁷ (yes/no)	18	r	0.49 ⁶	Senosy et al., 2009
Metricheck	VDS (0 – 3)	Cytobrush	% PMN ⁸	25 & 32	r	0.59	Senosy et al., 2009
Metricheck	VDS (0 – 5)	Cytobrush	≥ 6% PMN	35	κ	0.14 - 0.20	Dubuc et al., 2010a
Metricheck	VDS (0 – 5)	Cytobrush	≥ 9% PMN	28	κ	0.29	McDougall et al., 2010
Metricheck	VDS (0 – 5)	Cytobrush	≥ 7% PMN	42	κ	0.12	McDougall et al., 2010
Metricheck	VDS (0 – 5)	Cytobrush	≥ 8% PMN	28 to 41	κ	0.30	Peter et al., 2011
Vaginoscopy	PVD	Cytobrush	≥ 8% PMN	28 to 41	κ	0.52	Barlund et al., 2008
Vaginoscopy	VDS (0 – 3)	Cytobrush	≥ 5% PMN	21 to 27	r	0.30	Westermann et al., 2008
Vaginoscopy	VDS (0 – 3)	Cytobrush	≥ 18% PMN	21 to 27	r	0.30	Westermann et al., 2008
Vaginoscopy	PVD	Biopsy ⁹	Hist ¹⁰ score	28 to 35	r	0.36	Studer and Morrow
Vaginoscopy	PVD	Biopsy ¹¹	Hist score	28 to 35	r	0.42	Studer and Morrow
Ultrasound	IUF (> 3 mm)	Cytobrush	% PMN (2 cut-points) ¹²	20 to 42	κ	0.28	Kasimanickam et al., 2008
Uterine lavage	≥ 8% PMN	Cytobrush	≥ 8% PMN	28 to 41	κ	0.74	Barlund et al., 2008
Uterine lavage	≥ 8% PMN	Cytobrush	≥ 8% PMN	28 to 41	r	0.66	Barlund et al., 2008
Cervical LE ¹³	0 – 3 +	Uterine LE	0 – 3 +	21 to 47	κ	0.37	Couto et al., 2013
Metricheck	VDS (0 – 5)	Cytobrush	5 bacteria ¹⁴	29	κ	0.05	McDougall et al., 2010
Metricheck	VDS (0 – 5)	Cytobrush	5 bacteria	42	κ	0.00	McDougall et al., 2010
Vaginoscopy	PVD	Uterine swab	Bacteria	28 to 35	r	0.44	Studer and Morrow
Vaginoscopy	VDS (0 – 3)	Cytobrush	<i>T. pyogenes</i>	21 to 27	r	0.40	Westermann et al., 2008
Cytobrush	≥ 5% PMN	Cytobrush	<i>T. pyogenes</i>	21 to 27	r	0.42	Westermann et al., 2008
Cytobrush	≥ 18% PMN	Cytobrush	<i>T. pyogenes</i>	21 to 27	r	0.42	Westermann et al., 2008
Cytobrush	≥ 9% PMN	Cytobrush	5 bacteria	29	κ	0.14	McDougall et al., 2010
Cytobrush	≥ 7% PMN	Cytobrush	5 bacteria	42	κ	0.12	McDougall et al., 2010

Biopsy	Hist score	Uterine swab	Bacteria	28 to 35	r	0.27	Studer and Morrow
Biopsy	Hist score	Biopsy	<i>T. pyogenes</i>	26	r	0.25	Bonnett et al., 1993
Biopsy	Hist score	Biopsy	<i>T. pyogenes</i>	40	r	0.37	Bonnett et al., 1993
Biopsy	<i>T. pyogenes</i> ¹⁵	Biopsy	<i>T. pyogenes</i>	40	r	0.63	Bonnett et al., 1993
Biopsy	<i>T. pyogenes</i> ¹⁵	Biopsy	Hist lesions	40	r	0.27	Bonnett et al., 1993

¹The Kappa statistic (κ) gives a value between -1 and 1, where ≤ 0 no agreement and 1 is perfect agreement beyond chance. The correlation coefficient (r) gives a perfect negative association and 1 is perfect positive association. Complete independence has a value of 0.

²PVD: presence of purulent vaginal discharge with a dichotomous outcome (positive or negative).

³'At risk' cows (i.e. those with RFM, metritis, or twins i.e. a population in which a high prevalence of PVD would be expected) were assessed 35 days prior to the s

⁴VDS (0 - 5): vaginal discharge score = no mucus to > 50% purulent vaginal discharge and odor.

⁵VDS (0 - 3): vaginal discharge score = clear or translucent mucus to > 50% purulent vaginal discharge.

⁶Agreement only in cows with a corpus luteum.

⁷IUF: Intrauterine fluid.

⁸PMN: Polymorphonuclear leucocytes.

⁹Biopsy taken from right horn.

¹⁰Hist: Histopathological.

¹¹Biopsy taken from left horn.

¹²2 cut-points: > 18% PMN at 21-33 DIM and > 10% PMN at 34-47 DIM.

¹³LE test: Leucocyte esterase test [0 (no leukocytes), trace, + (small), 2 + (moderate), and 3 + (large)].

¹⁴*Trueperella pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica*, *Proteus* spp. or *Escherichia coli*.

¹⁵*Trueperella pyogenes* cultured at 26 DIM.

2, 7, 1, 1, 2, and 1 papers, respectively (Table 2.3). Agreement measures (i.e. κ and r) compare diagnostic methods, irrespective of the tests being correct or not. Sensitivity and Sp are measurements used for validation of diagnostic methods. Seven papers reported Se and Sp, with other diagnostic tests as the reference method (Table 2.4). These reference methods were previously validated as being associated with reproductive performance. The Bayesian approach for TAGS was used in one study to determine Se and Sp, and reported reasonable to high Se and Sp for vaginoscopy and Metricheck (Table 2.4).

Studies comparing vaginoscopy, Metricheck and ultrasonography with cytobrush as the reference test report overall low Se and moderate to high Sp (Table 2.4), indicating a high number of false negatives and a low number of false positives. For example, approximately half of the cows were PVD negative by vaginoscopy, but had a PMN score $> 8\%$ (Barlund et al., 2008). Se and Sp for vaginoscopy and Metricheck with cytobrush as the reference were similar, but were only described in 2 papers. The Sp in these 2 papers was slightly higher than calculated with the TAGS approach; whereas, Se analyzed with the TAGS approach was considerably higher. There is inconsistency regarding Se (poor to high) and Sp (mediocre to high) of ultrasonography relative to cytology (Table 2.4).

Controversy exists about the magnitude and direction of association between the bacterial species isolated from the uterus and the different diagnostic methods for PVD and PMN% as detected by the cytobrush technique. In some studies isolation of any bacteria, or even specific bacterial species, is not directly or consistently associated with the degree of inflammation within the uterus or vagina (Table 2.3); whereas, others found positive associations between isolation of specific bacterial species and PVD score (Studer and Morrow, 1978; Williams et al., 2005). Similar and positive correlations are reported for the agreement between the inflammation score for biopsies and isolation of bacteria (Table 2.3). The potential lack of association between bacteriology and other tests may not be surprising given the

different biological bases upon which the tests are based (i.e. assessing different elements of the immune or inflammatory response to bacterial infection and tissue trauma).

Table 2.4. Sensitivity (Se) and specificity (Sp) of tests for reproductive-tract disease relative to other, validated diagnostic tests reported in papers included in a systematic review (n = 7)

Diagnostic method	Reference method/outcome	DIM	Se	Sp	Reference
Vaginoscopy (≥ flecks of pus)	Bayesian TAGS ¹ approach	33 ± 16	72	87	McDougall et al., 2007
Vaginoscopy (≥ muco-purulent)	Cytobrush (> 8% PMN ²)	28 to 41	54	96	Barlund et al., 2008
Metricheck (≥ flecks of pus)	Bayesian TAGS approach	33 ± 16	96	78	McDougall et al., 2007
Metricheck (≥ flecks of pus)	Cytobrush (> 8% PMN)	28 to 41	44	89	Peter et al., 2011
Ultrasound (ET ³ > 7 mm)	Cytobrush (> 8% PMN)	28 to 41	23	75	Barlund et al., 2008
Ultrasound (ET > 8 mm)	Cytobrush (> 8% PMN)	28 to 41	4	89	Barlund et al., 2008
Ultrasound (IUF ⁴ > 1mm)	Cytobrush (> 8% PMN)	28 to 41	39	78	Barlund et al., 2008
Ultrasound (IUF > 3mm)	Cytobrush (> 8% PMN)	28 to 41	31	93	Barlund et al., 2008
Ultrasound (IUF present)	Cytobrush (2 cut-points ⁵)	21 to 47	88	62	Meira et al., 2012
Ultrasound (cervix > 5.0 cm)	Cytobrush (2 cut-points)	21 to 47	56	73	Meira et al., 2012
Biopsy (score < 15)	Cytobrush (2 cut-points)	21 to 47	44	92	Meira et al., 2012
Combination ⁶	Cytobrush (2 cut-points)	21 to 47	44	97	Meira et al., 2012
Uterine lavage (> 8% PMN)	Cytobrush (> 8% PMN)	28 to 41	92	94	Barlund et al., 2008
ULOSD ⁷ (> 0.058)	Pus IUF ⁸	34 to 36	76	78	Machado et al., 2012
ULOSD (> 0.059)	Uterine lavage (> 18% PMN)	34 to 36	100	82	Machado et al., 2012
LE ⁹ (> 2 +)	Cytobrush (> 10.2% PMN)	21 to 47	69	73	Couto et al., 2013
LE (> 2 +)	Uterine lavage (> 10% PMN)	40 to 60	77	52	Cheong et al., 2012
pH (7.0)	Uterine lavage (> 10% PMN)	40 to 60	45	79	Cheong et al., 2012
Protein (3 +)	Uterine lavage (> 10% PMN)	40 to 60	58	56	Cheong et al., 2012
LE (3 +) and pH (7.0)	Uterine lavage (> 10% PMN)	40 to 60	19	97	Cheong et al., 2012
NEFA (serum; ≥ 0.5 mmol/L)	Metricheck (PVD)	-7 to -1	54	53	Dubuc et al., 2010b
NEFA (serum; ≥ 1.0 mmol/L)	Metricheck (PVD)	1 to 7	41	66	Dubuc et al., 2010b
NEFA (serum; ≥ 0.9 mmol/L)	Metricheck (PVD)	8 to 14	43	64	Dubuc et al., 2010b
BHBA (serum; ≥ 1,100 μmol/L)	Metricheck (PVD)	1 to 7	28	84	Dubuc et al., 2010b
BHBA (serum; ≥ 700 μmol/L)	Metricheck (PVD)	8 to 14	59	48	Dubuc et al., 2010b
Haptoglobin (serum; ≥ 0.8 g/L)	Metricheck (PVD)	1 to 7	39	80	Dubuc et al., 2010b
Haptoglobin (serum; ≥ 0.3 g/L)	Metricheck (PVD)	8 to 14	47	67	Dubuc et al., 2010b

¹TAGS: Test(s) in absence of a gold standard.

²PMN: Polymorphonuclear leucocytes.

³ET: Endo thickness.

⁴IUF: Intrauterine fluid.

⁵2 cut-points: > 18% PMN at 21-33 DIM and > 10% PMN at 34-47 DIM.

⁶Combination: Ultrasound (intrauterine fluid present), ultrasound (cervix diameter > 5.0 cm) and biopsy (score < 15).

⁷ULOSD: Uterine lavage sample optical density.

⁸Pus IUF: Gross uterine discharge in uterine lavage fluid.

⁹LE: Leucocyte esterase test.

In the search for systemic diagnostic tests for reproductive-tract diseases, studies compared outcomes of reproductive tract-based diagnostic methods (e.g. PVD, cytology) with indirect or systemic tests such as hematology and biochemistry (Green et al., 2009), local and circulating concentrations of cytokines (Ishikawa et al., 2004; Kim et al., 2005; Fischer et al., 2010), acute phase proteins (Williams et al., 2005; Dubuc et al., 2010b), NEFA and BHBA (Dubuc et al., 2010b; Senosy et al., 2012) and hormones (e.g. progesterone and prostaglandin F₂α metabolite; Seals et al., 2002; Senosy et al., 2011). Although associations may be found among these tests, the direction of causality is not clear. For example, reproductive-tract inflammation may directly result in elevated acute phase protein concentrations. Alternatively, systemic inflammation may stimulate release of pro-inflammatory cytokines and acute phase proteins, which may contribute to impaired immune defenses or reproductive-tract inflammation. Hence, where such associations are found, these outcomes may not be specific enough to be a predictive test for reproductive-tract diseases. Additionally, intrauterine concentrations of cytokines may be more closely associated with uterine disease than the circulating concentrations (Galvão et al., 2011).

2.4.8. Complications with Comparison Among Tests

The determination of PMN% is most commonly used as the 'near-gold standard' test for the calculation of Se and Sp (Table 2.4). However, there are no data available that support this, and it seems that endometritis assessed by cytology is only one element of reproductive-tract inflammatory disease, along with PVD and cervicitis (Dubuc et al., 2010a; Deguillaume et al., 2012). Also, the use of different cut-points makes it difficult to compare test validation studies. Inflammation can also be evaluated by histopathological assessment of tissue obtained by biopsy (Bonnett et al., 1991). The extent of variability in intra- and interobserver repeatability of both tests in cows is unknown. Most papers do not describe how often, and with what pressure, the cytobrush is rolled on the cervix or endometrium. It can be hypothesized that this may influence the PMN:epithelial cell ratio depending on the depth of cells that

are removed. Moreover, the cytobrush and biopsy techniques sample only a small area. In horses, diagnosing endometritis using these techniques was not representative for the entire endometrium and seemed to have a low within-horse repeatability (Overbeck et al., 2013). It is unclear if this is also true for the bovine uterus. It has also been reported that up to 41% of the biopsies taken can be unsatisfactory for histological evaluation (Meira et al., 2012). This high failure rate may create bias; cows with more severe intrauterine pathology might be less likely to yield biopsy material that is considered assessable.

Uterine lavage may sample a larger area than the cytobrush. However, the period which fluid is left in the uterus, or how often and with what pressure the uterus is massaged have not been clearly reported. Similar to the pressure used with the cytobrush, it may be difficult to measure the pressure used while massaging the uterus in vivo. In one study, the operator was unable to recover uterine lavage fluid in 17% of the cases (Kasimanickam et al., 2005). In contrast, others have reported 100% successful sampling (Gilbert et al., 2005; Galvão et al., 2009). Kasimanickam et al. (2005) also reported a larger number of deformed cells recovered by uterine lavage in comparison to the cytobrush technique. Even though both techniques (cytobrush and uterine lavage) report the same outcome, PMN%, they are different methods. The cytobrush likely removes adhered endometrial and inflammatory cells, whereas uterine lavage may collect proportionally more cells that are free within the uterine lumen. Neither test is designed to evaluate the endometrium. The reported intra- and inter-cytology slide reader agreements suggest that the reading and scoring aspects of these techniques are robust (Table 2.2). Also, one study reported a high Se and Sp comparing uterine lavage with cytobrush as the reference test (Table 2.4). Unfortunately, no cytology slide reading protocols are described, which may influence comparison of test results between various studies.

Considering these limitations, the cytobrush, uterine lavage and biopsy technique are not perfect diagnostic methods. Therefore, when reference tests, such as PMN% determined by cytobrush or uterine lavage, and histopathological results by biopsy are used and errors of these tests are ignored, bias in the evaluation of the accuracy of test under evaluation is likely. Also, it is not surprising that the overall level of agreement between tests was only fair to moderate given the basis of the tests is different (Table 2.3). The low agreement in this case may be interpreted that one test is a poor surrogate for the other, likely because, despite previous assumptions, these techniques assess different aspects of reproductive-tract disease.

2.4.9. Test Validation Using Reproductive Outcomes

Although it could be argued that histopathological findings are the gold standard against which a clinical test should be assessed, there has been limited validation of histopathological techniques, either against other tests or against reproductive outcomes. For veterinary practitioners and producers, the use of reproductive outcomes either as a dichotomous outcome, e.g. pregnancy by an economically-based target interval, or as a continuous variable (e.g. calving to conception interval) seems the preferred reference for cut-point analysis and validation of reproductive-tract diagnostic methods where no gold standard exists. Diagnostic tests for reproductive-tract diseases in production animals have limited utility if there is no clinical impact or economic rationale. However, reproductive performance is not only influenced by reproductive-tract diseases. Confounders may have a negative impact on reproduction, e.g. poor heat detection, older cows, poor semen quality, diseases other than those of the reproductive-tract, management systems, and nutrition. Thus, these confounders need to be taken into account when using biological outcomes from field trials to validate diagnostic tests.

Tests for Grossly Evident Purulent Material in the Vagina. Six papers compared reproductive performance in cows with PVD to unaffected ones. Impaired reproduction outcomes were described in 5

of the 6 papers diagnosing PVD by vaginoscopy (n = 5), Metricheck (n = 1), and transrectal palpation determining the diameter of the cervix (n = 1) mainly between 14 and 35 days, but ranging from 7 and 60 DIM.

Cows with PVD required more inseminations per pregnancy (LeBlanc et al., 2002; Gautam et al., 2009), and had a decreased first service conception risk (LeBlanc et al., 2002; Runciman et al., 2009). A positive correlation has been reported between purulence score and time to conception ($r = 0.22$; $P < 0.05$; Studer and Morrow, 1978). A decrease in the proportion of cows pregnant by 6 wk after the mating start date (0.32 vs. 0.55; $P = 0.005$; Runciman et al., 2009), and an increase in time to conception of 119 to 151 days ($P = 0.001$; LeBlanc et al., 2002) and 120 to 325 days ($P < 0.001$; Gautam et al., 2009) was found in PVD negative vs. PVD positive cows, respectively. Cows with PVD had an increased time to pregnancy (LeBlanc et al., 2002; Gautam et al., 2010), and decreased pregnancy by 210 DIM (Gautam et al., 2009). No unfavorable reproduction effect of PVD was reported by one study, where PVD was diagnosed with a gloved hand between 18 and 52 DIM in 243 pasture-based cows (Plöntzke et al., 2011). Although this is only one study conducted in 3 herds, it highlights that further validation of this commonly used diagnostic method may be required, particularly relative to the timing of examination to calving and to first insemination.

The Se and Sp for various diagnostic tests validated with reproductive outcomes were reported in 6 papers and are described in Table 2.5. The reproductive outcome in all studies was the pregnancy status, but this was assessed at different DIM. It is often unclear whether pregnancy or non-pregnancy was used as the reference outcome. Only moderate Se (61 and 65%) and Sp (63 and 61%) are reported for vaginoscopy and Metricheck, respectively, in seasonal calving herds when using reproductive outcomes as the reference (Table 2.5). For example, the false positive and false negative rates following classification of cows based on PVD were approximately 40% using non-pregnancy by 6 wk after the

start of breeding season as the outcome variable. In contrast, poor Se (15 and 18%) and high Sp (90 and 92%) were found in non-seasonal herds for Metricheck at two different time points (Table 2.5). The combination of measuring cervical diameter by transrectal palpation and vaginoscopy had a poor Se (20%), i.e. only a few cows that were detected positive by either transrectal palpation or vaginoscopy were not pregnant by 120 DIM, and a high Sp (88%), i.e. many test negative cows were pregnant by 120 DIM (Table 2.5).

Table 2.5. Sensitivity and specificity of tests for reproductive-tract disease with reproductive outcomes as the reference outcome reported in papers included in a systematic review (n = 6)

Diagnostic method	Outcome	DIM	Se	Sp	Reference
Vaginoscopy (\geq muco-purulent)	Pregnant by 6 wk after PSM ¹	7 to 28	61	63	Runciman et al., 2009
Metricheck (\geq muco-purulent)	Pregnant by 6 wk after PSM	7 to 28	65	61	Runciman et al., 2009
Metricheck (\geq muco-purulent)	Pregnant by 120 DIM	35 \pm 3	18	90	Dubuc et al., 2010a
Metricheck (\geq muco-purulent)	Pregnant by 120 DIM	56 \pm 3	15	92	Dubuc et al., 2010a
Vaginoscopy and cervix diameter ²	Nonpregnancy beyond 120 DIM	20 to 33	20	88	LeBlanc et al., 2002
Cytobrush ($>$ 6.7% PMN ³)	Pregnant by 90 DIM	21 to 47	86	42	Couto et al., 2013
Cytobrush ($>$ 6% PMN)	Pregnant by 120 DIM	35 \pm 3	24	86	Dubuc et al., 2010a
Cytobrush ($>$ 4% PMN)	Pregnant by 120 DIM	56 \pm 3	17	91	Dubuc et al., 2010a
Cytobrush ($>$ 18% PMN)	Pregnant by 132 DIM	20 to 33	36	94	Kasimanickam et al., 2004
Uterine lavage ($>$ 10% PMN)	Pregnant by 210 DIM	40 to 60	79	43	Cheong et al., 2011

¹PSM: Planned start of mating; start of breeding season.

²Vaginoscopy and cervix diameter: Cut-points are muco-purulent discharge at $>$ 26 DIM diagnosed by vaginoscopy and cervix diameter $>$ 7.5 cm at $>$ 20 DIM diagnosed by transrectal palpation.

³PMN: Polymorphonuclear leucocytes.

Depending on the management system, i.e. seasonal and non-seasonal calving systems, different levels of Se and Sp may be optimal. In seasonally managed herds, cows not detected pregnant at the end of the breeding period will likely be culled. Therefore, using a diagnostic test with a high Se may be more important in seasonal herds than in non-seasonal herds as there is limited time to intervene. Hence, accepting a high false positive rate may be more cost effective than a high Sp. A diagnostic method with a low Se (for example, defining a high Metricheck score threshold as test positive) will result in fewer cows being test positive, and more truly diseased cows being incorrectly defined as test negative (i.e.

this cut-point increases the proportion of false negative results). These cows will not have the benefit of intervention and may be at higher risk of not conceiving by the end of the breeding program and thus being culled. In a non-seasonal herd, the same cow may get more time to conceive meaning the lack of Se may be less critical. The optimal cut-point for tests thus depends on the cost of the test, the Se and Sp of that test, the economic impact of false negative and false positive results, and the cost and efficacy of the treatments.

Cytological and Histopathological Testing Methods. Twelve papers reporting on diagnostic methods measuring inflammatory response by cytology or histopathology compared with reproductive outcomes were included in this review. Diagnosis was made by biopsy, cytobrush, uterine lavage, and leucocyte esterase test in 1, 6, 4, and 2 papers, respectively.

Of 6 papers diagnosing reproductive-tract inflammation by cytobrush, 5 reported impaired reproduction outcomes associated with increased proportions of PMN. Of these papers, one included cervical and uterine inflammation in the reproductive analysis (Deguillaume et al., 2012), whereas the other papers diagnosed uterine inflammation only (Kasimanickam et al., 2004; McDougall et al., 2011; Senosy et al., 2012; Couto et al., 2013). Cows were examined between 20 and 49 DIM. Cut-points for inflammation were between 5 and 9% in 4 papers, whereas, 1 paper derived higher cut-points (i.e. 10 and 18%) between 20 to 33 and 34 to 47 DIM (Kasimanickam et al., 2004).

Between 7 and 19 percentage-point reductions in first service conception risk are described in cows diagnosed with endometritis by cytobrush (Kasimanickam et al., 2004; Senosy et al., 2012). Cows diagnosed with endometritis took longer to conceive from start of breeding (13 to 23 days) and from calving (29 to 62 days) compared with unaffected cows (Kasimanickam et al., 2004; McDougall et al., 2011). A decrease in proportion pregnant by the end of the breeding season was described in cows with endometritis diagnosed by cytobrush at 28 and 42 DIM compared to those unaffected at these days

(McDougall et al., 2011). Also, in non-seasonal systems, reduced pregnancy rates were reported (Kasimanickam et al., 2004; Deguillaume et al., 2012; Couto et al., 2013). In contrast, in one study in pasture-based herds, no differences in reproductive performance were found between cows affected with endometritis diagnosed by cytobrush compared to those unaffected (Plöntzke et al., 2010).

All 4 studies on endometritis diagnosed by uterine lavage reported unfavorable reproduction outcomes. Cows were examined between 21 and 60 DIM. Cut-points for inflammation were between 4 and 10% PMN (Gilbert et al., 2005; Galvão et al., 2009; Bacha and Regassa, 2010; Cheong et al., 2011). A 25 percentage-point reduction in first service conception risk was observed in cows with endometritis diagnosed by uterine lavage (Gilbert et al., 2005; Bacha and Regassa, 2010). Also, affected cows took 30 to 88 days longer to conceive (Gilbert et al., 2005; Galvão et al., 2009; Cheong et al., 2011). Cows with endometritis were less likely to be pregnant by 180 and 300 DIM (Gilbert et al., 2005; Bacha and Regassa, 2010).

Couto et al. (2013) found no associations between a leucocyte esterase test performed on cytobrush samples taken from the cervix and the uterus on reproductive outcomes over a range of cut-points. The testing procedure involved suspending the cytobrush in a small volume of saline. Subsequently, a leucocyte esterase test strip was dipped in the saline. It is unclear what the dilution effect may have been using this method. In contrast, multiparous cows that were test-positive ($\geq 3+$) following testing of uterine lavage fluid with leukocyte esterase test took 39 days longer to conceive compared with cows below this cut-point (Cheong et al., 2012). Further validation of the leucocyte esterase test is required.

Complications with Cytological and Histopathological Testing Methods. The use of different cut-points and diagnostic techniques makes it challenging to compare the association between PMN% and reproductive outcomes. Moreover, no consistent reproductive outcome variable is reported, partially because of the use of different outcomes in different management systems. It is also unclear when an

outcome variable was not reported that this implied that no difference was found, and therefore was assumed not to be a valuable result, or if that the variable was not analyzed. This problem exists due to lack of clarity in reporting study methods. Additionally, several studies have excluded cows diagnosed with PVD. Using methods to diagnose 2 potentially different diseases (Dubuc et al., 2010a; b), this effectively is testing cows in series (i.e. performing diagnostic tests one after the other). Therefore, measurements of reproductive outcomes will be biased by excluding cows with PVD. Future validation studies on diagnosing endometritis using only cytobrush or uterine lavage should include all cows but specifically report on those affected with PVD. In contrast, Se and Sp of identifying cows with reproductive-tract disease may increase by combining tests (Barlund et al., 2008; Dubuc et al., 2010a); however, limited data are available on this. The optimal test strategy in clinical practice may be to use a rapid, inexpensive method with high Se for whole herd testing, followed by testing disease positive cows with a more specific test.

The only study that did not find any effect of increased endometrial PMN on reproductive outcomes (Plöntzke et al., 2010) excluded cows with PVD, and the chosen cut-point (5% PMN) at 18 to 38 DIM may be too low. However, studies that did find a difference in some, but not all, reproductive outcomes used similar cut-points at similar sampling times (8.5, 5, and 5% PMN diagnosed at 3, 4, and 5 wk postpartum, respectively; Galvão et al., 2009; Bacha and Regassa, 2010; Senosy et al., 2012). Of these studies, Galvao et al (2009) included cows with PVD and found examination at 35 and 49 DIM using 6.5 and 4.0% cut-points, respectively, to be predictive for reproductive failure. Another study that excluded cows with PVD did not find a cut-point for PMN% that affected pregnancy by 90 DIM, when cows were examined between 21 and 31 DIM, whereas those examined between 32 and 47 DIM with > 6.7% PMN had a decreased pregnancy rate (Couto et al., 2013).

Variation exists in the reported Se and Sp of the cytobrush and uterine lavage techniques assessed against the proportion of cows pregnant at given times. Poor (< 36%) Se and good (> 86%) Sp for predicting pregnancy by 120 and 132 DIM were found for cytobrush results at 3 different cut and time-points. Predicting pregnancy by 90 DIM by cytobrush or by 210 DIM by uterine lavage had reasonable sensitivities (79 and 86%), but poor specificities (42 and 43%; Table 2.5). Only one study calculated Se and Sp of uterine lavage with pregnancy status at 210 DIM as the reference outcome, whereas 4 studies measured Se and Sp for the cytobrush technique with similar reference outcomes at different DIM. Also, different cut-points for endometritis were used. With the available data, it is not possible to determine which of these 2 diagnostic tests is a better predictor of reproductive performance. However, the present data indicate that > 5% PMN in the uterus after 4 wk postpartum was associated with worse reproductive performance. Before this time-point, many cows may be included with physiological inflammation that might be associated with the process of postpartum uterine involution.

Bacteriological Tests. Uterine bacterial growth was compared with reproduction performance in 2 papers included in this systematic review. Isolation of *Trueperella pyogenes*, coliforms, or *Streptococci* from the uterus increased the number of services required per conception (3.53, 3.45, and 3.36, respectively) in comparison with no bacterial growth (2.14; $P < 0.05$; Studer and Morrow, 1978). Conversely, bacterial infection of the uterus with *T. pyogenes*, *Fusobacterium necrophorum*, *Prevotella melaninogenica*, *Proteus spp.* and *Escherichia coli* did not influence reproductive performance in a study conducted 3 decades later (McDougall et al., 2011). However, technical difficulties with bacteriology of uterine samples (i.e. presence of multiple bacterial species including aerobes and anaerobes as well as Gram-positive and negative isolates) and the generally small number of cases assessed mean that associations may have been missed. Recent development of (meta)genomic tests that allow multiple bacterial species to be detected without the cost and time associated with culture may allow re-assessment of these relationships (Santos and Bicalho, 2012). Additionally, studies not selected for this

systematic review have reported decreased reproductive performance in cows with uterine infection with *T. pyogenes* (Bonnett et al., 1993; Huszenicza et al., 1999). In contrast, others did not find these associations, and only described decreased reproductive performance in cows infected with *E. coli* possessing certain virulence factors (Bicalho et al., 2010; Bicalho et al., 2012). Further investigations on bacteriological tests and the association between bacterial isolation and reproductive failure are needed.

2.4.10. Practical Applications of Diagnostic Tests

The utility of a test will depend on the purpose of the test, e.g. if the test is being used in a research or clinical context, and if an effective therapy is available. Treatment efficacy will also influence the time of examination. The use of tests which require penetration of the cervix are likely to have more limited application in the clinical environment due to the time and skill required and the requirement for laboratory support for subsequent testing. In the search for new diagnostic methods, a cow-side test, e.g. on milk or blood, using systemic and reproductive specific biomarkers appear attractive options for further investigation. Costs, sampling time, on-farm convenience of the diagnostic method, requirement for laboratory skills, laboratory costs, and time to report are important considerations to justify examination of reproductive-tract disease in clinical practice. For example, the Metrichick method is faster and easier than a vaginal speculum (McDougall et al., 2007; Runciman et al., 2009). Unfortunately, there are no data available on the economics of various diagnostic methods. However, it is likely that some reduction of Se and Sp in the clinical environment is acceptable to reduce the costs and test-result turn-around time.

2.5. CONCLUSIONS

Various reproductive-tract diseases and diagnostic methods have been described in the literature. However, the quality of reporting of disease definitions, validation and diagnostic methods is

inconsistent and generally low. The majority of the papers reviewed did not contain enough information to thoroughly assess of the validity of the tests used. Hence future authors are encouraged to use a checklist for quality of design and reporting as a guide to improve the clarity, completeness and utility of their manuscripts.

Based on the evaluated literature, vaginoscopy or Metricheck are likely to remain the preferred cow-side diagnostic methods for detecting reproductive-tract disease in the clinical environment. However, further studies are required that meet the criteria of high quality research. The ideal time for diagnostic examination and which cut-point to use, may depend on management system (e.g. seasonal and non-seasonal calving systems). Cytological assessment of endometritis using current methodology may be better suited to research, where economical and time factors may be less stringent. However, development and validation of simple, inexpensive “point-of-care tests” would be highly desirable. Between 35 and 40 DIM, uterine cytology with > 5% PMN cut-point is generally associated with impaired reproductive outcomes. It was outside the scope of this review to perform a meta-analysis on cut-points and time of examination, but as more data become available, that may be helpful.

No gold standard test is available for reproductive-tract disease. Therefore, the use of reproductive outcomes in clinical trials is the most logical way to validate tests. To improve comparisons between studies, authors are encouraged to report more reproductive outcomes including those without significant differences. Use of newer statistical techniques, like the Bayesian approach for TAGS, may be a potential path to improve the understanding of the validity of current and future tests. Furthermore, more data on intra- and interobserver agreement are needed to determine the precision and sources of variability of the evaluated diagnostic methods. Additionally, further work is needed to more clearly optimize the timing of diagnosis relative to calving and to breeding, and to establish diagnostic cut-points and criteria in this context. Cut-points may vary between different management systems (e.g.

housing conditions and milk yield), and approaches that define the optimal Se and Sp in the different production systems are topics for future research. To be able to improve uterine health and reproductive performance in dairy cows, a better understanding of diagnostic methods is required. Such progress will be aided by rigorous, comprehensive, clear, and consistent reporting of study methods and outcomes.

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2.7. CONFLICTS OF INTEREST

Nothing to disclose.

2.8. REFERENCES

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Chapter 3

Associations between intrauterine bacterial infection, reproductive tract inflammation and reproductive performance in pasture-based dairy cows

Submitted to:

Theriogenology

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3.1. ABSTRACT

Reproductive tract bacterial infections, particularly those caused by *Escherichia coli* and *Trueperella pyogenes* can have a negative impact on reproductive performance. It has been hypothesised that presence of *E. coli* early postpartum may increase the risk of isolation of *T. pyogenes* later postpartum. The objective of the current study was to examine associations between intrauterine bacterial infections with *E. coli* and *T. pyogenes* and any bacterial growth (irrespective of bacterial species), purulent vaginal discharge (PVD), cytological evidence of endometritis (an increased proportion of polymorphonuclear cells (PMN)) and reproductive performance. Dairy cows (n = 272) from six herds were examined at Days 0 (median: 2 d in milk), 21 and 42 postpartum. From each cow two intrauterine samples were collected via triple guarded cytobrush at Days 0 and 21. The first cytobrush was used for bacteriological culture. *Escherichia coli* and *T. pyogenes* were isolated by culture, and *E. coli* isolates were assigned to 1 of 4 phylogenetic groups using a two-step triplex polymerase chain reaction (PCR). In addition, *T. pyogenes* was confirmed by PCR. The second cytobrush was used to prepare a cytology slide. Nucleated cells (n = 200) were categorized as epithelial cells, PMN or macrophages. Cows were also assessed for body condition score, PVD score, presence of a corpus luteum and pregnancy. Statistical analysis was performed using multivariable models. There was no association between presence of *E. coli* at Day 0 and probability of isolation of *T. pyogenes* 3 wk later, however *E. coli* positive cows at Day 0 were more likely to be diagnosed with *E. coli* at Day 21 (RR = 2.0, P < 0.01). *E. coli* at Day 0 or *T. pyogenes* at Day 21 increased the risk of PVD diagnosis 3 wk later (RR = 1.9; P = 0.04 and RR = 3.0; P = 0.05, respectively). Cows with any bacterial growth at Day 21, irrespective of species, were less likely to conceive within 3 wk after the start of the seasonal breeding program (RR = 0.8; P = 0.05). Interestingly, cows with $\geq 25\%$ PMN at Day 0 had shorter time to pregnancy (HR = 1.32; P = 0.05). Intrauterine bacterial infection may impair reproductive performance but presence of *E. coli* was not associated with isolation of *T. pyogenes*

3 wk later. Increased endometrial flux of PMN in cows early postpartum may be a physiological process and improve reproductive performance.

Keywords: Escherichia coli, Trueperella pyogenes, purulent vaginal discharge, endometritis, polymorphonuclear cells, conception

3.2. INTRODUCTION

Postpartum, most cows have bacteria present in the uterus. However, a majority of cows undergo self-resolution with time (McDougall et al., 2007). A wide diversity of bacterial species are present in the uterus of cows, but differences in bacterial species isolated occur between healthy cows and those with reproductive tract disease (Santos and Bicalho, 2012). Bacteria which are considered potentially pathogenic to uterine tissue include *Escherichia coli* and *Trueperella pyogenes* (Williams et al., 2005). Risk factors for uterine disease include low body condition score (BCS) at calving, hyperketonemia, and peripartum disease (McDougall et al., 2007; Dubuc et al., 2010b).

Endometritis may be defined as cytological evidence of inflammation of endometrial tissue which is associated with reduced reproductive performance (De Boer et al., 2014a). Recent studies have demonstrated that purulent vaginal discharge (PVD) may be associated with, but is not identical to endometritis (Dubuc et al., 2010a). Therefore, a diagnosis of PVD is applied to cows with grossly evident purulent material in the vagina, whether or not there is evidence of endometritis as defined by cytology (De Boer et al., 2014a). Purulent vaginal discharge can be diagnosed by retrieval of purulent material from the vagina by placement of a gloved hand (Plöntzke et al., 2011) or Metricheck™ device (McDougall et al., 2007) into the vagina, or visualization by vaginoscopy (Leutert et al., 2012). The Metricheck device is the quickest and most straightforward technique (McDougall et al., 2007). Endometritis may be diagnosed by endometrial biopsy (Bonnett et al., 1991). Uterine cytology following

sample collection by cytobrush or uterine lavage are now frequently used, at least in the research environment (De Boer et al., 2014a). The cytobrush technique collects cells for cytology by rolling a small brush against the endometrium (Kasimanickam et al., 2005).

The same sample collected to define the degree of uterine inflammation may also be used for bacteriology (Bonnett et al., 1991; Santos and Bicalho, 2012; Werner et al., 2012). Isolation of some species of bacteria from the uterus early postpartum have been associated with reduced dominant follicle growth rate, reduced oestradiol concentrations and a smaller corpus luteum following ovulation (Williams et al., 2007). However, not all studies have found associations between isolation of bacteria and reproductive outcomes (McDougall et al., 2011). Specific strains or virulence factors of *E. coli* seem to be more pathogenic than others (Sheldon et al., 2010; Bicalho et al., 2012), and intrauterine presence of a specific virulence factor for *E. coli* (*fimH*) was associated with reduced pregnancy risk (Bicalho et al., 2012). Cows infected with *T. pyogenes* took longer to conceive compared to those without *T. pyogenes* (Bonnett et al., 1993). The presence of *T. pyogenes* has also been associated with an increased influx of inflammatory cells in uterine tissue (Bonnett et al., 1991) and a higher risk of PVD (Williams et al., 2005).

The prevalence of *E. coli* decreases and that of *T. pyogenes* increases with time postpartum (Williams et al., 2007; Werner et al., 2012). Additionally, a small study in which approximately half of the cows had retained foetal membranes (RFM) found an association between *E. coli* within 2 d postpartum and *T. pyogenes* at 14 DIM (days in milk; Dohmen et al., 2000).

The primary objective of this study was to test the hypothesis that the presence of *E. coli* within the first wk postpartum increases the risk of subsequent isolation of *T. pyogenes*. Secondary objectives were to assess associations between early isolation of *E. coli*, *T. pyogenes* or any bacteria (i.e. isolation of any bacterial species irrespective of type) and subsequent isolation, reproductive tract inflammation measured by PVD or polymorphonuclear cells (PMN) in endometrial cytology, and reproductive

performance. The final objectives were to assess associations between early reproductive tract inflammation and subsequent inflammation, and reproductive performance.

3.3. MATERIALS AND METHODS

Animal ethics approval was obtained prior to study commencement (AgResearch Animal Ethics Committee; Ruakura; Hamilton; New Zealand; application number: 12395).

3.3.1. Herds and Cows

Dairy cows ($n = 272$) from six commercial herds in the Waikato region of New Zealand were enrolled between 18 July and 12 August 2011 (Table 3.1; Figure 3.1). Data collection ended 7 March 2012, the date of the last pregnancy diagnosis. The enrolment of herds was on a convenience basis, with herds being recruited based on willingness to participate, agreeing to the study protocol and allowing access to herd- and cow-level records. Cows were managed in spring-calving, predominantly pasture-fed herds. At each enrolment day (Day 0), herd owners were asked to present multiparous cows calved between 1 and 4 d for veterinary examination. Cows with peripartum disease (e.g. dystocia, retained foetal membranes) were included, but cows were excluded if an antibiotic or anti-inflammatory treatment had been administered ≤ 30 d before enrolment or if a rectal temperature > 39.5 °C was recorded at the initial examination. When ≤ 25 cows were available on a given enrolment day (maximum two days for each herd), all cows that met the enrolment criteria were enrolled. When > 25 were available, the first or second cow in the row was selected for exclusion by flip of a coin. Thereafter, every second cow in the row was excluded from the study until in total 25 cows remained. Study cows ($n = 16/272$) that received any antimicrobial or anti-inflammatory treatment between Days 0 and 21 were excluded.

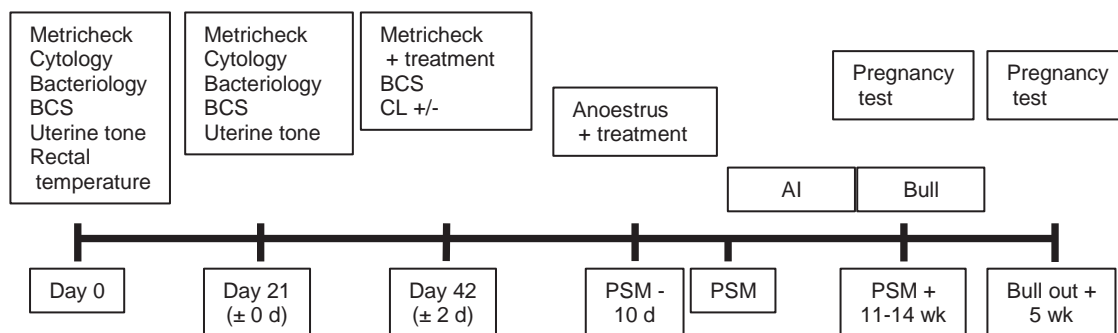


Figure 3.1. Study design to assess associations between intrauterine bacterial isolation (Bacteriology), reproductive tract inflammation as diagnosed by Metricheck and cytobrush (Cytology), and reproductive performance in pasture-based dairy cows (n=272) from six herds bred from the start of the seasonal breeding programme (planned start of mating; PSM).

3.3.2. Sampling Methods

Cows were sampled on Days 0, 21 (range: ± 0 d) and 42 (± 2 d). Day 21 cytobrush samples were taken by one of two veterinarians (MdB and SL), while all other samples, including all Day 0 and 42 samples, were taken by the one veterinarian (MdB). On Day 0, the following measurements and samples were collected from each study cow: rectal temperature, uterine tone score (firm vs. large/flaccid without striations), BCS on a 1 to 10 scale (Roche et al., 2004), vaginal discharge score (VDS) evaluated using a Metricheck device (Simcro Tech, Hamilton, New Zealand) and two intrauterine samples using cytobrushes (Ebos Group Ltd, Auckland, New Zealand). A VDS of 0 was given when no mucus was visible; a score of 1, 2, 3, 4, or 5, was given when clear mucus, flecks of purulent discharge, more than flecks but less than 50% purulent discharge, more than 50% purulent discharge, more than 50% purulent discharge plus odour, respectively, was visualized (McDougall et al., 2007). Two intrauterine samples were collected per cow using a triple-guarded cytobrush as previously described (Barlund et al., 2008). The first cytobrush was used for bacterial isolation and was immediately placed in transport media (Amies Agar Gel, Fort Richard, Auckland, New Zealand) and stored on ice. After moving the catheter slightly

forward, a second cytobrush sample was taken for cytology. After retraction, this brush was rolled gently along a clean microscope slide. All measurements, with the exception of rectal temperature were repeated on Day 21. On Day 42, BCS and VDS were recorded, and presence of a CL was determined using transrectal ultrasonography (BCF Ultrasound, Auckland, New Zealand). The presence and stage of gestation was determined using transrectal ultrasonography between 10-12 wk after the PSM and again at approximately 6 wk after the bulls were removed from the herd.

On Day 42, following common practice in New Zealand (McDougall et al., 2007), each study cow with a VDS ≥ 2 was treated with 500 mg cephalixin i.u. (MSD Animal Health, New Zealand; Table 3.1). Day 42 was a median of 29 d (range: 19 to 37 d) before the start of the seasonal breeding program (planned start of mating; PSM). Cows not detected in oestrus by removal of tail paint 10 d before PSM were treated with a combination of the Ovsynch protocol and a vaginal progesterone insert at the PSM (anoestrus treatment), followed by set time AI (McDougall, 2010). From PSM cows were artificially inseminated based on detection of oestrus using tail paint for 4 to 8 wk, followed by a period where bulls were run with the herds (Table 3.1).

3.3.3. Bacteriology

The first cytobrush was used to streak a 0.1% esculin, 5% sheep blood agar plate and a MacConkey agar plate (Fort Richard, Auckland, New Zealand) on the day of sampling. The blood agar plate was placed into a sealed GasPack™ container (BD, Auckland, New Zealand) with a CO₂-enriched atmosphere. The MacConkey plate was incubated aerobically. Bacterial growth of each plate was assessed after 2 d of incubation at 37 °C. Culture positive plates, irrespective of bacterial species were recorded as having bacterial growth. Of the cultured bacteria, only *E. coli* and *T. pyogenes* were typed. The identity of presumptive *E. coli* and *T. pyogenes* was confirmed on the basis of colony morphology, Gram stain and biochemical tests (Quinn et al., 1999). Following subculture, each purified isolate was inoculated into 2

Table 3.1. Descriptive statistics of number of cows (%) used for intrauterine sample collection at Day 0 (median 2 d in milk), Day 21 and Day 42 in six commercial spring-calving dairy herds in Waikato, New Zealand

Variable	Herd						Total
	1	2	3	4	5	6	
Herd size ^a	560	329	430	515	690	830	3354
Cows enrolled	50	25	47	50	50	50	272
Cows included in analyses	47	23	41	48	49	45	253
Cow age (years)							
Min	3	3	4	3	3	3	3
Median	6	5	5	5.5	5	5	5
Max	13	13	11	12	11	10	13
Breed							
Friesian	31 (66)	20 (87)	10 (24)	16 (33)	0 (0)	40 (89)	117 (46)
Jersey	2 (4)	0 (0)	3 (7)	6 (13)	45 (92)	0 (0)	56 (22)
Crossbreed	14 (30)	3 (13)	28 (68)	26 (54)	4 (8)	5 (11)	80 (32)
DIM ^b at Day 0							
Min	1	1	1	1	1	1	1
Median	3	2	2	2	2	2	2
Max	5	3	5	5	7	4	7
PSC to calving ^c							
Min	4	6	8	7	3	3	3
Median	6	7	14	12	6	10	10
Max	14	8	22	16	13	14	22
Diagnosed with PVD ^d							
Day 0	7 (15)	-	1 (2)	9 (19)	4 (8)	3 (7)	24 (10)
Day 21	4 (9)	1 (4)	6 (15)	10 (21)	12 (25)	4 (9)	37 (15)
Day 42	5 (11)	1 (4)	2 (5)	3 (6)	3 (6)	4 (9)	18 (7)
CL Day 42	22 (47)	14 (61)	31 (76)	24 (50)	19 (39)	27 (60)	137 (54)
Breeding ^e (d)	89	96	80	88	98	92	NA
AI ^e (d)	40	55	30	32	35	46	NA
Bull ^e (d)	49	41	50	56	63	46	NA
Anoestrus ^f	23 (49)	4 (17)	4 (10)	6 (13)	14 (29)	3 (7)	54 (21)
AI ≤3 wk ^g	19 (79)	14 (74)	35 (95)	40 (95)	33 (94)	36 (86)	177 (89)
Preg ≤3 wk ^h	30 (64)	14 (61)	25 (61)	28 (58)	30 (61)	23 (51)	150 (59)
Conc to S1 ≤3 wk ⁱ	29 (69)	14 (78)	21 (54)	27 (59)	27 (57)	23 (59)	141 (61)
Preg ≤6 wk ^h	35 (75)	17 (74)	36 (88)	38 (79)	39 (80)	33 (73)	198 (78)
Final preg ^j	43 (92)	22 (96)	40 (98)	46 (96)	47 (96)	41 (91)	239 (95)

^a Number of dairy cows at the start of the calving period.

^b DIM = days in milk.

^c Days from planned start of the calving period to actual calving of the enrolled cows.

^d PVD = Purulent vaginal discharge (flecks of pus or more) detected by Metrichcek™ device.

^e Total length of the breeding program (Breeding; median: 91 d) consisted of a period of AI (median: 37.5 d), followed by natural breeding (Bull; median: 50 d).

^f Cows not detected in oestrus by tail paint removal within one month prior to the start of the breeding program.

^g Cows submitted for AI within the first 3 wk of the start of the breeding program. Cows treated for anoestrus are not included in this variable.

^h Pregnant within the first 3 or 6 wk of the breeding program.

ⁱ Conception to first service within the first 3 wk of the breeding program.

^j Pregnant by the end of breeding program.

ml brain heart infusion broth with 20% glycerol and stored at -20 °C for further analyses. To determine phylogenetic groups A, B1, B2 and D, *E. coli* isolates were subjected to a two-step triplex PCR using primers targeting the genes *chuA* and *yjaA* and the DNA fragment TspE4.C2 (Table 3.2). Confirmation of *T. pyogenes* isolates was undertaken using PCR using primers targeting the genes *plo* and *sodA*, and the 16S-23S rDNA intergenic spacer region (Table 3.2).

3.3.4. Cytology

Slides were stained with a modified Wright-Giemsa stain (Diff-Quick, New Zealand Veterinary Pathology Ltd, Hamilton, New Zealand), air dried, and stored. Two hundred nucleated cells (i.e. epithelial cells, PMN and macrophages; MAC) were counted at a 400x magnification by one veterinarian (MdB). Percentages of PMN (PMN%) and MAC (MAC%) were calculated by dividing the number of PMN or MAC from the total number of the identified nucleated cells. A subset of slides (n=53; 10%) were randomly selected and evaluated twice to assess intraobserver repeatability.

3.3.5. Power Statistics

It was anticipated that the prevalence of intrauterine *E. coli* within 4 d postpartum would be 25% (Griffin et al., 1974), and that there would be a critical difference of 20% points in prevalence of *T. pyogenes* between exposed (*E. coli* positive) and non-exposed (*E. coli* negative), i.e., 57% *T. pyogenes* in cows that had *E. coli* just after calving and 37% *T. pyogenes* in non-exposed (i.e. $0.25 \times 57\% + 0.75 \times 37\% = 42\%$; Griffin et al., 1974). With a ratio of 25%:75% = 1:3 of exposed vs. non-exposed, a sample size of 220 cows was needed yielding 55 exposed and 165 non-exposed (80% power, P = 0.05, 2-sided).

3.3.6. Analysis

Data were collected in a purpose-built SQL database (Microsoft SQL Server Management Studio 2008, Seattle, WA) and transferred for statistical analyses into Stata 13.1 (StataCorp, College Station, TX, USA).

To assess any potential bias, cows removed from the study were compared with those not removed using ANOVA and χ^2 statistics for continuous and categorical variables, respectively, for herd, age, breed, DIM at Day 0, and presence of *E. coli*, *T. pyogenes*, PMN%, and MAC% at Days 0 and 21. Intraobserver variability of the estimate of PMN percentage from the cytology slides was assessed using Lin's concordance coefficient.

Table 3.2. PCR primers used for the phylogenetic grouping of *E. coli* and the identification of *T. pyogenes*

Primer	Direction	Sequence (5' – 3')	Size of PCR product (bp)	References
chuA	Forward	GACGAACCAACGGTCAGGAT	279	Clermont et al. 2000
	Reverse	TGCCGCCAGTACCAAAGACA		
yjaA	Forward	TGAAGTGTCAGGAGACGCTG	211	Clermont et al. 2000
	Reverse	ATGGAGAATGCGTTCCTCAAC		
TSPE4.C2	Forward	GAGTAATGTCGGGGCATTCA	152	Clermont et al. 2000
	Reverse	CGCGCCAACAAAGTATTACG		
plo	Forward	GGCCCGAATGTCACCGC	270	Jost et al. 2002
	Reverse	AACTCCGCCTCTAGCGC		
sodA	Forward	CGAGCTCGCCGACGCTATTGCT	199	Hijazin et al. 2011
	Reverse	GAGCATGAGAATCGGGTAAGTGCCA		
ISR ^a	Forward	GTTTGCTTGATCGTGGTGGTTATGA	122	Ulbeigi-Mohyla et al. 2010
	Reverse	AAGCAGGCCACGCGCAGG		

^a ISR = 16S-23S rDNA intergenic spacer region.

For multivariable analyses, the following predictor variables were considered: age (categorized into 3 and 4, 5 and 6, >6 years); breed (categorized into Friesian (>11/16th Friesian), Jersey (>11/16th Jersey) and the remaining defined as crossbreed); time from the herd's planned start of calving to the cow's actual calving date (days); DIM at Day 0; rectal temperature at Day 0; uterine tone (categorized as firm or large/flaccid) at Days 0 and 21; BCS (categorized as ≤ 4 and > 4) at Days 0, 21 and 42; presence or absence of *E. coli* at Days 0 and 21; presence or absence of *T. pyogenes* at Days 0 and 21; presence or absence of any bacterial growth, irrespective of species, at Days 0 and 21; PVD categorized as absent

(i.e. VDS ≤ 1) or present (i.e. VDS >1) at Days 0, 21 and 42; PMN% at Days 0 and 21; MAC% at Days 0 and 21; presence or absence of a CL at Day 42, and detection of oestrus (yes or no) by PSM. Isolated *E. coli* were categorised into phylogenetic groups A, B1, and B2/D. Phylogenetic groups B2 and D are extra-intestinal pathogenic strains with multiple virulence factors (Picard et al., 1999), and due to the low proportion of group B2, this group was grouped with group D strains for analysis. Dystocia was not included in the analyses, because one herd (n=45) failed to record this and due to the low incidence in the remaining herds (<1%; n=2/208).

Reproductive tract infection was defined as isolation of *E. coli*, *T. pyogenes*, or any bacterial growth (irrespective of species), whereas reproductive tract inflammation was assessed by the variables PVD or PMN%. The following associations were assessed: uterine infection status at each sample day with infection status on subsequent days, with subsequent reproductive inflammation, with anoestrus treatment and reproductive performance. Additional assessed associations were reproductive tract inflammation at each sample day with subsequent days, anoestrus treatment and reproductive performance. The Days 21 and 42 and the reproduction variables listed in Figure 3.2 were considered as the outcome variables for multivariable analyses. Variables were added to multivariable binary logistic and linear regression models using manual forward stepwise regression and retained if the P-value was ≤ 0.10 or if it caused a change in one of the coefficients by $> 20\%$. Model fit was determined using Akaike's information criterion. Herd was included as a fixed effect in each model. Interaction terms were evaluated where biologically plausible. Relative risk (RR) is preferred over odds ratios (OR) when the outcomes are common, because OR can be a considerable overestimation of associations (McNutt et al., 2003). Poisson regression was used to compute RR for binary outcomes (McNutt et al., 2003).

Survival analysis was used to analyse the PSM to conception interval. Cows removed during the breeding period were right censored on the day of removal, and those not pregnant at the end of the breeding

period were right censored on the day of bull removal. Independent variables as described above ($P \leq 0.10$) were offered to Cox's proportional hazards models using manual forward stepwise regression. The analysis was performed using previously published cut-points for PMN% of 5% (Gilbert et al., 2005), 10% and 18% (Kasimanickam et al., 2004), and 25% (Hammon et al., 2006). Significance was declared at $P \leq 0.05$, and a tendency at $P \leq 0.10$.

3.4. RESULTS

3.4.1. Descriptive Statistics

Retained foetal membranes were diagnosed in two cows, of which one was pyrexia at Day 0, treated with antimicrobials and consequently excluded prior to enrolment. The second cow with RFM was enrolled, but excluded because of antimicrobial treatment four days later. Including the enrolled cow with RFM, 16/272 cows (6%) had received antimicrobial or anti-inflammatory treatment before Day 21. One cow was diagnosed with a gastrointestinal tract accident and was culled, and two cows enrolled without appropriate herd identification and were lost to follow up. Of the 272 study cows sampled, 19 (7%) were excluded after enrolment, leaving 253 cows for analysis (Table 3.1).

3.4.2. Microbiology and Cytology

The prevalence of isolation of any bacteria, i.e. culture positive irrespective of bacterial species, was 93% at Day 0 and 79% at Day 21. Overall, the prevalence of *E. coli* decreased from 42% at Day 0 to 24% at Day 21 (Table 3.3). The distribution of the *E. coli* phylogenetic groups is presented in Table 3.4. At Day 0, *T. pyogenes* was not detected in four of the six herds and the overall prevalence was low (1% of cows) but increased to 9% by Day 21. The distribution of uterine bacteria varied amongst herds (Table 3.3).

The overall prevalence of PVD was 10%, 15% and 7% at Days 0, 21, and 42, respectively (Table 3.1). The prevalence of PVD at Day 21 varied between herds ($P \leq 0.05$). Of the 527 slides assessed for cytology,

two slides at each of Days 0 and 21 were of poor quality (i.e. low number of cells or distorted cells) and were excluded from further analysis. Intraobserver repeatability of PMN% assessment was found to be high (PMN%: $r_c = 0.94$, $P < 0.001$, and MAC%: $r_c = 0.89$, $P < 0.001$). Polymorphonuclear cells were identified in the majority of cows (96% and 87% at Day 0 and Day 21, respectively). The median PMN% were 41% (range: 0-95%) and 5% (0-84%) at Days 0 and 21, respectively (Figure 3.3a and b). In total, 89% (range between herds: 80-94%), 79% (72-84%), 68% (60-73%) and 62% (53-73%) of cows at Day 0, and 50% (42-58%), 36% (28-41%), 26% (13-34%) and 19% (4-26%) of cows at Day 21 had a PMN% above 5%, 10%, 18%, and 25%, respectively. Macrophages were identified in 78% and 38% cows at Days 0 and 21, respectively. The median MAC% were 7% (range: 0-82%) and 0% (0-67%) at Days 0 and 21, respectively.

Table 3.3. Number of cows (n) and proportion (% of cows enrolled in that herd) with *E. coli*, *T. pyogenes* or any bacterial growth irrespective of species (Any bacteria) at Days 0 (median 2 d in milk) and 21, by study herd^a

Herd	Cows (n)	<i>E. coli</i> (n; %)		<i>T. pyogenes</i> (n; %)		Any bacteria (n; %)	
		Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
1	47	32 (68)	13 (28)	2 (4)	8 (17)	45 (96)	42 (89)
2	23	5 (22)***	4 (17)	0 (0)	1 (4)#	20 (87)	17 (74)
3	41	10 (24)***	7 (17)	0 (0)	3 (7)	40 (98)	34 (83)
4	48	20 (42)*	7 (15)	1 (2)	3 (6)	43 (90)*	35 (73)#
5	49	15 (31)***	18 (37)	0 (0)	4 (8)	44 (90)#	41 (84)
6	45	23 (51)	10 (22)	0 (0)	4 (9)	43 (96)	33 (73)#
Total	253	105 (42)	59 (24)	3 (1)	23 (9)	235 (93)	202 (79)

^aBetween herd differences calculated from multivariable models with Herd 1 as the referent herd: # $P \leq 0.10$, * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

3.4.4. Bacteriological Multivariable Associations

A diagram of all associations ($P \leq 0.10$) is presented in Figure 3.2. Isolation of *E. coli* at Day 0, while accounting for breed and DIM at enrolment, was not associated with the isolation of *T. pyogenes* at Day 21 ($P = 0.53$). Additionally, there were no associations ($P > 0.10$ for each group) between the phylogenetic group of *E. coli* isolated at Day 0 and isolation of *T. pyogenes* at Day 21. However, isolation

of *E. coli* at Day 0 increased the risk of isolation of *E. coli* or of any bacterial growth at Day 21, with 34/105 (32%) with *E. coli* and 25/148 (17%) without *E. coli* at Day 0, having *E. coli* isolated at Day 21 ($P < 0.01$); and 91/105 (87%) cows with *E. coli* and 111/148 (75%) of cows without *E. coli* at Day 0, having any bacterial growth at Day 21 ($P = 0.03$).

Table 3.4. Number of cows (n) and proportion (%) among cows with *E. coli* isolated from the uterus stratified by phylogenetic groups, at Days 0 (median 2 d in milk) and 21 postpartum, by study herd

Herd	Day 0					Day 21				
	Cows with <i>E. coli</i> (n)	Phylogenetic group (n; %)				Cows with <i>E. coli</i> (n)	Phylogenetic group (n; %)			
		A	B1	B2	D		A	B1	B2	D
1	32	6 (19)	14 (44)	2 (6)	10 (31)	13	4 (31)	7 (54)	0 (0)	2 (15)
2	5	1 (20)	3 (60)	0 (0)	1 (20)	4	0 (0)	4 (100)	0 (0)	0 (0)
3	10	5 (50)	4 (40)	0 (0)	1 (10)	7	5 (71)	2 (29)	0 (0)	0 (0)
4	20	4 (20)	14 (70)	0 (0)	2 (10)	7	1 (14)	4 (57)	0 (0)	2 (29)
5	15	8 (53)	6 (40)	0 (0)	1 (7)	18	6 (33)	8 (44)	0 (0)	4 (22)
6	23	5 (22)	16 (70)	0 (0)	2 (9)	10	4 (40)	5 (50)	0 (0)	1 (10)
Total	105	29 (28)	57 (55)	2 (2)	17 (15)	59	20 (33)	30 (51)	0 (0)	9 (15)

Cows with *E. coli* at Day 0 and those with *T. pyogenes* at Day 21 were more likely to be diagnosed with PVD 3 wk later, with 20/105 (19%) with *E. coli* and 17/148 (11%) without *E. coli* at Day 0, being diagnosed with PVD at Day 21 ($P = 0.04$); and 4/23 (17%) cows with *T. pyogenes* and 14/230 (6%) of cows without *T. pyogenes* at Day 21, being diagnosed with PVD at Day 42 ($P = 0.05$). In contrast, those with *E. coli* at Day 21 tended to be less likely to be diagnosed with PVD at 42, with 1/59 (2%) with *E. coli* and 17/194 (9%) without *E. coli* at Day 21, being diagnosed with PVD at Day 42 ($P = 0.10$). Associations between bacteriological variables and reproduction outcomes are presented in Figure 3.2.

3.4.5. Associations with Reproductive Tract Inflammation

A standard deviation increase over the mean of PMN% at Days 0 or 21 increased the probability to be diagnosed with PVD 3 wk later from 13 to 21% ($P < 0.01$), and from 7 to 9% ($P = 0.07$), respectively.

Polymorphonuclear cell percentage at Day 0 was significantly associated with PMN% at Day 21 as calculated by linear regression ($P < 0.01$; Figure 3.2). Associations between inflammatory variables and reproduction outcomes are presented in Figure 3.2.

3.4.6. Variables Affecting Time from the Start of Breeding Program to Conception

Cows with *E. coli* isolated at Day 21 tended to conceive later than those without (hazard ratio = 0.8 (95% CI: 0.5-1.0); $P = 0.08$, Figure 3.4). Conception occurred earlier in cows with $\geq 25\%$ PMN at Day 0 than those with $< 25\%$ PMN (hazard ratio = 1.3 (95% CI: 1.0-1.7); $P = 0.05$, Figure 3.5).

3.5. DISCUSSION

The primary objective of this study was to assess whether isolation of *E. coli* from the bovine uterus within the first wk postpartum would predispose to isolation of *T. pyogenes* 3 wk later. Additionally, associations between early isolation of *E. coli*, *T. pyogenes* or any bacteria (i.e. isolation of any bacteria irrespective of species) and subsequent bacterial isolation, reproductive tract inflammation, and reproductive performance were assessed. Finally, effects of early postpartum reproductive tract inflammation and subsequent inflammation, and reproductive performance were evaluated.

Primiparous cows were excluded from this study. Differences in reproductive tract inflammation between primiparous and multiparous cows exist, and therefore it has been suggested that these parity groups should be assessed separately (Sens and Heuwieser, 2013). Further, this study involved multiple herds, whereas others did not. It was expected that associations between uterine pathogens would vary between herds and that the external validity of the study would be improved by enrolling multiple herds. However, no interactions were observed between those associations and herd, possibly due to considerable variation in natural pathogen challenge (i.e. prevalence within herd; Table 3.3) or limited statistical power. Nevertheless, many previous studies examining uterine bacteriology in dairy cows

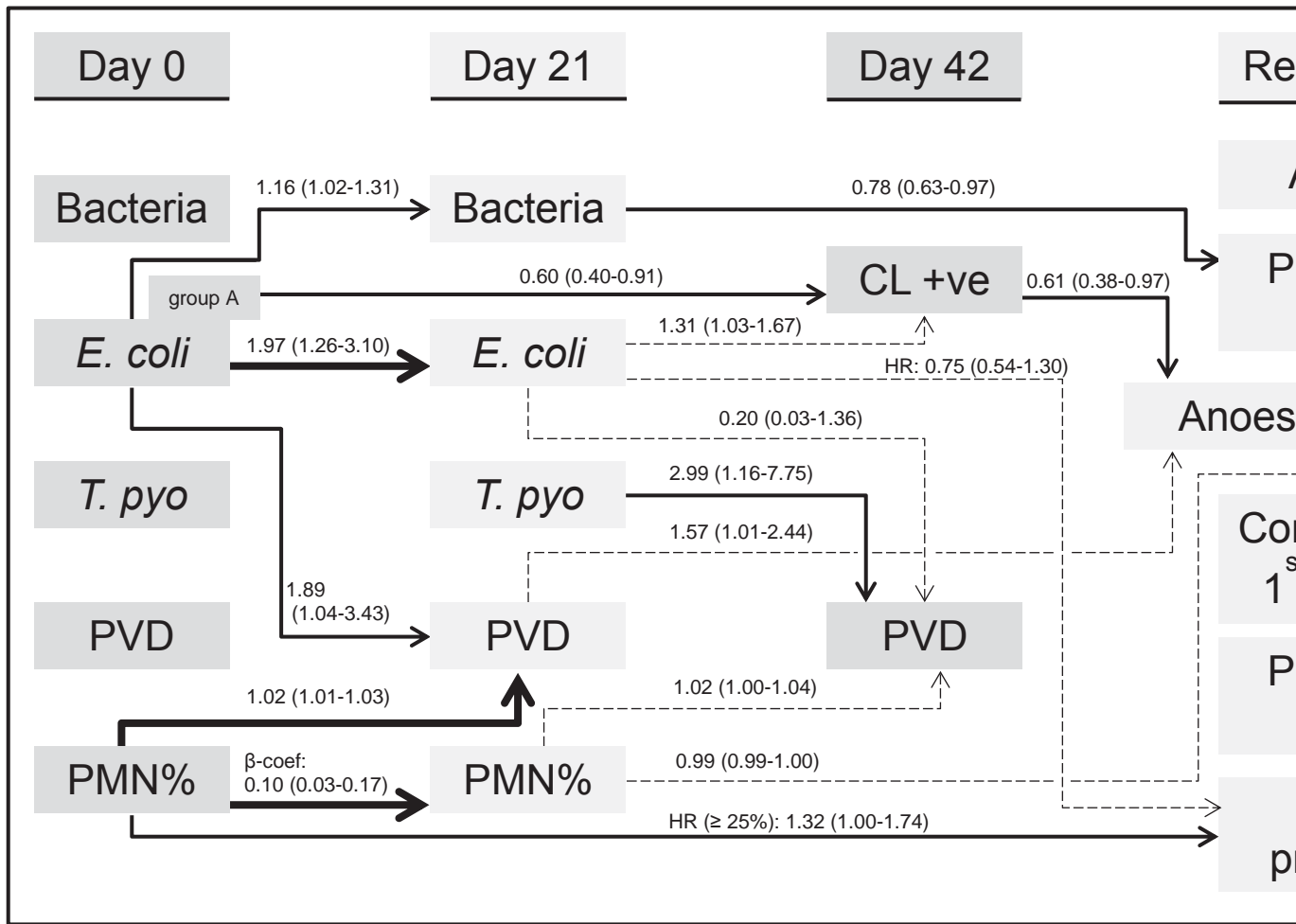


Figure 3.2. Diagram of associations between-sample days (i.e. Days 0, 21 and 42) found between *E. coli* (including phylogenetic group A) and *T. pyo* isolated from the uterus, culture positive irrespective of bacterial species (bacteria), purulent vaginal discharge (PVD), peritoneal neutrophil counts (PMN%), anoestrus treatment (anoestrus) and reproductive outcomes of 253 dairy cows from six herds. Each association is presented as hazard ratio (HR; 95% CI), except when specified as β -coefficient (β -coef; 95% CI) from linear regression or hazard ratio (HR; 95% CI) from Cox's proportional hazards model. Arrow weights are giving by the weight of the arrows (dashed: $P \leq 0.10$, solid thin: $P \leq 0.05$, and solid thick: $P \leq 0.01$).

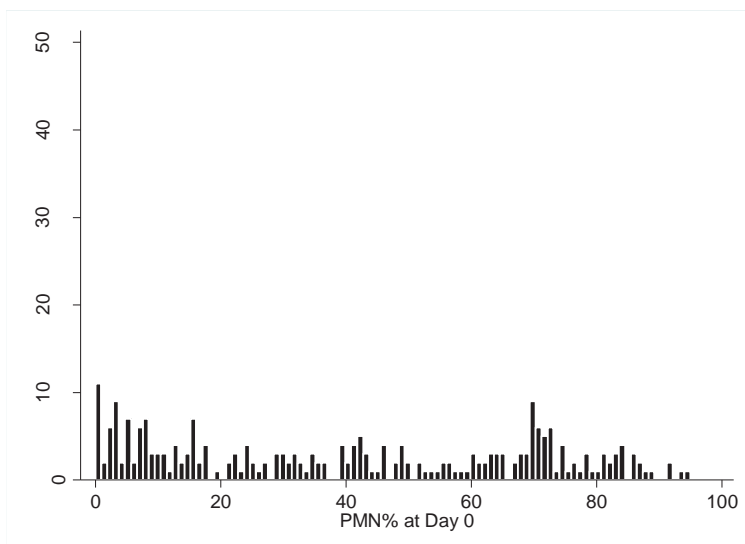
have used only one herd (Williams et al., 2005; McDougall et al., 2011; Sens and Heuwieser, 2013), hence extrapolation of these results to dairy cows in general must be undertaken with caution.

The decreasing prevalence of *E. coli* and increasing prevalence of *T. pyogenes* over time resulted in the suggestion that *E. coli* early postpartum predisposes cows to infection with *T. pyogenes* (Williams et al., 2007). Similar to others using traditional culturing techniques (Werner et al., 2012), such an association was not found in this study. Additionally, no significant association was found in the current study between any of the phylogenetic groups of *E. coli* and subsequent isolation of *T. pyogenes*. It has been reported that particular genotypes within *E. coli* phylogenetic group B1 isolated early postpartum were significantly associated with subsequent isolation of *T. pyogenes* (Sheldon et al., 2010). However, no data on additional subdivision of the phylogenetic groups were available in the current study. Even though *T. pyogenes* affected few cows, its presence seemed to be of clinical importance and was associated with reproductive tract inflammation and impaired reproduction (Bonnett et al., 1991; Bonnett et al., 1993; Werner et al., 2012). Besides decreasing *E. coli* and increasing *T. pyogenes* proportions over time, little evidence, including the present data, is currently available supporting the notion that presence of *E. coli* predisposes to *T. pyogenes* infection. However, more cows may be needed in future natural exposure studies to prove this hypothesis, as the power to test the primary hypothesis of this study was only moderate. Post hoc power calculation showed that the sample size would have to have been 3-fold greater to define the differences observed in the current study as significant. The current study used natural exposure to test the primary hypothesis. An alternative methodology to test this hypothesis would have been to experimentally infect cows postpartum with *E. coli*.

A study using culture independent techniques, i.e. genomic tests, recently reported that *E. coli* was less prevalent compared to *Fusobacterium necrophorum* (Bicalho et al., 2012). That same study found that presence of *F. necrophorum*, but not *E. coli*, increased the odds of subsequent detection of *T. pyogenes*. Similarly, another study also failed to find an association between *E. coli* and *T.*

pyogenes using culture independent techniques, but found that *F. necrophorum* was highly prevalent in metritic cows, but not detected in healthy cows (Santos and Bicalho, 2012). In the current study, assessing the association between *E. coli* and *T. pyogenes* was the main objective and thus these were the only bacterial species typed. Therefore, it is unknown whether *F. necrophorum* predisposes *T. pyogenes* infection in pasture-based cows and further research is required. Sensitivity of culture independent bacteriology techniques seems to be higher than traditional culturing

(a)



(b)

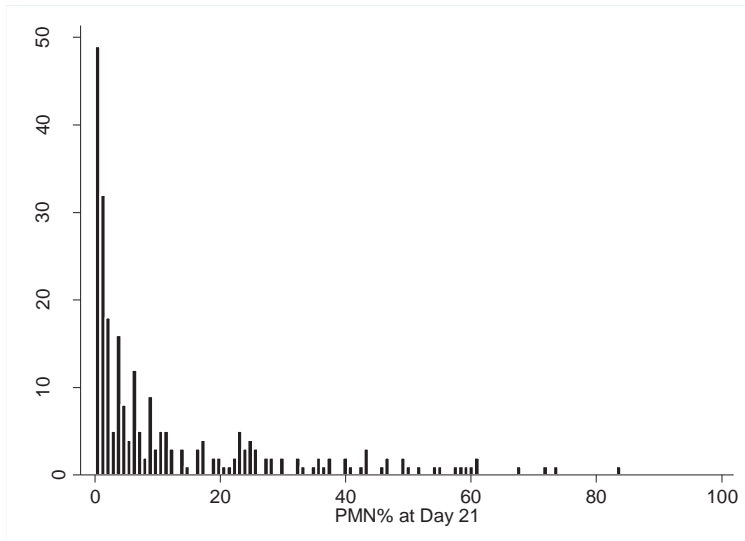


Figure 3.3. Frequency distribution of the percentage of intrauterine polymorphonuclear cells (PMN%) diagnosed at (a) Day 0 and (b) Day 21 from 253 cows from six New Zealand dairy herds.

techniques, although specificity may be lower because of the possibility of detecting related or non-viable bacteria (Preziuso and Cuteri, 2012). Accuracies of culture dependent versus independent techniques have not yet been compared for intrauterine bacteriological tests (Madoz et al., 2014). The current study used traditional culturing technique and may have underestimated the true prevalence of *E. coli* and *T. pyogenes*. Further studies are required to compare both techniques. Due to the lack of a gold standard, such studies may use reproductive performance outcomes for comparing and validating tests (De Boer et al., 2014a).

The presence of *E. coli* may truly not actually predispose to *T. pyogenes* infections, and anaerobic pathogens, such as *F. necrophorum* may be more clinically important early postpartum. Other underlying aetiologies, such as infection with bovine herpes virus type 4 (BoHV-4) may be associated with increased risk of *T. pyogenes*. A relationship between BoHV-4, *E. coli*, and *T. pyogenes* uterine infections has been suggested by Sheldon et al. (2009), with *E. coli* and *T. pyogenes* promoting the replication of BoHV-4, resulting in greater pathology to endometrial cells and more inflammation. Antibodies against BoHV-4 are present in New Zealand dairy cows (De Boer et al., 2014b), but contribution of this virus to reproductive infection was not the objective of current study, hence further research is needed.

The association between intrauterine bacterial growth and reproductive tract inflammation has been a subject of interest for many years and controversy still exists (Bonnett et al., 1991; McDougall et al., 2011). Similar to Deguillaume et al. (2012), this study did not find an association between bacterial intrauterine presence and MAC%. The PMN% at Day 21 in this study was numerically higher compared with others sampling cows up to 29 DIM (McDougall et al., 2011; Sens and Heuwieser, 2013). This study found no association between the bacteriological variables at Day 0 and increased PMN% 3 wk later. This stands in contrast to another study, where cows without PVD and with *T. pyogenes* isolated at 10 ± 1 DIM had increased odds of PMN (>18%) 2 wk later (Sens and Heuwieser, 2013). That study, similar to the current study, did not find an association between *E. coli* and

PMN%. McDougall et al. (2011) reported little agreement between one of five ‘major’ bacteria and PMN% at 29 DIM in pasture-based cows. Besides exclusion of PVD, other differences in cytological outcomes like variations in sampling protocol, number of cells counted, protocol of cytology slide assessment, and examined populations complicate comparisons between studies (De Boer et al., 2014a). Based on data from this study and others (McDougall et al., 2011), it appeared that bacteria isolated from the uterus of pasture-based cows were not associated with PMN%. It may be hypothesized, cows calving in the pasture environment may be contaminated with different species of bacteria compared to those calving in confined housing; hence differences may be present between different studies.

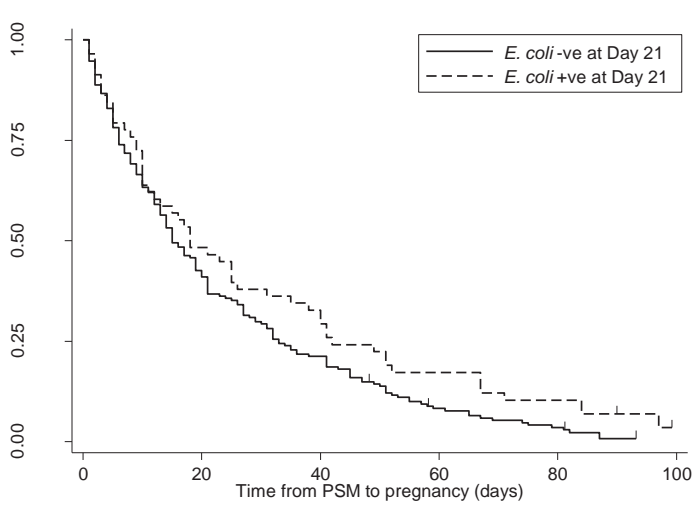


Figure 3.4. Survival curves for time to pregnancy up to the end of the seasonal breeding program in 253 dairy cows from six herds and categorized by intrauterine presence of *E. coli* (n = 59) at Day 21 (median: 23 DIM). Median days from the start of the seasonal breeding program to pregnancy (95% CI; P-value) were 14 (12-18) and 17 (9-24; 0.08) for cows without and with *E. coli* isolation, respectively.

Cows identified with *E. coli* at Day 0 (attributable fraction = 0.40) or *T. pyogenes* at Day 21 (attributable fraction = 0.65) were at increased risk of being diagnosed with PVD 3 wk later, confirming findings of others (Williams et al., 2005; Werner et al., 2012). Specific bacterial genotype or phenotype may be more important as a cause of reproductive tract inflammation than the presence of a single bacterial species (Bicalho et al., 2012). The current study did not find any

associations between *E. coli* phylogenetic groups and reproductive tract inflammation. In contrast, one study described an association between some genotypes within the phylogenetic groups A and B1 and PVD, but failed to report if the association was significant or not (Sheldon et al., 2010). Interestingly, PVD at Day 42 tended to be diagnosed less often when *E. coli* was isolated at Day 21, however caution needs to be taken interpreting this non-plausible result as causal, because of the low proportion of cows (2%) with *E. coli* at Day 21 and being diagnosed with PVD at Day 42. Hence, it may be a type 1 error. *Escherichia coli* endotoxin lipopolysaccharide disrupts ovarian function by interfering with the hypothalamus-pituitary axis (Williams et al., 2007), and switches endometrial production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to prostaglandin E_2 (PGE_2 ; Herath et al., 2009). PGE_2 suppresses immune function (Xu et al., 2008), but it is unclear if this hormone affects leucocyte extravasation. Thus, questions remain to why, unexpectedly, *E. coli* isolated at Day 21 decreased the probability of PVD diagnosis and increased the probability of the presence of a CL at Day 42, yet was associated with longer time to pregnancy.

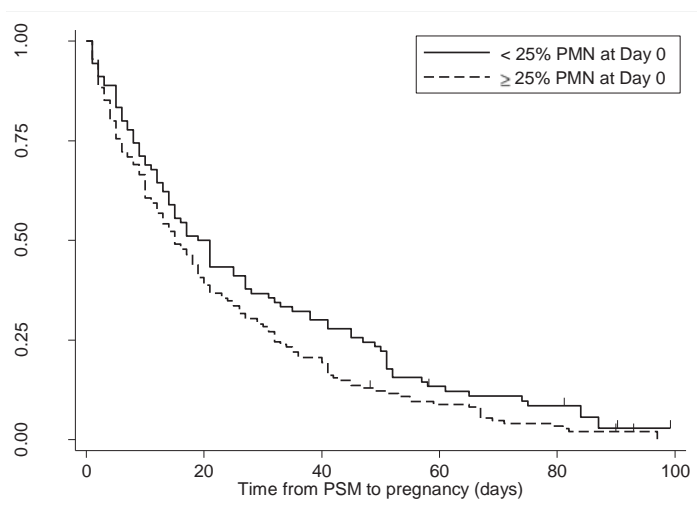


Figure 3.5. Survival curves for time to pregnancy up to the end of the seasonal breeding program in 253 dairy cows from six herds and categorized by affected by polymorphonuclear cells (PMN; $\geq 25\%$; $n = 157$) at Day 0 (median: 2 d in milk). Median days from the start of the seasonal breeding program to pregnancy (95% CI; P-value) were 16 (12-24) and 14 (11-18; 0.05) for cows with $< 25\%$ and those with $\geq 25\%$ PMN, respectively.

Isolation of *E. coli* at Day 21 or any bacteria irrespective of species, was associated with increased time to pregnancy and reduced risk of pregnancy within 3 wk from PSM, respectively (Figure 3.4 and 3.3). In contrast, no associations were found between isolation of one or more of five 'major' bacterial species, including *E. coli* and *T. pyogenes*, at 29 DIM and reproductive outcomes in another study in New Zealand (McDougall et al., 2011). In contrast, *T. pyogenes* at 26 d postpartum reduced risk of pregnancy by 119 days postpartum (Bonnett et al., 1993). Similarly, *E. coli* possessing the virulence factor *fimH* was associated with increased time to pregnancy (Bicalho et al., 2012). A recently reported reduction in reproductive tract disease by prepartum vaccination against *E. coli* and other putative uterine pathogens (Machado et al., 2014) supports the notion that reduction of uterine pathogen load or its effects in the early postpartum period is a relevant goal.

New Zealand dairy cows are commonly tested for PVD about a month before PSM. In a large study, those diagnosed with PVD but not treated were at higher risk of reproductive failure (McDougall et al., 2007). Therefore, cows with PVD on Day 42 (approximately one month before PSM) were treated at that time in the present study, thereby diminishing a potential negative affect of PVD on reproductive performance. Many studies have reported impaired reproductive performance in cows with increased PMN% at various time points (Kasimanickam et al., 2004; Barlund et al., 2008). The protective effect of PMN% at Day 0 on time to conception from PSM appears to be a novel finding from this study (Figure 3.5). In addition, Gilbert *et al.* (2007) reported that the presence of bacteria and the risk of endometritis as determined by PMN% was reduced by the influx of PMN early postpartum. It appears from that data and this study that an influx of PMN may be part of the physiological process in early postpartum cows and required to achieve optimal reproductive performance. Thus, early postpartum treatment with anti-inflammatory drugs in non-systemically ill cows may potentially be contraindicated. Recently, no benefit was found following early postpartum treatment with a non-steroidal anti-inflammatory drug (Meier et al., 2014). Negative energy balance, as measured by elevated concentrations of β -hydroxybutyric acid (BHBA) and non-esterified fatty acids, reduced PMN functionality (Hammon et al., 2006). A large proportion (17%) of early

postpartum New Zealand cows have elevated BHBA concentrations (Compton et al., 2014). This underlines the importance of the correct nutritional status at calving (Compton et al., 2014), because when innate immunity is disturbed long term reproductive effects may be observed.

In conclusion, this study demonstrated no association between culturing *E. coli* from the uterus at Day 0 and *T. pyogenes* 3 wk later, while presence of *E. coli* did increase the probability of subsequent re-isolation of the same bacterial species. Although the presence of *E. coli*, *T. pyogenes*, or any bacterial species, did not influence PMN% 3 wk later, the clinical importance of detection of *E. coli* at Day 0 and *T. pyogenes* at Day 21 was the association with the later diagnosis of PVD. Additionally, cows with intrauterine isolation of *E. coli* or culture of any bacteria irrespective of species had poorer reproductive performance. Purulent vaginal discharge is currently treated in New Zealand around a month before PSM. This study may support earlier treatment of PVD positive cows or vaccination against these two pathogens. Interestingly, cows with PMN \geq 25% at Day 0 were pregnant sooner compared to those below this cut-point, indicating that an early influx of PMN may be part of the cow's normal and desired physiological response of uterine involution. Conversely, PMN% at day 21 was negatively associated with pregnancy with 3 wk from PSM, indicating inflammation at that stage is detrimental.

3.6. ACKNOWLEDGEMENTS

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Chapter 4

Minimum inhibitory concentrations of a range of antimicrobials against *Escherichia coli* and *Trueperella pyogenes* of bovine uterine origin

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4.1. ABSTRACT

Minimum inhibitory concentrations (MIC) of nine antimicrobials for isolates of two common bovine intrauterine bacterial pathogens, i.e. *Escherichia coli* (n = 209) and *Trueperella pyogenes* (n = 35) were determined using broth microdilution methodology. The isolates were recovered from 575 dairy cows from seven herds using the cytobrush technique. The identity of the pathogens was initially performed using phenotypic techniques. Additionally, PCR was used to confirm the identity of *T. pyogenes* isolates and to categorise the *E. coli* isolates into phylogenetic groups A, B1, B2, and D. Minimum inhibitory concentrations in excess of published cut-points or bimodal distributions of MICs indicated potential antimicrobial resistance for ampicillin, cefuroxime, cephalixin and oxytetracycline for *E. coli*, and oxytetracycline for *T. pyogenes*. Of the antimicrobials tested, ticarcillin/clavulanic acid, ceftiofur and enrofloxacin had the lowest MICs for these two pathogens and may be candidates for future intrauterine therapy in cattle. Differences in MICs of some antimicrobials were found between herds, age, breeds and *E. coli* phylogenetic groups ($P \leq 0.05$). Isolation of *E. coli* with an MIC ≥ 8 $\mu\text{g}/\text{mL}$ of oxytetracycline at 23 days post-partum was associated with a lower probability of pregnancy within 6 weeks of commencement of breeding compared with those isolates with an MIC < 8 $\mu\text{g}/\text{mL}$ (RR = 0.66; $P = 0.09$). While MICs for uterine pathogens were determined for isolates from New Zealand dairy cows, the interpretation of these MICs remains unclear in the absence of either epidemiological or clinical interpretive criteria. Further studies are required to define interpretative criteria for uterine isolates, including determination of pharmacokinetic and pharmacodynamic profiles for antimicrobials.

Key words: minimum inhibitory concentration, antimicrobial susceptibility, *Escherichia coli*, *Trueperella pyogenes*, uterine infection, dairy cows

4.2. INTRODUCTION

A wide range of aerobic and anaerobic bacteria can be found in the postpartum uterus of cows (Santos et al., 2011). Many species have been isolated including *Escherichia coli*, *Trueperella pyogenes*, *Prevotella* spp, *Fusobacterium necrophorum*, *Bacillus licheniformis*, *Clostridium perfringens*, *Pasteurella multocida*, *Staphylococcus* spp. and *Streptococcus* spp. (Williams et al., 2005). Commonly, multiple bacterial species are isolated from a single cow at a single time point. These bacteria have been classified into three different categories of pathogenicity, with *E. coli* and *T. pyogenes* purportedly pathogenic for the endometrium while the others are classified as opportunist or potential pathogens (Williams et al., 2005). Also *in vitro* and *in vivo* results suggest isolation of *E. coli* after calving may predispose cows to infections with other bacteria or viruses (Dohmen et al., 2000; Donofrio et al., 2008).

Broad spectrum antimicrobial therapy is usually recommended and deemed appropriate for uterine infections shortly after calving (Azawi, 2008), but its efficacy depends on pathogen susceptibility. A variety of methods are used to evaluate antimicrobial sensitivity of pathogens, including agar zone (or disc) diffusion assays, broth dilution methods or modifications to this, and procedures to find penicillin resistant genes (Watson et al., 1991; Salmon et al., 1995; Vintov et al., 2003). Compared with the zone diffusion system, the broth dilution method is less frequently used due to it being more complex, time consuming and costly. There is a high correlation between the zone diffusion and broth dilution methods (Watson et al., 1991). However, since zone diffusion assays only categorize laboratory outcomes as susceptible, intermediate susceptible or resistant, it is not possible to quantify resistance by actual concentrations of the antimicrobial. The advantage of knowing the minimum inhibitory concentration (MIC; obtained by broth dilution) is that resistance can be quantified more accurately and where required, antimicrobial concentrations can be modified to achieve therapeutic serum or tissue concentrations where pharmacokinetic/-dynamic data are available (Turnidge and Paterson, 2007).

Interpretive cut-points for MICs are available from the Veterinary Antimicrobial Susceptibility Testing subcommittee of the Clinical and Laboratory Standards Institute (CLSI, 2013) for a range of veterinary pathogens and antimicrobials. Cut-points derived from human trials with human pathogens and antimicrobials, as determined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org), have also been used to assess veterinary antimicrobials (Bengtsson et al., 2009). However, no MIC interpretive criteria have been established for bovine uterine derived *E. coli* or *T. pyogenes*.

Only a few studies have specifically examined the MIC of antimicrobials for pathogens isolated from the bovine uterus, particularly for *E. coli* (Sheldon et al., 2004; Santos et al., 2010b) and *T. pyogenes* (Sheldon et al., 2004; Liu et al., 2009; Santos et al., 2010a) and there is no data from isolates from pasture-based systems as commonly used in New Zealand. The primary aim of this study was to determine the MICs for *E. coli* and *T. pyogenes* from pasture-based dairy cows and secondly, to evaluate associations between MICs and demographic factors (such as herd and age), and reproductive performance.

4.3. MATERIALS AND METHODS

4.3.1. Herds, Cows and Isolate Collection

The *E. coli* and *T. pyogenes* isolates used were from bovine uterine samples collected via a triple guarded cytobrush (Barlund et al., 2008) from cows in two studies. The isolates from Study 1 were collected from cows (n = 272) sampled twice at a mean (\pm SD) of 2 (\pm 1.2) days in milk (DIM) and 23 (\pm 1.2) DIM from herds (n = 6) in the Waikato region, New Zealand during spring 2011 (De Boer et al., 2014, in preparation). Isolates from Study 2 were obtained from cows (n = 303) from one herd sampled twice at a mean (\pm SD) of 29 (\pm 2.4) and 43 (\pm 2.3) DIM in the Taranaki region, New Zealand, during spring 2008 (McDougall et al., 2011). The cows in each study calved in spring, were managed on pasture and were predominantly fed on rye grass/white clover pasture.

4.3.2. Microbiology

Collection and storage of isolates have been previously described (De Boer et al., 2014, in preparation; McDougall et al., 2011). In Study 1, the cytobrushes were used to streak a 0.1% esculin, 5% sheep blood agar plate and a MacConkey agar plate (Fort Richard, Auckland, New Zealand), and those collected in Study 2 were used to streak two agar plates containing 0.1% esculin, 5% sheep blood (Fort Richard, Auckland, New Zealand). The MacConkey agar (Study 1) and one of the esculin sheep blood agar plates (Study 2) were incubated for 2 days at 37 °C in an aerobic environment, where the remaining esculin, sheep blood agar plates were incubated for 2 days at 37 °C in sealed containers with CO₂. *Escherichia coli* and *T. pyogenes* isolates were selected based on colony morphology, shininess/glossiness, colour, lactose fermentation (*E. coli*), haemolysis patterns, and Gram stain. Isolates were confirmed by a range of biochemistry tests; lactose, maltose, mannitol, motility, OUMI (ONPG; an enzymatic test for ortho-nitrophenyl-β-galactosidase, urase, motility, indol), Simmons citrate and TSI (triple sugar iron) tests for presumptive *E. coli* isolates, and lactose, maltose, mannitol, nutrient gelatin and ONPG for presumptive *T. pyogenes* isolates. Each purified isolate was inoculated in 2 mL brain heart infusion (BHI) broth including 20% glycerol and stored at -80 °C for subsequent analysis. The identity of the *T. pyogenes* isolates from each study was confirmed by PCR using previously described primers (Jost et al., 2002; Hassan et al., 2008; Hijazin et al., 2011), while *E. coli* isolates were divided into phylogenetic groups A, B1, B2, and D by triplex PCR (Clermont et al., 2000).

4.3.3. Susceptibility Testing

In total, 209 *E. coli* and 35 *T. pyogenes* isolates were evaluated for susceptibility testing. The antimicrobial susceptibility tests were undertaken following guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2013). A custom-made plate for the broth microdilution method (Sensititre, Trek Diagnostic Systems LTD, East Grinstead, UK) was used for the susceptibility testing of Ampicillin, Cloxacillin, Ticarcillin/Clavulanic Acid (constant 2), Ceftiofur, Cefuroxime, Cephapirin,

Enrofloxacin, Clindamycin, and Oxytetracycline. All antimicrobials were tested ranging from 0.0625 to 32 or 64 µg/mL in doubling dilutions (i.e. 10 or 11 dilutions). Prior to susceptibility testing, isolates from BHI broth were revived on Mueller Hinton agar containing 5% of sheep blood (Fort Richard, Auckland, New Zealand), and incubated at 37 °C; *E. coli* isolates aerobically for 24 hours, and *T. pyogenes* isolates in a CO₂-enriched environment for 48 hours. Revived colonies were emulsified in 5 mL demineralised water (Trek Diagnostic Systems LTD, East Grinstead, UK) to a turbidity of 0.5 McFarland standards. In total, 15 µL of the suspension was mixed in 11 mL Mueller Hinton broth (Trek Diagnostic Systems LTD, East Grinstead, UK). Each well of the plate was inoculated with 50 µL of inoculated Mueller Hinton broth. For the *T. pyogenes* isolates, prior to this step, 2% lysed horse blood was added to the Mueller Hinton broth to support the growth of these fastidious isolates (CLSI, 2013). Plates were incubated at 37 °C for 24 and 48 hours for *E. coli* and *T. pyogenes* inoculums, respectively. Incubation times specified by the CLSI guidelines were increased to 48 hours for *T. pyogenes* inoculums, due to the fastidious growth of these bacteria as previously recommended (Sheldon et al., 2004). The well containing the lowest concentration without visible growth was classified as the MIC for that isolate.

To assess the number of colony forming units (CFU)/mL of the inocula, 1 µL undiluted and 1 µL 1:50 diluted inocula of the reference cultures and some unknown inocula were streaked over entire Mueller Hinton agar plates and incubated at 37 °C for 24 and 72 hours for *E. coli* and *T. pyogenes*, respectively, and the number of colonies were counted.

Prior to analysis of any wild type isolates, MICs were determined for 20 replicates of the reference *E. coli* (ATCC 25922) and 40 of the reference *T. pyogenes* (ATCC 19411). Additionally, a reference culture was tested in parallel with each group of wild type isolates tested. Available reference ranges for the reference type cultures provided by the microdilution plate manufactures (Trek Diagnostic Systems LTD, UK) were used as the normal ranges. For the antimicrobials (i.e. cloxacillin and clindamycin for the reference strain *E. coli* ATCC 25922 and all antimicrobials for the *T. pyogenes*

ATCC 19411 reference strain) where no reference range data from either the broth microdilution plate manufacture, or from CLSI was available, reference ranges were calculated as previously described (Turnidge and Bordash, 2007; Table 4.1).

4.3.4. Statistical Analysis

The concentration that inhibited 50% (MIC₅₀) and 90% (MIC₉₀) of the isolates were calculated for each antimicrobial. Minimum inhibitory concentrations were graphed and tabulated. Where interpretive cut-points for MICs have been established for some pathogens and antimicrobials by CLSI and EUCAST, these were used to interpret the results of the current study. Where no interpretive cut-points were available from CLSI or EUCAST, the MICs were plotted on frequency histograms and normality of distribution was tested using Shapiro-Wilk test (Razali and Wah, 2011). When distribution was not normal and clearly a bimodal distribution, cut-points were visually created.

Further analyses were undertaken on a subset of *E. coli* (n = 175) and *T. pyogenes* (n = 26) isolates from Study 1. Associations between MIC values (log₂-transformed) with herd, age (categorized as 3-4, 5-6, and ≥7 years), breed (categorized as Friesian, Jersey, and crossbreed), sample day (2 or 23 DIM), and phylogenetic grouping for *E. coli* were assessed using Fisher's exact analyses. Binary logistic regression was undertaken to evaluate the association between MIC of antimicrobials and reproductive performance. The outcome variables were submission for artificial insemination (AI) within three weeks after the start of the seasonal breeding programme (planned start of mating; PSM), pregnant within 3 or 6 weeks after PSM, and conception to first AI within 3 weeks after PSM. The MIC of each antimicrobial separately (categorized by binary cut-point available from CLSI, EUCAST; Table 4.2, or from a possible bimodal distribution) was included in each model to evaluate the causal effect on each outcome variable. Herd was included as a fixed effect in each model. Variables were retained if the P-value was ≤0.10, or if it caused a change in one of the coefficients by

>20%. Relative risk (RR) was calculated using Poisson regression. Data analysis was undertaken using Stata 13.1 (StataCorp, College Station, TX, USA).

4.4. RESULTS

4.4.1. Quality Assurance

A total of 27 *E. coli* ATCC 25922 and 43 *T. pyogenes* ATCC 19411 reference strains were tested, and of the 189 individual tests two (1.1%) were not within the available reference ranges. For those antimicrobials where no reference range data were available, 8 (1.8%) of the 441 individual tests were not within the calculated reference ranges (Table 4.1). The MIC determinations were repeated for 12 of 244 (5%) wild type isolates as growth in the plate was not acceptable (i.e. >1 'skips' of growth within a sequence of dilutions within an antimicrobial occurred) or the positive controls had no growth. A total of 92.3% (n = 36/39) of inoculums were between 5×10^4 and 5×10^5 CFU/mL, the optimum inoculum (CLSI, 2013).

Table 4.1. The CLSI, the broth microdilution plate manufacturer (Trek Diagnostic Systems LTD, UK) suggested and calculated diagnostic reference ranges for *E. coli* and *T. pyogenes* ATCC® type cultures used as quality control

Antimicrobial	<i>E. coli</i> ATCC® 25922		<i>T. pyogenes</i> ATCC® 19411	
	CLSI	Trek	Calculated	Calculated
Ampicillin	2-8	2-8	-	0.03-0.125
Ceftiofur	0.25-1	0.25-1	-	0.5-2
Cefuroxime	-	2-8	-	0.03-0.125
Cephapirin	-	8-32	-	0.06-0.25
Clindamycin	-	-	32-128	0.03-0.125
Cloxacillin	-	-	32-128	0.25-1
Enrofloxacin	0.008-0.03	0.008-0.03	-	0.5-2
Oxytetracycline	-	0.5-2	-	0.125-0.5
Ticarcillin/Clavulanic acid	4/2-16/2	4/2-16/2	-	0.03-0.125

4.4.2. Frequency Distribution and MIC₅₀ and MIC₉₀

Ampicillin and oxytetracycline had the highest MIC and the widest range (1 to ≥ 128 $\mu\text{g}/\text{mL}$) for *E. coli*, whereas enrofloxacin had the lowest detected maximum MIC (0.25 $\mu\text{g}/\text{mL}$). The MICs were ≥ 64 $\mu\text{g}/\text{mL}$ for cloxacillin and clindamycin for *E. coli* as no isolates were inhibited even at the highest concentration tested. The frequency distributions of MICs for *E. coli*, including the MIC₅₀ and MIC₉₀ are presented in Table 4.2. In total, 1.9%, 1.4%, 6.7% and 4.8% of the *E. coli* isolates were resistant (based on the interpretive criteria from human-derived data) for ampicillin, cefuroxime, cephalixin and oxytetracycline, respectively (Table 4.2).

All antimicrobials inhibited growth of *T. pyogenes* isolates by at least one of the concentrations tested, except for oxytetracycline where 2.9% of the isolates were not inhibited at the highest concentration tested. Oxytetracycline also had the widest range (0.25 to ≥ 128 $\mu\text{g}/\text{mL}$) for *T. pyogenes*, whereas ampicillin and ticarcillin/clavulanic acid had the lowest detected maximum MIC (0.13 $\mu\text{g}/\text{mL}$). The frequency distribution of MICs for *T. pyogenes*, including the MIC₅₀ and MIC₉₀ are presented in Table 4.3. In contrast to *E. coli*, no CLSI or EUCAST interpretive criteria are available for *T. pyogenes* isolated from any host species, including humans, or infection site.

4.4.3. Variables Associated with MIC

Variation in distribution of MIC values of some antimicrobials for *E. coli* and *T. pyogenes* amongst herds, age-groups, and breeds were found (Table 4.4). The heterogeneity in MICs of ceftiofur ($P = 0.01$) and oxytetracycline ($P = 0.05$) observed amongst herds are presented in frequency distribution diagrams (Figure 4.1a and b, respectively). Differences ($P = 0.04$) amongst age-groups and MIC values of ceftiofur for *T. pyogenes* are visualized in Figure 4.2. Additionally, differences ($P \leq 0.05$) were found between the distribution of MIC of some antimicrobials for *E. coli* and sample day and genetic differentiation into phylogenetic groups (Table 4.4).

Of all MIC cut-points tested, only *E. coli* isolates at 23 DIM with an MIC ≥ 8 $\mu\text{g/mL}$ of oxytetracycline (6/10; 60%) tended to be less likely (RR = 0.66 (95% CI: 0.38-1.15); P = 0.09) to conceive within 6 weeks than those with MIC < 8 $\mu\text{g/mL}$ (39/50; 78%).

4.5. DISCUSSION

This study has provided MIC data of 9 antimicrobials for 2 common bovine intrauterine pathogens, *E. coli* and *T. pyogenes* (Williams et al., 2005). There is limited data available on MIC values for these pathogens from the postpartum bovine uterus, and this is the first time these data are generated for isolates of intrauterine origin from pasture-based cattle. Those cows may be exposed to different bacterial species and strains, due to the fact that they calved on pasture, rather than in a housed environment. For example, pasture-based cows have a lower prevalence of mastitis caused by *E. coli* to those in housed systems (McDougall, 2002). Additionally, it appears that associations between the MICs of intrauterine pathogens and cow demographics (such as herd and age), and between MICs and reproductive outcomes have not been published before for any management system.

The MIC₅₀ and MIC₉₀'s reported in this study differ from those from previous studies in some instances. For ceftiofur, the MICs for *E. coli* isolates were similar to those of previous studies (Sheldon et al., 2004; Santos et al., 2010b). However, for ampicillin and oxytetracycline, the MICs found in this study were 2 to 3 dilutions lower than reported by others (Sheldon et al., 2004; Santos et al., 2010b), and for cephalosporin, the MIC₅₀ was 2 dilutions higher and MIC₉₀ was 1 dilution higher compared to MICs found by Sheldon et al. (2004). The MICs of ceftiofur and enrofloxacin for *T. pyogenes* described in current study were similar to concentrations reported in the UK, USA and China, while MICs for ampicillin, clindamycin and oxytetracycline were lower in the current study than in those studies (Sheldon et al., 2004; Liu et al., 2009; Santos et al., 2010a). In contrast, the MICs for cephalosporin were two dilutions higher in comparison to MICs previously reported from *T. pyogenes* of uterine origin (Sheldon et al., 2004). The differences between MIC₅₀ and MIC₉₀ of bacteria from pasture-based cows and cows calving in pens may be attributable to laboratory or cow

Table 4.2. Frequency distribution (% of all isolates; n = 209) of minimum inhibitory concentrations (MIC) for a range of antimicrobials (±1.2; SD) and 43 (±2.3) DIM from postpartum bovine uteri¹

Antimicrobial	Antimicrobial concentration (µg/mL)												MIC ₅₀ ²	MIC ₉₀ ³	Res% ⁴
	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128			
Ampicillin	0	0	0	0	2.9	23.4	69.4	1.9	0.5	0.5	0	1.4	4	4	1.9
Cloxacillin	0	0	0	0	0	0	0	0	0	0	100		64	64	
Ticarcillin/Clavulanic acid	0	0	0	0	9.1	67.5	22.0	0	1.4	0	0	0	2	4	0
Ceftiofur	0	1.0	52.2	44.5	1.9	0.5	0	0	0	0	0	0	0.25	0.5	0
Cefuroxime	0	0	0	0	0.5	11.5	76.1	10.5	0.5	0	1.0		4	8	1.4
Cephapirin	0	0	0	0	0	1.4	7.7	26.8	57.4	6.2	0.5		16	16	6.7
Enrofloxacin	97.1	1.9	1.0	0	0	0	0	0	0	0	0		0.06	0.06	0
Clindamycin	0	0	0	0	0	0	0	0	0	0	100		64	64	
Oxytetracycline	0	0	0	0	6.2	41.1	35.9	12.0	0	0.5	0.5	3.8	4	8	4.8

¹The light grey shading represents the intermediate susceptible zone, while the darker grey shading represents the resistant zone as suggested by the Clinical and Laboratory Standards Institute (CLSI) Committee on Antimicrobial Susceptibility Testing. Antimicrobials without shading have no interpretive criteria available. Those isolates not inhibited at the highest concentration tested are presented in the next highest dilution and reported as ≥ that MIC. Where there are results in the lowest concentration tested, the results are reported as ≤ than that MIC.

²MIC₅₀ = The MIC (µg/mL) that inhibited 50% of the isolates.

³MIC₉₀ = The MIC (µg/mL) that inhibited 90% of the isolates.

⁴Res% = Percentage resistant *E. coli* for antimicrobials using the interpretative criteria for *E. coli* isolated from other host species, i.e. humans and poultry, or from bovine mastitis.

factors. It is important to realize that regardless of management system, *in vivo* antimicrobial concentrations (i.e. pharmacokinetics) need to be above the MIC to have therapeutic effects, and therefore these differences may be clinically less meaningful.

There is a lack of veterinary interpretation criteria of MIC for many antimicrobials. For example, only ceftiofur has veterinary specific interpretative criteria for *E. coli* isolates from bovine origin, however, this criteria has not been validated for uterine isolates. Enrofloxacin has veterinary specific interpretative criteria for *E. coli* isolates from poultry origin, and cut-points for other antimicrobials (Table 4.2) were derived from human data (CLSI, 2013). Even where veterinary cut-points from animal trials were available, it is likely that human data, e.g. human pharmacokinetics, have been used to calculate cut-points for veterinary use (Turnidge and Paterson, 2007). Therefore, in the absence of pharmacokinetic/-dynamic data and clear evidence of a bimodal distribution of the MICs of these

Table 4.3. Frequency distribution (% of all isolates; n = 35) of minimum inhibitory concentrations (MIC) for a range of antimicrobials against *T. pyogenes* isolated between 2 (± 1.2 ; SD) and 43 (± 2.3) DIM from postpartum bovine uteri¹

Antimicrobial	Antimicrobial concentration ($\mu\text{g}/\text{mL}$)												MIC ₅₀ ²	MIC ₉₀ ³
	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128		
Ampicillin	97.1	2.9	0	0	0	0	0	0	0	0	0	0	0.06	0.06
Cloxacillin	14.3	5.7	48.6	22.9	5.7	0	2.9	0	0	0	0	0	0.25	0.5
Ticarcillin/clavulanic acid	91.4	8.6	0	0	0	0	0	0	0	0	0	0	0.06	0.06
Ceftiofur	0	0	11.4	14.3	48.6	22.9	2.9	0	0	0	0	0	1	2
Cefuroxime	74.3	17.1	5.7	2.9	0	0	0	0	0	0	0	0	0.06	0.12
Cephapirin	5.7	28.6	62.9	2.9	0	0	0	0	0	0	0	0	0.25	0.25
Enrofloxacin	0	0	0	34.3	65.7	0	0	0	0	0	0	0	1	1
Clindamycin	85.7	8.6	5.7	0	0	0	0	0	0	0	0	0	0.06	0.12
Oxytetracycline	0	0	20.0	48.6	22.9	2.9	0	0	2.9	0	0	2.9	0.5	1

¹ Those isolates not inhibited at the highest concentration of antimicrobial tested, are presented in the next highest dilution and reported \geq that MIC. Where there are results in the lowest concentration tested, the results are reported as \leq that MIC. There are no interpretive criteria available from the Clinical and Laboratory Standards Institute and European Committee on Antimicrobial Susceptibility Testing for antimicrobials against *T. pyogenes*.

² MIC₅₀ = The MIC ($\mu\text{g}/\text{mL}$) that inhibited 50% of the isolates.

³ MIC₉₀ = The MIC ($\mu\text{g}/\text{mL}$) that inhibited 90% of the isolates.

antimicrobials, no inferences can be drawn about the resistance in these isolates for the antimicrobials tested.

However, if potential resistance of *E. coli* isolates against the first generation cephalosporin, cephalixin were confirmed, this is of concern. Cephalixin is the commonly used first line antimicrobial for uterine infections in New Zealand, and these data indicate that at least some *E. coli* isolates may have elevated

Table 4.4. P-values (dark shaded: ≤ 0.05 ; light shaded: ≤ 0.10) of Fisher's exact analyses for the bivariate associations between herd (n = 6), age (categorized as 3 and 4, 5 and 6, and ≥ 7 years), breed (categorized as Friesian, Jersey, and crossbreed), days in milk (DIM) at sampling (day; categorized as 2 (± 1.2 ; SD) and 23 DIM (± 1.2)) and genetic differentiation of pathogen into phylogenetic group (phylo group; categorized as A, B1, and B2 and D) with the distribution of minimum inhibitory concentrations (MIC) of a range of antimicrobials for *E. coli* (n = 175) and *T. pyogenes* (n = 26) for bovine intrauterine origin

Pathogen	Antimicrobial	Herd	Age	Breed	Day	Phylo group
<i>E. coli</i>						
	Ampicillin	0.38	0.65	0.18	0.30	<0.01
	Cloxacillin	- ¹	-	-	-	-
	Ticarcillin/Clavulanic acid	0.39	0.46	0.68	0.84	0.03
	Ceftiofur	0.01	0.72	0.82	0.91	<0.01
	Cefuroxime	0.18	0.51	0.87	0.42	0.02
	Cephalexin	0.18	0.05	0.38	0.05	<0.01
	Enrofloxacin	1.00	1.00	1.00	0.35	1.00
	Clindamycin	-	-	-	-	-
	Oxytetracycline	0.05	0.16	0.09	0.60	0.03
<i>T. pyogenes</i>						
	Ampicillin	0.60	1.00	0.56	0.88	NA ²
	Cloxacillin	0.83	0.65	0.19	0.32	NA
	Ticarcillin/Clavulanic acid	0.01	0.30	0.01	0.67	NA
	Ceftiofur	0.93	0.04	0.65	0.81	NA
	Cefuroxime	0.08	0.84	0.84	0.65	NA
	Cephalexin	0.98	0.75	0.75	0.12	NA
	Enrofloxacin	0.48	0.59	0.77	0.24	NA
	Clindamycin	0.62	0.82	0.23	1.00	NA
	Oxytetracycline	0.16	0.10	0.66	0.72	NA

¹ -: All isolates have the highest MIC tested (≥ 64 $\mu\text{g/mL}$).

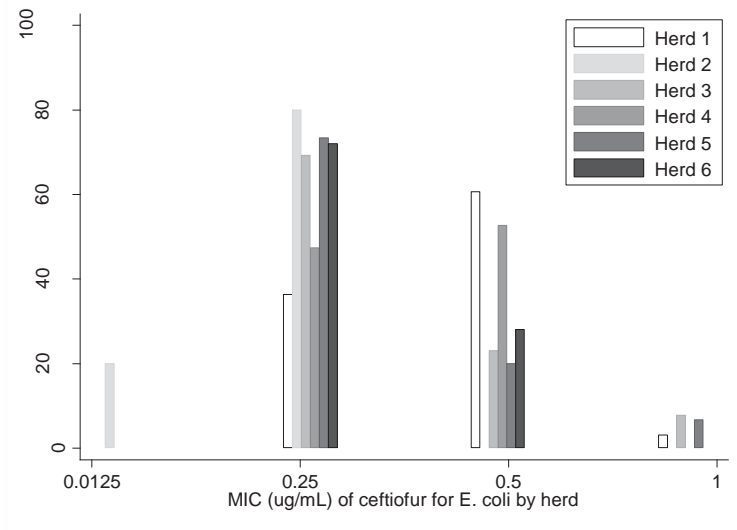
² NA: not available, because no genetic differentiation was done for *T. pyogenes*.

MICs for this antimicrobial. As *E. coli* is present >3 weeks postpartum in a proportion of New Zealand cows (De Boer et al., 2014, in preparation; McDougall et al., 2011) and as some of these cows may be diagnosed with purulent vaginal discharge postpartum, at least some cows with an elevated MIC are likely to be treated with this antimicrobial. In the current study, no associations were found between an elevated MICs for cephapirin and any of the reproductive outcomes tested, suggesting that the MIC is observed were not of biological significance. No pharmacokinetic data are available for cows following intrauterine infusion of cephapirin. However, simple calculations (i.e. mass of cephapirin infused/uterine luminal volume) suggests that current on-label cephapirin treatment will likely achieve concentrations above the MICs reported in the current study. Additionally, it is not clear what effect uterine inflammation will have on the concentrations of antimicrobial following intrauterine infusion. Studies have shown that concentrations of some antimicrobials may be higher in infected tissue than in non-infected tissue (Clarke et al., 1996). Further studies are required to determine if the approximately 20% of cows found to have purulent vaginal discharge 14 days post-treatment with cephapirin (McDougall et al., 2013) are associated with *E. coli* with elevated MICs for cephapirin, related to presence of other bacterial species, reinfection or due to ongoing inflammation in the absence of bacteria.

No interpretative criteria are available for any of the evaluated antimicrobials against *T. pyogenes* and clinicians are thus unable to determine optimal doses and frequencies for these compounds when treating uterine and other infections. None of the antimicrobials tested had an MIC₉₀ of <1 µg/mL against both *E. coli* and *T. pyogenes*. Ceftiofur is a third generation cephalosporin, and relatively low MICs against both pathogens were found in the current study. It has been used in a number of studies that have assessed the efficacy for uterine infections, particularly for early postpartum metritis (Drillich et al., 2001; Risco and Hernandez, 2003) and cows with grossly evident purulent discharge in the vagina (Kaufmann et al., 2010). In all these studies, ceftiofur was administered by subcutaneous injection. None

of the *E. coli* isolates and 2.9% of *T. pyogenes* isolates had MICs greater than the reported maximum mean

(a)



(b)

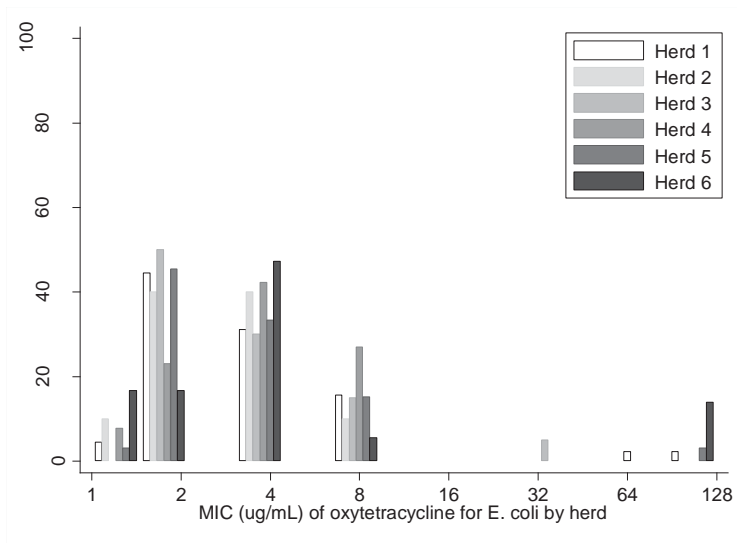


Figure 4.1. Distribution (percentage of isolates within herd) of the minimum inhibitory concentration (MIC) of (a) ceftiofur and (b) oxytetracycline for *E. coli* from intrauterine origin (n = 175) by herd.

concentration of ceftiofur ($\leq 2 \mu\text{g/g}$) in endometrial tissue in early postpartum cows (Okker et al., 2002; Drillich et al., 2006). However, in cows with uterine disease, the highest mean concentration ($2.02 \mu\text{g/g}$) occurred 50 hours after the initial start of treatment, i.e. 2 hours after the third administration with ceftiofur hydrochloride (1 mg/kg ; Drillich et al., 2006). The compliance in completing courses of antimicrobial therapy is reportedly low, with only 24% of surveyed dairy producers in the USA completing antimicrobial treatment courses (Sawant et al., 2005). The mean concentrations of ceftiofur in endometrial tissue of cows with uterine disease and treated once daily for only 2 days was $<1.5 \mu\text{g/g}$ (Drillich et al., 2006). Hence, had cows been treated for only 2 days, 0.5% *E. coli* isolates and 25.8% *T. pyogenes* isolates in the current study could be expected to have had an MIC above the maximum concentration achieved with this treatment regime. Additionally, the highest mean concentration of ceftiofur of $2.33 \pm 1.63 \mu\text{g/g}$ was reached in endometrial tissue of healthy cows treated with the long-acting antimicrobial drug ceftiofur crystalline free acid (CCFA; 6.6 mg/kg s.c) at 24 hours after initial treatment, whereas 3 to 7 days post treatment, all mean concentrations were $<2 \mu\text{g/g}$ (Witte et al., 2011). Thus, it appears that concentrations of ceftiofur in endometrial tissue will only be sufficiently high when correct treatment regimens are used, at least for the isolates of the current study. No data on the pharmacokinetics or of clinical efficacy following intrauterine infusion of ceftiofur are apparently publicly available. Additionally, combinations of systemic and intrauterine infusion treatment may result in a better distribution of antimicrobials across all layers of the uterus (Braun et al., 2009). Hence, substantially more research is required on the pharmacokinetics/-dynamics and clinical efficacy of the use of ceftiofur in this way. At present, this would be an off-label use for the currently commercially licensed products.

Ticarcillin is a carboxypenicillin susceptible to beta-lactamases, hence it is used in combination with clavulanic acid. It has been used for therapy of endometritis in humans (Faro, 1991) and horses (LeBlanc, 2009). Intrauterine infusion of 1 or 3 g of ticarcillin results in relatively low ($<4 \mu\text{g/mL}$) serum

concentrations, indicating little systemic absorption in the mare (Threlfall and Keefe, 1983). Intrauterine infusion resulted in a higher endometrial tissue concentration than an i.v. administration in the mare, however, the half-life of the formulation used was relatively short (Van Camp et al., 2000). There appear to be no studies on the clinical efficacy of ticarcillin for uterine infections in cattle.

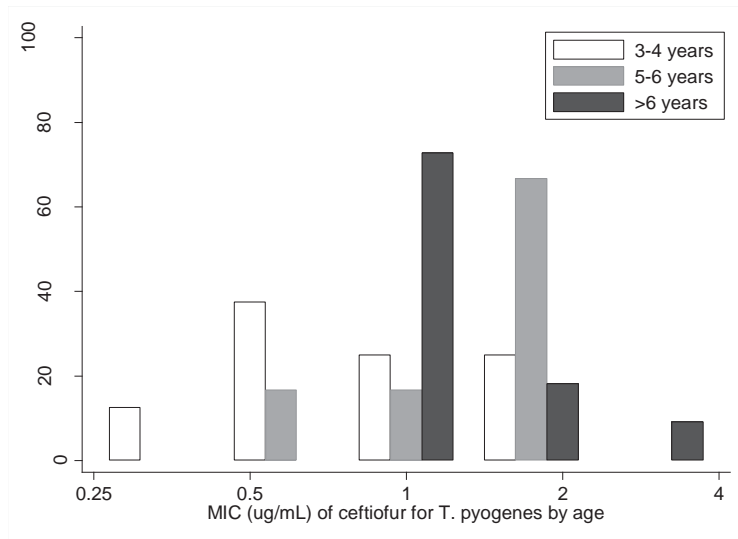


Figure 4.2. Distribution (percentage of isolates within age category) of the minimum inhibitory concentration (MIC) of ceftiofur for *T. pyogenes* from intrauterine origin (n = 26) categorized by age.

The MIC values of some antimicrobials differed between herds, age-groups and breed. Similar findings have been reported for bacteria isolated from milk samples (McDougall et al., 2014), but not for those from uterine origin. Differences in clinical severity, the diagnostic approach, antimicrobial therapeutic choices or culling decisions may have accounted for the observed differences in MICs amongst herds and age-groups. The reason for the difference between breeds and the MIC values of ticarcillin/clavulanic acid for *T. pyogenes* is unclear. Differences in *T. pyogenes* virulence factors have been found between isolates from different cows (Santos et al., 2010a). It is possible that the variation in immune response amongst cow breeds may result in selection of *T. pyogenes* with varying virulence and microbial resistance factors. In the current study, *E. coli* was differentiated into phylogenetic groups,

and these were associated with MIC values for many of the tested antimicrobials. In contrast, one study found no difference between *E. coli* phylogenetic groups and disk diffusion results (Sheldon et al., 2010). The heterogeneity in MICs observed amongst herds of the present study suggests that testing of sensitivity of uterine isolates may need to be considered for individual herds before prescribing decisions are made.

The only elevated MIC with a potential association with impaired reproductive performance was ≥ 8 $\mu\text{g}/\text{mL}$ of oxytetracycline for *E. coli* isolates. This cut-point was derived from human isolates for intermediate susceptible ($8 \mu\text{g}/\text{mL}$) and resistant ($\geq 16 \mu\text{g}/\text{mL}$) *E. coli* isolates (CLSI, 2013). Reproductive tract infections in early lactation are commonly treated with oxytetracycline (Azawi, 2008). Intrauterine treatment of oxytetracycline by infusion or solid form, i.e. pessaries, appears to result in a poor distribution to serum and milk (Bretzlaff et al., 1983; Roncada et al., 2000), suggesting that the therapeutic compound is retained within the lumen and endometrial tissue (Azawi, 2008). The mean concentrations of oxytetracycline in the endometrium as described in two 30 year old studies appear to be approximately $23 \mu\text{g}/\text{g}$ after intrauterine infusion (Bretzlaff et al., 1983) and $\leq 4 \mu\text{g}/\text{g}$ after intramuscular injection (Masera et al., 1980). Nonetheless, in the current study a cut-point of $\geq 8 \mu\text{g}/\text{mL}$ of oxytetracycline may be of clinical significance, suggesting that oxytetracycline may not be the drug of choice in reproductive tract infections, with 16.8% of the *E. coli* isolates above this criterion.

4.5.1. Conclusions

There are limited data available on MICs of antimicrobials against pathogens of bovine uterine origin. This study provided MICs of a range of antimicrobials for *E. coli* and *T. pyogenes* from intrauterine origin. This is the first study to report these data from pasture-based cows, and some differences with studies using cows in housed systems seem to exist. The absence of interpretive criteria for these bovine isolates and of pharmacokinetic and pharmacodynamic data, limits interpretation. Thus, further work is

required on susceptibility testing of antimicrobials for use in veterinary medicine; in particular for bovine reproductive tract infections. In addition, future study designs should evaluate cows from multiple herds and age-groups, as differences in MICs were found amongst herds and age-groups. Finally, only *E. coli* isolates with a high MIC ($\geq 8 \mu\text{g/mL}$) for oxytetracycline had poorer reproductive performance than cows from which *E. coli* with lower MICs were isolated. Validation of MIC cut-points using reproductive outcomes may be more clinically meaningful in bovine reproductive tract infections compared to cut-points selected using human pharmacokinetic or pharmacodynamic data.

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Chapter 5

Detection of bovine herpesvirus type 4 antibodies and bovine lymphotropic herpesvirus in New Zealand dairy cows

According to:

Short communication: Detection of bovine herpesvirus type 4 antibodies and bovine lymphotropic herpesvirus in New Zealand dairy cows. *New Zealand Veterinary Journal*, 2014, in press

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5.1. ABSTRACT

AIM: To detect the presence of bovine herpesvirus type 4 (BoHV-4) in New Zealand dairy cows with clinical metritis.

METHODS: Serum samples taken from 92 dairy cows diagnosed with clinical metritis, each from a different farm, were tested for the presence of antibodies against BoHV-4 using a commercially available, indirect ELISA. Peripheral blood mononuclear cells (PBMC) were collected from ten BoHV-4 seropositive cows, and PBMC were examined by a pan-herpesvirus nested PCR to detect herpesvirus. PCR products were sequenced directly and a proportion of the PCR products were cloned and sequenced to identify the virus present.

RESULTS: BoHV-4 antibodies were detected in 23/92 (25%) serum samples. The pan-herpesvirus PCR was positive in 8/10 PBMC samples. Cloning and sequencing identified that all of the eight PCR positive PBMCs contained bovine lymphotropic herpesvirus (BLHV); no BoHV-4 DNA was detected.

CONCLUSIONS: This study reports the finding of the presence of apparent antibodies to BoHV-4, and BLHV DNA in New Zealand dairy cows affected by metritis.

CLINICAL RELEVANCE: Bovine herpesvirus type 4 and BLHV are reported to have the potential to cause reproduction failure in cows. This is the first report of apparent BoHV-4 antibodies, and BLHV in New Zealand. The significance and epidemiology of these viruses in cattle in New Zealand requires further investigation.

KEY WORDS: Bovine herpesvirus type 4, BoHV-4, bovine lymphotropic herpesvirus, BLHV, metritis, dairy cows, detection, ELISA, cloning, peripheral blood mononuclear cells

5.2. INTRODUCTION

Postpartum uterine infection and inflammation is common in dairy cattle (McDougall et al., 2007; McDougall et al., 2011). Multiple studies have identified a range of bacterial species associated with such infections including some regarded as pathogenic: *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum* and *Prevotella* spp. (Sheldon et al., 2002). Additionally, bovine herpesvirus type 4 (BoHV-4) has been isolated from cattle with metritis (Monge et al., 2006; Bilge-Dagalp et al., 2010; Welchman et al., 2012), endometritis (Frazier et al., 2001) and from animals that have aborted (Czaplicki and Thiry, 1998; Deim et al., 2006). Bovine herpesvirus type 4 is the type species in the genus of Rhadinovirus, subfamily Gammaherpesvirinae, family Herpesviridae (McGeoch et al., 2005). Aborting cows and repeat breeding cows have a higher probability (OR=1.9 and 2.8, respectively) of being seropositive for BoHV-4 than those without these conditions (Czaplicki and Thiry, 1998; Gür and Dogan, 2010). *In vitro*, the virus has been shown to be trophic for bovine endometrial and stromal cells and to induce cytopathic effects (Donofrio et al., 2007). There is also a BoHV-4 dose dependent increase in prostaglandin E₂ production by stromal cells (Donofrio et al., 2007). A vicious cycle between pathology of endometrial tissue, bacterial uterine infection and BoHV-4 reactivation has been suggested by Sheldon *et al.* (2009). Reported BoHV-4 seroprevalences can be high. In a survey of 49 dairy herds and 51 beef herds in Northern Ireland, 29 (59%) of the dairy herds and 35 (69%) of the beef herds contained one or more seropositive cows, and 33.3% of tested dairy cows and 23.3% of the beef cows were positive (Graham et al., 2005). Diagnosis of BoHV-4 infection can be by serology, histology or PCR (Frazier et al., 2001). Although postpartum uterine infection and inflammation is common in dairy cows in New Zealand, the presence of BoHV-4 in New Zealand dairy cows has not been investigated.

The aim of the study was to determine whether BoHV-4 is present in New Zealand dairy cows suffering from clinical metritis.

5.3. MATERIALS AND METHODS

Dairy cows (n=375) from 125 commercial herds diagnosed with severe clinical metritis, (i.e. foetid, thin/watery, red/pink to chocolate brown vaginal discharge and signs of being systemically ill) were enrolled as part of a clinical study in the Waikato region, New Zealand during spring 2010. Cows were a median of 9 days in milk (DIM) at enrolment (range: 0–18 DIM). After clinical examination by a veterinarian, a 10 ml blood sample was collected from the coccygeal vessel using an evacuated tube without an anticoagulant (BD, Franklin Lakes, NJ, USA). Serum samples were separated and stored at -20°C at Cognosco, Anexa Animal Health, Morrinsville, until transportation on ice to the Hopkirk Research Institute, Palmerston North for further analysis.

Randomly selected serum samples from 92 animals, each from a different farm, were tested for BoHV-4 antibodies using a commercially available, indirect ELISA (BIO-X BHV-4 ELISA KIT, Bio-X Diagnostics Jemelle, Belgium) according to the manufacturer's instructions. Briefly, a 1:100 dilution of each serum sample was added to two wells; one containing the BoHV-4 virus and one containing a control antigen. Results were expressed as a Sample to Positive control ratio (S/P%) using the formula: $S/P\% = 100 \times \frac{[\text{sample optical density at 450 nm (OD)} - \text{negative control OD}]}{[\text{positive control OD} - \text{negative control OD}]}$. Reactivity of the samples was assigned a score of 0–5+ as instructed by the manufacturer (Table 5.1).

Of the 23 ELISA-positive cows, ten were revisited at approximately nine months after initial serum collection (median: 288.5 DIM, range: 269–332 DIM) and a blood sample was collected from the coccygeal vessel using a 10 ml evacuated tube containing sodium heparin (BD, Franklin Lakes, NJ, USA; Table 5.2). The blood samples were stored chilled on ice and transported from Cognosco, Anexa Animal Health, Morrinsville to the Hopkirk Research Institute, Palmerston North overnight. Within 24 hours, peripheral blood mononuclear cells (PBMC) were isolated from these samples using Lymphoprep™

(density: 1.077 g/ml, Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's instructions, and the purified PBMC were stored at -80°C until further analyses.

Table 5.1. Interpretation of the Sample to Positive control ratio (S/P%) of the indirect ELISA to detect bovine herpes type 4 antibodies in serum as assigned by the manufacturer (BIO-X BHV-4 ELISA KIT, Bio-X Diagnostics Jemelle, Belgium)

ELISA result	S/P%
0	< 10.22
1+	10.22 < 34.10
2+	34.10 < 57.98
3+	57.98 < 81.85
4+	81.85 < 105.73
5+	≥ 105.73

DNA samples from the PBMC were purified using a Roche, High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH Mannheim, Germany), according to the manufacturer's instructions. DNA concentrations ranged from 13.25–20.38 ng/μL. A modified nested PCR was executed as described by VanDevanter et al. (1996). Briefly, the first round was performed with 20 μL reaction mixture containing 2 μL of the template, 1x PCR buffer, 0.2mM deoxynucleotides, 1 μM of each primer (DFA, ILK and KG1; Table 5.3), and 0.5 U Taq DNA polymerase. A DNA sample purified from BoHV-1 infected Madin-Darby bovine kidney cells were used as a PCR positive control. An initial incubation was executed at 94°C for 2 minutes, followed by a thermo profile, which was repeated for 45 cycles: 94°C for 30 seconds for denaturation, 46°C for 1 minute for annealing, and 72°C for 1 minute for strand extension. The final extension was at 72°C for 7 minutes, and held at 4°C prior to gel analysis. The secondary PCR was performed under the same conditions as the primary, with the exceptions that 50 μL reaction mixture were used containing 1.25 μL of the primary PCR product and 1 μM of each primer (TGV and IYG; Table 5.3), and the thermo profile was repeated for 40 cycles. PCR products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide and viewed under UV light.

The products showing the expected band (VanDevanter et al., 1996) were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics GmbH), according to the manufacturer's instructions. The PCR products were sequenced directly using TGV primer at the New Zealand Genomic Limited (NZGL) sequencing facility at Massey University, Palmerston North, New Zealand. Basic Local Alignment Search Tool (BLAST) was used to compare the sequences of the PCR products against viral sequences in the public domain (Vector NTI Advance 11, Invitrogen, Carlsbad, CA, USA). Four PCR products with the brightest bands, as visualised with UV light, were cloned into T vectors using pGEM-T Easy Vector System (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. The successful cloning of the PCR products was confirmed by colony PCR. Briefly, DNA template was prepared by boiling part of an *E. coli* colony containing the plasmid in 100 μ L DNA/RNA free H₂O at 100°C for 10 minutes, and performing PCR in a 20 μ L reaction mixture containing 3 μ L DNA template, 0.4 μ M of each primer (pUC/M13 forward and reverse; Table 5.3), 1x PCR buffer, 0.2 mM deoxynucleotides, and 0.5 U Taq DNA polymerase. An initial incubation of 2 minutes at 94°C was executed, followed by a thermo profile, which was repeated for 30 cycles: 94°C for 10 seconds for denaturation, 58°C for 20 seconds for annealing, and 72°C for 20 seconds for strand extension. The final extension was at 72°C for 7 minutes, and held at 4°C. PCR products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide and viewed under UV light. Plasmids from overnight *E. coli* cultures containing the PCR products were purified using a High Pure Plasmid Isolation Kit (Roche Diagnostics GmbH), according to the manufacturer's instructions. The DNA concentration ranged from 10.54–13.00 ng/ μ L. Sequencing was performed using the pUC/M13 forward primer (Table 5.3) at the NZGL sequencing facility at Massey University, Palmerston North, New Zealand.

5.4. RESULTS

Of the 92 serum samples from cows clinically affected by metritis, 23 samples (25%) were ELISA positive for BoHV-4 antibodies. One positive was classified as 3+ and the other 22 positives were considered 1+ (Table 5.2).

After performing the nested PCR on the PBMC, a band of approximately 230 base pairs (bp) was visualised in eight out of 10 PCR products, suggesting presence of herpesvirus in these samples.

Approximately 100 bp long sequences were obtained after sequencing the purified nested PCR products directly. The eight herpesvirus-like sequences were subjected to BLAST searches, and all of the eight sequences returned the same top hit sequence, the DNA polymerase gene of a bovine lymphotropic herpesvirus (GenBank accession number: AF327830). In total, one to four colonies from each cloning ligation were screened for plasmid inserts by PCR. Only three (n=3/13) assessed colonies contained plasmids with the expected inserts. The cloned PCR products were derived from two animals (sample ID 26 and 125). The three plasmids from these clones contained sequences that were 100% identical to each other and shared 99% identity (174/175 bp) with the DNA polymerase gene of BLHV, and only 56% with that of BoHV-4 (Figure 5.1). The presence of BLHV in the DNA samples purified from PBMC was further confirmed independently by the Investigation and Diagnostic Centre, Ministry of Primary Industries, New Zealand, using pathogen-specific PCRs (D. Pulford, unpublished data).

5.5. DISCUSSION

A number of reports have indicated that cattle seropositive for BoHV-4 are more likely to have reproduction problems (Czaplicki and Thiry, 1998; Frazier et al., 2001; Gür and Dogan, 2010); but, to date, the presence of antibodies to BoHV-4 in New Zealand cattle has not been documented. In the current study, serum samples were obtained from cows with reproduction problems (i.e. metritis) to

Table 5.2. Data from cows that were ELISA positive for bovine herpesvirus type 4 (BoHV-4) antibodies and the result of subsequent testing for the presence of bovine lymphotropic herpesvirus (BLHV) in peripheral blood monocytes (PBMC) samples collected from heparin blood samples

Sample ID	Breed	Age (years)	Calving date	DIM ^a at serum sampling	BoHV-4 ELISA result (1+ to 5+)	DIM at plasma sampling	Pan-herpesvirus PCR (BLHV)
4	Friesian	6	27/06/2010	12	1+	332	positive
21	Crossbred	2	21/06/2010	18	1+	NT ^b	NT
26	Crossbred	7	14/07/2010	5	1+	308	positive
27	Friesian	10	18/07/2010	8	1+	NT	NT
37	Jersey	2	13/08/2010	11	1+	279	positive
76	Friesian	8	29/08/2010	10	1+	NT	NT
111	Jersey	7	9/07/2010	0	1+	NT	NT
114	Crossbred	4	11/07/2010	9	1+	NT	NT
116	Crossbred	10	30/07/2010	6	3+	299	positive
125	Jersey	2	12/08/2010	13	1+	286	positive
168	Friesian	3	26/08/2010	1	1+	NT	NT
221	Friesian	2	18/07/2010	4	1+	NT	NT
227	Crossbred	2	23/07/2010	10	1+	299	positive
282	Friesian	2	29/07/2010	14	1+	NT	NT
286	Friesian	3	21/08/2010	11	1+	270	negative
292	Crossbred	2	22/07/2010	15	1+	NT	NT
293	Crossbred	4	12/08/2010	11	1+	NT	NT
307	Crossbred	6	23/08/2010	5	1+	269	positive
309	Crossbred	7	14/08/2010	16	1+	NT	NT
319	Jersey	2	1/08/2010	12	1+	290	positive
323	Friesian	6	1/08/2010	13	1+	NT	NT
343	Crossbred	2	5/08/2010	14	1+	287	negative
421	Crossbred	8	7/09/2010	8	1+	NT	NT

^a DIM is days in milk.

^b NT is not tested.

maximise the chance of detecting BoHV-4 antibodies in New Zealand dairy cows. Twenty-five per cent of the 92 cows, each from a different farm, were seropositive for BoHV-4. Timing of seroconversion could not be assessed, because single rather than paired serum samples were obtained. Blood monocytes are target cells for the latent stage of this herpesvirus (Ackermann, 2006); therefore, ten seropositive cows were revisited to collect blood for the detection of herpesvirus DNA in PBMC by a pan-herpesvirus PCR analysis. Herpesviruses have the capability to maintain themselves in a latent form in the host for the duration of that host's life, resulting in a long latent stage after the initial infection (Cohrs and Gilden,

2001). Therefore, it is likely that approximately 9 months after the initial BoHV-4 antibody testing, when the follow-up blood samples were taken, DNA would still be present in the PBMC of any animal that had been previously infected with BoHV-4.

Interestingly, the presence of BLHV DNA was detected by the pan-herpesvirus PCR. Only a limited number of peer-reviewed papers are available on this virus. Bovine lymphotropic herpesvirus was first described by Rovnak et al. in the USA (1998), and has since only been reported in the United Kingdom, Canada and Belgium (Cobb et al., 2006; Gagnon et al., 2010; Garigliany et al., 2013). There are suggestions of its association with reproduction failures; i.e. metritis and abortion in infected cows (Banks et al., 2008; Gagnon et al., 2010). However, further work is required to elucidate the true implications of infections with this herpesvirus in cows.

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BLHVnz TCGGTCCGGTTTGCTTCCTTGCTTGAAAATTGCAGAAACGGTCACTCTAC 50
BLHV -----
BoHV-4 ---T--A--AA-T--C--A---A-CCC---A-----T--A--AT---

BLHVnz AGGGGCGAACCATGCTGGAAAGAATAAGCACTACGTGGAAAGCTTACAG 100
BLHV -----
BoHV-4 ----AA----A---T----G-AGT-A---GCA-T-----G-TGA-*--C

BLHVnz CCCGTAGACTTGGAGCGCATTTGCCAGAGGCCCATTCAGTCGCAGAGGG 150
BLHV -----
BoHV-4 A--AG---GGCTA--T-ACA---T-TC-TA-----G-A-CT-GTG-CCCA-

BLHVnz GCACGCCGATCCTAGCTTGCGCGTG
BLHV --G-----
BoHV-4 ATG-CT-AT-TAG--*-A-*****

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Figure 5.1. The alignment of partial DNA sequences (175 bp) of the DNA polymerase gene of bovine lymphotropic herpesvirus of New Zealand isolates (BLHVnz) with that from isolates of a BLHV and a BoHV-4 (GenBank accession

numbers: AF031808 and AF031811, respectively). '-' denotes identical nucleotide with that of the BLHVnz, a letter includes an alternate base, and '*' denotes a gap.

The finding of BLHV instead of BoHV-4 in BoHV-4 seropositive cows in this study could be the result of several possibilities. Firstly, both BLHV and BoHV-4 are members of the ruminant rhadinoviruses (RuRV; Rovnak et al., 1998; McGeoch et al., 2005). There are four suggested viral types in RuRV based on the amino acid residual sequences of DNA polymerase gene fragments. As BLHV and BoHV-4 belong to Type 2 and Type 3 RuRV, respectively (Li et al., 2005), it is likely that the two viruses share cross-reactive antigens. Therefore, antibodies stimulated by the BLHV could cross-react with BoHV-4 antigens and, hence, cows were positive to the BoHV-4 ELISA. Another possible explanation is that the seropositive cows were infected by BoHV-4 in the past and the antibodies detected by the BoHV-4 ELISA were BoHV-4-specific; however, due to viral latency and analysing the target cells of BoHV-4 (i.e. PBMC), this may be a less likely possibility. Further elucidation of potential cross-reactivity between BoHV-4 and BLHV antigens would be helpful for the interpretation of the serological results of the current study as, although antibodies against BoHV-4 were detected, no evidence was found for the presence of BoHV-4 DNA in the PBMC of the subset of seropositive animals that were resampled. However, due to the use of pan-herpes consensus primers instead of specific BoHV-4 primers, there may be a small possibility that in the case of a mixed infection, one virus (i.e. BLHV) may have been preferentially amplified at the cost of another (e.g. BoHV-4). Therefore, the true absence of BoHV-4 infection cannot be confirmed by these analyses.

A relationship has been found by other researchers between serological evidence of BoHV-4 infection and both infertility and abortion (Czaplicki and Thiry, 1998; Gür and Dogan, 2010); however, the aim of the current study was not to investigate the role of BoHV-4 in metritis in New Zealand cows as samples were collected as part of an investigation on clinical metritis. Nonetheless, analyses were undertaken to assess the association between cows positive or negative on the BoHV-4 antibody ELISA and potential

explanatory variables, i.e. being dehydrated, rumen contractions (cut-point: 2 contractions/min), and rectal temperature at the time of enrolment. Chi-square tests and t-tests were used to assess the associations with the categorical and continuous explanatory variables, respectively. No associations between the putative explanatory variables and BoHV-4 antibody test status were found ($p>0.05$; data not shown). In the clinical study, which will be reported separately, serum samples were only collected from severely affected animals and not from unaffected cows. For this reason and the small study size, these results need to be interpreted with caution.

Table 5.3. Sequences of primers (DFA, ILK, KG1, TGV, and IYG) used for a pan-herpesvirus nested polymerase chain reaction (PCR), and of primers (pUC/M13) used to confirm a successful cloning of pan-herpesvirus PCR products and to sequence those products

Primer	Direction	Sequence (5' – 3')
DFA ^a	Upstream	GAYTTYGCNAGYYTNTAYCC
ILK ^a	Upstream	TCCTGGACAAGCAGCARNYSGCNMTNAA
KG1 ^a	Downstream	GTCTTGCTCACCAGNTCNAACCCYTT
TGV ^a	Upstream	TGTAACCTCGGTGTAYGGNTTYACNGGNGT
ITYG ^a	Downstream	CACAGAGTCCGTRTCNCCRTADAT
pUC/M13 ^b	Forward	GTTTTCCCAGTCACGAC
pUC/M13 ^b	Reverse	CAGGAAACAGCTATGAC

^a VanDevanter et al. 1996.

^b Promega Corporation, Madison, WI, USA.

The pathogenicity of BLHV is unclear at this stage. Reports have suggested that the virus might play a role in post-partum metritis non-responsive to treatment and in the stimulation of the disease caused by bovine leukemia virus (Rovnak et al., 1998; Banks et al., 2008; Garigliany et al., 2013). Bovine lymphotropic herpesvirus was detected in brain and lymph node tissue from an aborted foetus (Gagnon et al., 2010). However, this foetus was also *Neospora caninum* positive, therefore the role of the virus on abortion in cattle is unproven. A recent study in the United Kingdom compared vaginal swabs from 15 cows diagnosed with metritis and 15 cows without, and did not find an association between the presence of BLHV and cows having metritis (Welchman et al., 2012). In other studies, the prevalence of

BLHV has been high in both young and adult cows (Rovnak et al., 1998; Banks et al., 2008). However, the results of these studies need to be interpreted with caution as their small sample sizes and preselected sample collection protocols do not represent randomized population samples.

This study reports on the first detection of apparent antibodies to BoHV-4 in serum samples, and the presence of BLHV DNA in PBMC, originating from dairy cows in New Zealand. However, it was beyond the scope of this study to determine the prevalence and the pathogenicity of these viruses. Further studies on the cross-reactivity of the two viruses and test validation of the ELISA used are needed.

Furthermore, epidemiological studies with larger numbers of randomly selected cows are needed to determine the prevalences and the potential reproductive consequences of these two herpesviruses in New Zealand dairy cows.

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Chapter 6

General discussion

6.1. INTRODUCTION

Reproductive tract diseases of dairy cows are prevalent world-wide, decrease reproductive performance and cause production losses (McDougall, 2001a; Esslemont, 2003; Gilbert et al., 2005; McDougall et al., 2011). In the last decade, the overall decline in reproductive performance plateaued in New Zealand, but is still not improving (Brownlie, 2012). Aspects considered in this research were methods utilised in the diagnosis of reproductive tract infection and inflammation, known and unknown pathogens potentially associated with reproductive tract disease within New Zealand, and the bacterial susceptibility against a range of antimicrobials which may be used in the treatment of reproductive tract disease were evaluated.

Specific aims were to review the accuracy and applicability of diagnostic methods, diagnostic criteria and definitions, repeatability and agreement among methods for diagnosis of reproductive tract diseases in dairy cows, and to critically appraise the quality of design and reporting of the publications describing those diagnostic methods (Chapter 2). In a large field study, cows were sampled to assess whether isolation of *Escherichia coli* from the uterus in the first week postpartum increased the risk of *Trueperella pyogenes* 21 days later. Potential associations between bacteriological variables (e.g. *E. coli* early postpartum and any bacteria irrespective of species), reproductive tract inflammation (i.e. purulent vaginal discharge; PVD or endometritis diagnosed by cytology) and reproductive performance were assessed (Chapter 3). Further objectives were to generate data on minimum inhibitory

concentrations (MIC) for a range of antimicrobials used in the treatment of *E. coli* and *T. pyogenes* isolated from pasture-based cows. Potential associations between the MIC data and reproductive performance were also evaluated (Chapter 4). The final objective of the thesis was to determine whether bovine herpesvirus type 4 (BoHV-4) was present in New Zealand dairy cows (Chapter 5).

Each thesis-chapter has been prepared for submission to a peer-reviewed scientific journal. Discussions within each chapter on the results and study design were restricted to the particular chapter and the appropriate journal format. While not attempting to repeat previous chapters, this chapter discusses the study designs including potential biases, the utility of available diagnostic tests for reproductive tract diseases, therapeutic options for those diseases, the importance of reproductive tract pathogens, and the immune response in an overall context. It concludes with suggestions for future research.

6.2. STUDY DESIGN

As highlighted in Chapter 2, there has been considerable variation in the quality of reporting and design of a number of published papers on diagnostic methods for reproductive tract diseases in cows.

Similarly, sources of bias may also be present in this thesis. For example, the herds enrolled in studies of this thesis were selected on the basis of a willingness to participate; thus selection was non-random.

Many previous studies of reproductive tract diseases have selected herds on the basis of convenience (e.g. university or research herds; Sheldon et al., 2004; Priest et al., 2013). However, these herds may not have been managed in the same manner as commercial herds, potentially affecting the external validity of study results. Herds enrolled in studies reported in this thesis were privately owned and managed on a commercial basis and, therefore, relatively less biased. Moreover, a relatively greater number of herds were enrolled in the current studies relative to many other studies (Sheldon et al., 2004; Santos et al., 2010; McDougall et al., 2011; Sens and Heuwieser, 2013). However, enrolled herd

owners/managers may have had a particular interest in research and, therefore, potentially with a more than average interest in improving herd health.

In the 2012/2013 season, the mean herd-size in the Waikato region of New Zealand was 327 cows (Anonymous, 2013). To increase the likelihood of enrolling the required number of cows for the field study, as described in Chapter 3, enrolment of larger herds was preferred. Hence, herds with a greater than average number of cows (median: 538 cows; range: 329-830 cows; Table 3.1.) were enrolled and a bias towards larger herd size may be present. Management differences between small and large herds may occur; however, it is unclear whether there would be a difference in risk of disease. Furthermore, not all cows within each herd were randomly selected. The random enrolment of cows was only possible when more than 25 cows were present at each enrolment day.

Although the author of this thesis was involved in every step of each study (i.e. sample collection, sample analysis and statistical analysis), no blinding was applied. Therefore, the author could have been aware of, for example, the bacterial exposure status at Day 0 and an outcome status at a later sample day (Chapter 3). However, the outcomes were relatively objective and the likelihood of any resultant bias was found to be low (e.g. intraobserver agreement in reading of cytology slides was high). Despite this, a double-blinded trial would have been preferred.

6.3. DIAGNOSTIC METHODS FOR REPRODUCTIVE TRACT DISEASES

Chapter 2 discussed the quality of design and reporting of previous publications on diagnostic methods available for reproductive tract disease in dairy cattle, including study bias, in detail. Currently, the diagnosis of PVD on New Zealand farms is usually undertaken using the Metricheck® device approximately one month prior to the start of the breeding season (planned start of mating; PSM). This remains the preferred diagnostic method due to being relatively low cost and providing an instantly available result (Chapter 2). While a recent New Zealand study found the sensitivity of this method to be

high (Se = 0.96), the specificity was found to be moderate (Sp = 0.78; McDougall et al., 2007). Cows diagnosed with PVD by Metrichick had lower reproductive performance relative to test-negative cows. For example, cows detected with a vaginal discharge score (VDS) of 2 (i.e. having flecks of pus) to score 5 (i.e. > 50% pus and foul smell) had a lower risk of pregnancy within 28 days postpartum compared to cows with a VDS of 0 or 1 (no or clear mucus; RR = 0.87-0.68; P < 0.01; McDougall et al., 2007). This implies a high diagnostic utility of Metrichick for expected reproductive performance (e.g. for detecting cows with PVD that require treatment for reproductive tract disease).

Recently, a number of studies (Gilbert et al., 2005; Dubuc et al., 2010a; Deguillaume et al., 2012), including Chapter 3, have used the detection of polymorphonuclear cells (PMN) by cytobrush or uterine lavage to diagnose endometritis. It is now known that the presence of PVD does not equate to endometritis, but can coincide with endometritis (Dubuc et al., 2010b; Deguillaume et al., 2012; Lima et al., 2013) and that a recruitment of PMN into the endometrium within the first few days postpartum is likely to be part of the physiological uterine involution process (Gilbert et al., 2007; Chapter 3). As discussed in Chapter 2, the test characteristics (i.e. sensitivity and specificity) of both the cytobrush and uterine lavage techniques are inadequate to be considered as potential gold standard diagnostic tests for endometritis. Moreover, these methods, that require penetrating the cervix, are not practical in a commercial setting that requires rapid test completion and availability of results. Although previous publications have assessed potential alternative tests (Cheong et al., 2012; Couto et al., 2013), a less time consuming and invasive cow-side test, such as detection of a reproductive-specific biomarker in milk or blood, would be advantageous and is still to be developed (Chapter 2).

6.4. THERAPY OF REPRODUCTIVE TRACT DISEASES

Although broad-spectrum antimicrobials have been consistently used for the treatment of reproductive tract diseases of cattle, controversy over their use exists (Lefebvre and Stock, 2012). In pasture-based

dairy management systems, intrauterine infusion with cephalosporins has been found to result in improved reproductive performance (McDougall, 2001a; Runciman et al., 2008). A recent systematic review and meta-analysis concluded there was little evidence that treatment with prostaglandin F_{2α} (PGF_{2α}) against PVD resulted in an improved reproductive performance (Haimerl et al., 2012; Haimerl et al., 2013). In contrast, a recent New Zealand study found cows with PVD and a palpable corpus luteum (CL) had similar reproduction performance when treated with PGF_{2α} compared to cows treated with cephalosporins (McDougall et al., 2013). In a recent trial, intrauterine infusion with bacteriophages (Machado et al., 2012) did not improve reproductive performance.

Recently, heifers in a USA trial were vaccinated against *E. coli*, *T. pyogenes*, and *Fusobacterium necrophorum* in the prepartum period and found to have improved reproductive performance relative to their unvaccinated herdmates (Machado et al., 2014). However, it is unclear if this newly developed vaccine, which was assessed in housed heifers, will have similar effects in a pasture-based system. Different bacteriological species, strains, or virulence factors may be present in pasture-based cows compared to those being housed (McDougall, 2001b). It can be hypothesized that cows in confined systems often calve in a calving pen commonly used as a hospital pen, whereas New Zealand cows calve on pasture with a potentially lower pathogen density compared with calving or hospital pens. Differences in prevalence of *E. coli* and *T. pyogenes* were found between pasture-based and those being housed (Werner et al., 2012; Sens and Heuwieser, 2013; Chapter 3). Due to the seasonality of the New Zealand dairy system, a large number of cows calve within a reasonably short period of time, a number of which may require human intervention at calving. It could be hypothesized that this may result in an increased risk of between-cow transmission of reproductive tract pathogens due to human contamination. However, on an individual cow basis, pasture-based cows are less likely to be checked for dystocia or other peripartum disease than cows under confined management, making the latter population at higher risk of reproductive tract disease.

There is an increasing awareness of antimicrobial resistance in the general public and among producers, veterinary researchers and practitioners (Anonymous, 2010; Acar et al., 2012; Compton and McDougall, 2013). Prudent usage guidelines for antimicrobial therapy for reproductive tract disease suggest that an *a priori* assessment of the MIC of the antimicrobial is required. However, only limited data are currently available on the pharmacodynamics and -kinetics of commonly used antimicrobials for this purpose (Chapter 4). Appropriate uterine sampling for susceptibility testing requires time and is, in a commercial farm setting, impractical. Moreover, only limited data are available on the validity of diagnostic tests used for the isolation of bacteria from the bovine uterus. Although a high intra- and inter-laboratory agreement for bacterial culture from uterine swabs and cytobrushes has been suggested (Werner et al., 2012), statistical measurements of agreement were not provided. Further research is required on test characteristics of current and future diagnostic methods for isolating bacteria from the bovine uterus as well as the potential for contamination during each procedure. However, evaluation of the latter may be challenging due to the lack of a gold standard test. Often, multiple bacterial species can be isolated from the bovine uterus (Földi et al., 2006) and susceptibility testing for each isolated species prior to antimicrobial administration is unlikely to be cost effective, particularly in the case of a single affected cow. Hence, more research is needed to assess the aetiology and importance of potential uterine isolates.

A possible solution may be to tailor antimicrobial therapy to the individual farm rather than at the cow-level. Differences in the prevalence of both *E. coli* and *T. pyogenes*, and bacteria irrespective of species were detected between herds (Chapter 3) and differences in minimum inhibitory concentration for a range of antimicrobials were also found to be herd-specific (Chapter 4). This indicates that bacterial strains and antimicrobial susceptibility may be unique to each herd. To reduce antimicrobial resistance of bovine uterine pathogens, it may be possible to establish herd-specific concentrations (e.g. MIC₉₀) for the preferred antimicrobial therapy. However, more data need to be generated on the

pharmacokinetics of antimicrobials (i.e. effective antimicrobial concentrations in reproductive tract tissue).

6.5. IMPORTANCE OF PATHOGENS ON THE EFFECT OF REPRODUCTIVE TRACT DISEASES

Controversy still remains regarding the importance of specific bacteria, particularly *E. coli* and *T. pyogenes*, on the occurrence of bovine reproductive tract disease. A current lack of consensus amongst published authors indicates that no single bacterium may be a necessary cause of an inflammatory response in the reproductive tract. Although *T. pyogenes* has been consistently shown to be associated with the presence of reproductive tract disease and decreased reproductive performance in high producing cows in confined systems (Bonnett et al., 1991; Bonnett et al., 1993; Huszenicza et al., 1999; Dohmen et al., 2000; Sens and Heuwieser, 2013), some recent studies did not find a similar association (McDougall et al., 2011; Bicalho et al., 2012).

Bicalho *et al.* (2012) and Sens and Heuwieser (2013) found an association between *Fusobacterium necrophorum* and α -haemolytic streptococci with reduced reproductive performance. It has also been hypothesized that bacterial endotoxins in addition to the commonly researched lipopolysaccharides, such as lipoteichoic acid (LTA; from Gram-positive bacteria), may be more involved in reproductive tract disease than indicated in the current literature (LeBlanc, 2014).

Interestingly, healthy placentas from women who had experienced spontaneous term delivery have been found to contain intracellular Gram-positive and -negative bacteria, including *E. coli* and *Fusobacterium* spp. (Stout et al., 2013; Aagaard et al., 2014). This could imply that (at least in late stage pregnancy) bacteria can normally be present in the uterus. Hence, the mere presence of a specific bacterial species in the uterus may not be as significant as previously assumed. Sheldon et al. (2009) have postulated that bovine herpesvirus type 4 may have a synergistic effect on bacteria in the pathogenesis of reproductive tract disease. In addition, other viruses, such as bovine lymphotropic

herpesvirus (BLHV; Chapter 5), may have such an effect. Moreover, pathogens that have not been a focus of previous studies (e.g. fungi) could also have a potential effect, although, similar to anaerobic bacteria, these pathogens may be difficult to isolate. Recently metagenomic tests have been developed to detect the presence of fastidious bacteria (Bicalho et al., 2010; Bicalho et al., 2012). Culture-independent tests have the advantage of being more sensitive compared to culture-dependent tests (Prezioso and Cuteri, 2012). However, further analyses can be done on cultured pathogens, such as antimicrobial susceptibility tests (Prezioso and Cuteri, 2012). It is evident that more research is required on the causality of the different pathogens on bovine reproductive tract diseases.

6.6. IMMUNE RESPONSE AND REPRODUCTIVE TRACT DISEASE

Polymorphonuclear cells are the key inflammatory cell types during an infection (Ingvarsen and Moyes, 2013) and it has been suggested that endometritis is a persistent inflammatory process instigated by a previous infection (LeBlanc, 2014). Therefore, the unsuccessful shift from down-regulation of the immune system (as required during pregnancy) back to a fully competent and functioning immune system (required for uterine involution and removal of any pathogens postpartum) may be more important than the presence or absence of specific pathogens in the diagnosis of endometritis (LeBlanc, 2014). An influx of PMN into uterine tissue shortly postpartum has been found to be beneficial for the clearance of bacteria (Gilbert et al., 2007) and was associated with a reduced time from PSM to pregnancy (Chapter 3).

Cows with a negative energy balance, specifically an increase in non-esterified fatty acids (NEFA), have been shown to have reduced PMN functionality (i.e. the 'oxidative burst'; Hammon et al., 2006; Moyes et al., 2009). Although a number of studies have also found a similar effect when beta-hydroxybutyrate (BHBA) was increased (Hoeben et al., 1997; Suriyasathaporn et al., 1999), a recent study did not support this association (Ster et al., 2012). Cows affected by reproductive tract disease have also been shown to

have lower PMN glycogen concentrations compared with unaffected cows and, as glucose is necessary for cell performance and maintenance and mandatory for the prime immune response, this may result in reduced PMN function in these cows (Galvão et al., 2010; Ingvarlsen and Moyes, 2013). Supporting this hypothesis was a recent study that showed that Holstein heifers with high fertility genetics had higher glucose concentrations and were less likely to be diagnosed with PVD and endometritis compared with heifers with low fertility genetics (Moore et al., 2014).

Cows that fail to adapt to increased nutrient requirements during early lactation may be more likely to have peripartum disease. Over-fed cows (i.e. those with a high body condition) or those being under-fed (i.e. with a low body condition) could be at higher risk of impaired metabolic parameters (Ingvarlsen and Moyes, 2013). The New Zealand pasture-based system is mainly reliant on adequate seasonal pasture growth, with cows typically receiving a supplement (e.g. grass and maize silage) when grass growth is insufficient (Verkerk, 2003). Despite this, a recent New Zealand study found that subclinical ketosis, as measured by BHBA, was highly prevalent in the dairy industry, with significant between-herd differences (Compton et al., 2014).

Hormones, such as glucocorticoids, released during stressful periods (e.g. calving or insufficient nutrition) may also suppress the immune response in a dairy cow (Verbrugghe et al., 2012). In the New Zealand dairy system, new-born calves will generally be removed from the dams once or twice daily. Thus, in some cases, a calf can be with its dam for up to 24 hours where a dam-calf bond may be more strongly established compared to when the calf is removed immediately, potentially causing additional stress. Although more research on the reduction of reproductive tract disease through changed management, particularly around the transition period, is needed, it could be hypothesized based on current knowledge that the risk of disease may be minimized by the reduction of stress, abrupt ration changes or malnutrition.

6.7. TOPICS FOR FURTHER RESEARCH

Good reproductive performance of cows is vital for the dairy industry and novel data have been presented in this thesis that will fill gaps existing in current knowledge of bovine reproductive tract disease. However, several areas that would benefit from further research became clear, some of which have been discussed above (e.g. more research is required to determine the causality of the different pathogens on bovine reproductive tract diseases). There are many factors influencing reproductive performance (Figure 6.1) and it was not the scope of this thesis to cover all of them; therefore, only some suggestions for further research topics arising from the studies presented in this thesis are made here.

6.7.1. Diagnostic Tests

As mentioned earlier in this Chapter, the creation of a rapid and practical cow-side test for endometritis that has been validated using reproductive outcomes, such as getting pregnant within six weeks from PSM, would be highly useful to aid in the improvement of reproductive performance in New Zealand dairy cows. In the meantime, the calculation of intra- and interobserver agreement of the available diagnostic tests for reproductive tract disease (i.e. Metricheck, cytobrush and uterine lavage) are required.

Even though a number of studies have been conducted at different time intervals from calving, there are currently insufficient data to determine the most advantageous time to diagnose the various reproductive tract diseases. Although PVD in New Zealand dairy cows is currently typically diagnosed approximately one month prior to PSM, it was found in the field trial that the diagnosis of PVD as early as three weeks postpartum increased the risk of anoestrus treatment (Chapter 3). Therefore, earlier diagnosis may be beneficial, but future research is required to further evaluate this hypothesis. Self-cure of disease (i.e. without intervention) is likely to be influential on the timing of diagnosis and, diagnostic

plans are also likely to depend on the sensitivity and specificity of the test of interest and the farm management system (e.g. seasonal vs. year-round calving systems). An economic evaluation of the optimal timing of a diagnostic test, taking into account the above variables, would be beneficial.

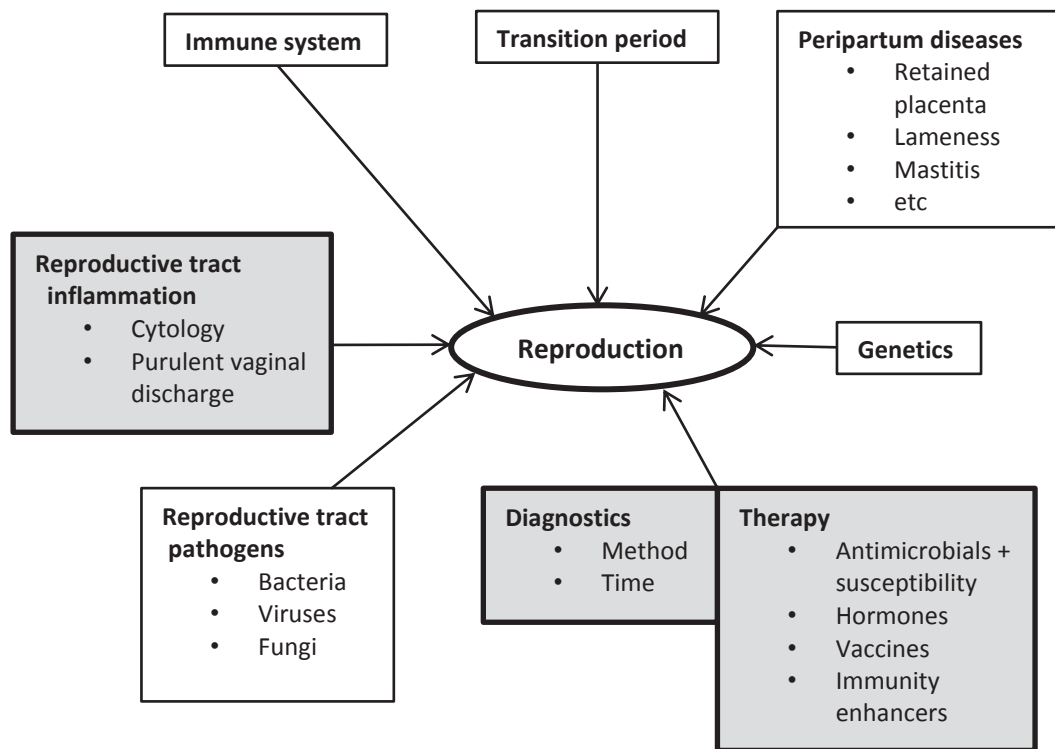


Figure 6.1. Diagram of some possible variables influencing reproductive performance of dairy cows. The grey shading represents specific areas addressed in this thesis, either partially or in detail.

The level of agreement between different bacteriological tests used in the diagnosis of bovine reproductive tract disease is currently unknown, with the exception of the level of agreement between the use of a cytobrush and uterine swab, both culture-dependent tests (Werner et al., 2012). Currently, there are no data available on the level of agreement between culture-dependent and culture-independent tests used for this purpose. These would be necessary data for interpretation of previous published results and for the enhanced development of future study designs. Due to the lack of a gold

standard test for reproductive tract disease, culture-dependent and culture-independent tests should be validated using reproductive performance (as described in Chapter 2).

6.7.2. Involvement of Pathogens in Reproductive Tract Diseases

Further research is required to evaluate the causal effect of bacteria, such as *E. coli*, *T. pyogenes*, anaerobes like *Fusobacterium* sp. and *Prevotella* sp., on the pathogenesis of bovine reproductive tract inflammation. This research should incorporate an evaluation of the effect of potential pathogens on reproductive performance, such as pregnancy within six weeks from the PSM. Moreover, the postulated synergistic effects between particular bacteria or viruses need to be confirmed if future therapy against a specific pathogen or combinations of pathogens (e.g. vaccination) is to be considered.

There is a dearth of New Zealand studies assessing bovine intrauterine bacterial flora using genomic tests, such as RAPD (random amplification of polymorphic DNA) PCR or MLST (multilocus sequence typing), as has been previously described in cows housed in confined systems (Sheldon et al., 2010). Such testing would improve our knowledge of flora specific to New Zealand pasture-based cows and shed light on the aetiology of those bacteria within reproductive tract infections. Further, this will determine whether there are different bacterial subspecies present in pasture-based cows compared to cows that are housed and calve in calving or hospital pens, and whether endometrial pathogenic *E. coli* (EnPEC) are present in New Zealand. The presence of intracellular bacteria in the healthy human placenta (Stout et al., 2013; Aagaard et al., 2014) refutes the hypothesis that bovine uteri may be sterile during pregnancy. To investigate this further, cows subjected to elective caesareans may be used for sterile sample collection.

It was postulated in Chapter 5 that the BoHV-4 antibody ELISA may cross-react with other herpesviruses, such as BLHV. This would need to be clarified prior to further studies on BoHV-4 using this commercial available ELISA. Although PCR may be preferred over the ELISA antibody test, detection of acute

infection by seroconversion is an advantage of serology. The effect of BLHV on cow health and reproductive performance is unknown and, because this virus may have a similar level of pathogenicity to endometrial tissue as BoHV-4 (Donofrio et al., 2007), further research would be beneficial.

6.7.3. Treatment

More research on antimicrobial treatment of reproductive tract diseases is warranted. A recent study found a 20% treatment failure of PVD and further investigation of the potential reasons for and economic consequence of this is needed (McDougall et al., 2013). There are no data available on the success of re-treatment of those cows found to be clinically affected with PVD two weeks after an initial treatment. Thus, further studies will be beneficial to determine whether re-treatment improves reproductive performance and, if so, which treatment would be preferred (i.e. the same or an alternative treatment).

More data are also needed to establish interpretive cut-points for minimum inhibitory concentrations (MIC) of antimicrobials for bacteria isolated from the bovine uterus. The current use of a first generation cephalosporin as the most common treatment for PVD may not be optimal and other antimicrobial treatment options need to be explored. For example, prostaglandin $F_{2\alpha}$ in cows with a CL affected by PVD was found to have a similar therapeutic effect compared with an intrauterine infusion of cephalosporin (McDougall et al., 2013) and the administration of ultra-filtered bovine whey from hyperimmunized cows and recombinant bovine granulocyte colony stimulating factor increased the functionality and proportion of circulating PMN, respectively (Roth et al., 2001; Kimura et al., 2014).

6.8. CONCLUSION

The overall reproductive performance of the New Zealand dairy industry, for example measured by pregnancy risk, has been either declining or plateaued as seen in the rest of the world and reproductive tract disease represents a significant economic burden to New Zealand dairy producers. Research has

been undertaken world-wide and variations in incidence of reproductive tract diseases have been found. It is not clear how important differences between farm systems are in incidence, severity, and outcome of these diseases.

Final conclusions of this thesis are as follows. A number of diagnostic methods for reproductive tract disease have been reviewed. For the detection of PVD and, in part, of endometritis, the use of the Metricheck device or vaginoscopy is currently still preferred in the commercial setting. Overall, the Metricheck device is easier and faster and, therefore, continues to be the favoured method for the New Zealand veterinary practitioner. Some treatment options for PVD are currently available and described. In spite of the fact that intrauterine infusion with cephalosporin is the commonly used empirical treatment for PVD in New Zealand dairy cows, this antimicrobial still appears to be effective against *E. coli* and *T. pyogenes*, but the high MIC may be of concern for future effectiveness. In contrast to what is implied by other researchers, no evidence was found that intrauterine presence of *E. coli* within the first week postpartum predisposed to isolation of *T. pyogenes* three weeks later. The presence of BoHV-4 could not be proven, however, the detection of BLHV in New Zealand is novel and this virus may possibly play a role in severity of reproductive tract diseases in dairy cows. The influx of PMN early postpartum, which is likely to be part of the physiological uterine involution, decreased time to pregnancy.

It is evident that increasing reproductive performance of dairy cows is not simply a matter of improving knowledge of reproductive tract pathogens, diagnostic methods or therapy (Figure 6.1.). A holistic approach utilising, in part, the knowledge generated in this thesis is required from all stakeholders within the New Zealand dairy industry to achieve its future reproductive goals.

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Appendices

Appendix 1

Template modified from the Cochrane Handbook (Higgins and Green 2011) to extract data from papers for a systematic review of diagnostic methods for reproductive tract diseases in dairy cows (Chapter 2)

Topic	Item id	Item to report
Source	1	Reviewer ID
	2	Report ID
	3	Title
	4	Year of study publication
Eligibility	5	Confirm eligibility for review
	6	Reason for exclusion
Study	7	Study design
Participants	8	Total number of herds
	9	Inclusion criteria Exclusion criteria
	10	Selection process of cows/herds
	11	Location (country)
	12	Calving pattern (year round, or seasonal calving)
Disease	13	Which of the following were studied? Vaginitis Cervicitis PVD ¹ Endometritis Salpingitis Oophoritis Other
	14	Were cows excluded that were diagnosed with another reproductive tract disease other than the study's disease of interest (e.g. studies on endometrium cytology, but cows with PVD were excluded)? If yes, which disease(s) were excluded? If yes, was the exclusion bias accounted for?
	15	How was (were) the reproductive tract disease(s) of interest defined (reference used, new definition provided and statistical evidence provided, not defined)?

		Which reference (first author, year of publishing) or what definition of the studied reproductive tract disease(s) was used?
Diagnostic criteria	16	Which of the following diagnostic tests were used? Gloved hand Vaginoscopy Metricheck Ultrasound Cytology by cytobrush Cytology by uterine lavage Biopsy Systemic tests If systemic tests were used, what test? Other diagnostic test described
	17	What was used to validate the diagnostic criteria (i.e. time to pregnancy; clinical cure, etc.)?
	18	How many times was each diagnostic test performed on each cow?
	19	At what time point(s) was (were) the diagnostic test(s) performed relative to calving and/or breeding?
Interventions	20	Were interventions administered after diagnostic tests were done?
	21	Total number of intervention groups.
	22	What interventions were administered?
	23	How were interventions controlled for (study design i.e. diagnostic assessment only in the untreated group, or analytical)?
Outcomes	24	Outcome definition (with diagnostic criteria if relevant)
	25	Rationale for the cut-points/how are cut-points defined?
Bias	26	Was blinding used? If yes, what method of blinding was used? If no blinding was used, was another method used to prevent bias? If yes, what method(s)?
Results	27	Number of participants allocated to each diagnostic/intervention group For each outcome of interest:
	28	Sample size
	29	Missing participants If present, was missing data explained?
	30	Summary data for each intervention group (e.g. 2x2 table for dichotomous data; means and SD's for continuous data)
	31	Are Se ² and Sp ³ reported? If yes, what was the reference outcome for Se and Sp? If yes, record Se and Sp for each test.
	32	If analyzed, what was intra-user agreement? If analyzed, what was inter-user agreement?
	33	Repeatability outcomes
Discussion	34	Are test characteristics discussed? If yes, describe

	35	Are cut-points discussed? If yes, describe
	36	Are possible sources of error discussed? If yes, describe
Miscellaneous	37	Funding source
	38	Key conclusions of the study authors
	39	Miscellaneous comments from the study authors
	40	Miscellaneous comments by the review author

¹PVD: Purulent vaginal discharge

²Se: Sensitivity is the probability of a positive test result in a disease-positive animal

³Sp: Specificity is the probability of a negative test result in a non-diseased animal

Appendix 2

Checklist modified from the STRADAS-paraTB checklist for diagnostic methods for Johne's disease in ruminants (Gardner et al. 2010). Responses in a systematic review for quality of papers (n = 51) on diagnostic methods for reproductive tract diseases in dairy cows were scored by two assessors. In case of a 'one grade' disagreement between the two assessors the most negative response was selected. In case of a 'multiple grade' disagreement, the response in between was selected and when necessary the most negative response.

Section	Item	Criterion	strongly	agree	neutral	disagree	strongly	not	Kappa ¹ (ind ²)
			agree				disagree	determined	
Title/ abstract/ keywords	1	The article is well identifiable as a study of diagnostic test performance or evaluation.	10 (20) ⁴	13 (26)	13 (26)	9 (18)	6 (12)	-	0.38***
Introduction	2	The research question or study aims are stated, such as estimation of diagnostic accuracy or comparison of accuracy in a specified matrix (specimen type) for a defined purpose at the animal or herd level.	9 (18)	34 (67)	7 (14)	-	1 (2)	-	0.04
Materials and Methods	3	Study sampling frame is well described: The source population and inclusion and exclusion criteria setting and location of data were collected for all relevant levels of the study sample (animals and herds).	1 (2)	19 (37)	17 (33)	9 (18)	5 (10)	-	0.25**
	4	Selection of animals and herds is well described: Sample selection methods (random, convenience, etc.) within each sampling hierarchy (e.g. regions, farms, barns, cows) including exclusion criteria and number of study animals and herds.	-	10 (20)	15 (29)	14 (28)	12 (24)	-	0.34***
5	Diagnostic test and/or sampling protocol are well described for well-defined reproductive tract infection and influenza. References are cited, or statistical evidence is provided (e.g. ROC curves), or a new definition is provided: The diagnostic methodology and/or sample collection, specimen size, transportation, handling and storage of specimens prior to testing of the TUE ⁵ .	2 (4)	21 (41)	18 (35)	7 (14)	3 (6)	-	0.26**	
6	Study design is well described.	1 (2)	16 (31)	26 (51)	8 (16)	-	-	0.2*	

7	Is data collection planned before the TUE were performed (prospective study), instead of after (retrospective study)?	44 (86)			2 (4)	5 (10) ⁶	0.03	
8	TAGS ⁷ are used, and its rationale is described.	8 (16)	11 (22)	17 (33)	12 (24)	3 (6)	-	0.16**
9	Technical specifications of materials and methods involved are well described, including how and when (i.e. number of days before breeding) measurements were taken, and/or references were cited for TAGS. Quality control samples for TUE and quality control samples for TAGS and specimen/analytical unit size of tested samples are specified.	-	19 (37)	22 (43)	9 (18)	1 (2)	-	0.26**
10	The outcome measure and rationale for the cut-offs and/or categories of the results of the TAGS are well described.	7 (14)	15 (29)	17 (33)	6 (12)	6 (12)	-	0.28***
11	The number, training and expertise of persons executing the TUE/TAGS, including the name, location, and qualification of the laboratory, are well described.	-	3 (6)	3 (6)	15 (29)	30 (59)	-	0.22*
12	The readers of the TUE/TAGS are blind (masked) to the results of the other test, and any individual or herd level information available to the readers is described.	2 (4)	2 (4)	2 (4)	4 (8)	41 (80)	-	0.39***
13	If blinding was not performed, methods are described to prevent bias, e.g. variability between operators, repeat testing, etc.	-	2 (4)	2 (4)	2 (4)	41 (80)	4 (8)	0.07
14	Methods are described for calculating or comparing measures of diagnostic accuracy, and are statistical methods that account for uncertainty (e.g. 95% confidence intervals).	5 (10)	14 (28)	12 (24)	13 (26)	7 (14)	-	0.28***
15	Methods for calculating test repeatability and reproducibility are done and well described.	1 (2)	2 (4)	7 (14)	4 (8)	37 (73)	-	0.24**
Results	It is well described when study was done, including beginning and end dates of recruitment.	9 (18)	19 (37)	4 (8)	8 (16)	11 (22)	-	0.45***
17	Demographic and other biologically relevant characteristics of the study sample at the individual (e.g. age, sex, breed) and at the herd levels (e.g. production system) are well reported.	-	26 (51)	12 (24)	11 (22)	2 (4)	-	0.19*
18	The number of animals and herds satisfying the criteria for inclusion that did or did not undergo the TUE and/or TAGS, including why animals and herds failed to receive either test.	-	14 (28)	22 (43)	13 (26)	2 (4)	-	0.02
19	Interventions were administered after the (collection of samples for the) TUE/TAGS are reported clearly.	4 (8)	10 (20)	14 (28)	7 (14)	9 (18)	7 (14)	0.22*

20	If interventions were administered after evaluation of the TUE. The interventions were controlled for in the anal design (e.g. intervention was included as a covariate in the diagnostic analyses) or the TUE are assessed in the un group?	4 (8)	8 (16)	11 (22)	6 (12)	3 (6)	19 (37)	0.33***	
21	Distribution of severity of disease or stage of infection, and other relevant diagnoses or treatments in animals in are reported clearly.	3 (6)	15 (29)	11 (22)	19 (37)	3 (6)	-	-0.06	
22	A cross tabulation of the results of the TUE (including indeterminate and missing results) by the results of another for continuous results, the distribution of the test results by the results of another TUE.	5 (10)	21 (41)	11 (22)	9 (18)	5 (10)	-	0.26**	
23	Any adverse events from performing the TUE/TAGS are reported.	-	-	-	7 (14)	44 (86)	-	0 ⁸	
24	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals) are repo	4 (8)	17 (33)	5 (10)	12 (24)	13 (26)	-	0.37***	
25	Indeterminate results, missing responses and outlier values of the TAGS are handled well. If additional testing of is done to resolve discrepant results, the rationale and approach (a flow diagram is strongly recommended) are c	-	1 (2)	20 (39)	23 (45)	7 (14)	-	0.14*	
26	If more than one diagnostic test is assessed on each animal, order bias is accounted for (e.g. when two cytobrush taken, the first sample might interfere with the second sample).	1 (2)	2 (4)	2 (4)	6 (12)	30 (59)	10 (20)	0.29***	
27	If done, estimates of variability of diagnostic accuracy between relevant subpopulations, readers, or testing sites	2 (4)	8 (16)	4 (8)	3 (6)	5 (10)	29 (57)	0.29**	
28	If done, estimates of test repeatability and reproducibility are reported well.	5 (10)	2 (4)	2 (4)	4 (8)	4 (8)	34 (67)	0.12	
Discussion	29	The utility of the TUE/TAGS in various settings (clinical, research, surveillance etc.) in the context of the currently are discussed.	1 (2)	21 (41)	8 (16)	16 (31)	5 (10)	-	0.28**

¹Weighted kappa statistic, †P < 0.1, *P < 0.05, **P < 0.01, ***P < 0.001. The Kappa statistic gives a value between -1 and 1, where ≤ 0 no agreement and 1 is perfect agreement between both assessors

²Agreement was calculated for each individual score

³Agreement was calculated combining the scores "strongly agree" and "agree", and the scores "disagree" and "strongly disagree"

⁴Values within parentheses are percentages

⁵TUE = test(s) under evaluation

⁶For five papers, there was discrepancy between assessors whether the study was prospective or retrospective. None of the assessors responded to this criterion

⁷TAGS = Test(s) in absence of gold standards

⁸The kappa value = (observed agreement – expected agreement)/(1 – expected agreement). For item no 23 observed and expected agreement between the assessor and combined responses, respectively, hence the kappa was 0

Appendix 3

Description of papers (n = 51) included for a review of diagnostic methods for reproductive tract diseases in dairy cows after excluding 1,549 papers using a systematic selection process (Chapter 2)

Authors ¹	Year	Source	Location	Herds	Sample Size
Couto et al.	2013	Theriogenology	Canada	5	285
Ghanem et al.	2013	Theriogenology	Japan	3	209
Bicalho et al.	2012	Veterinary Microbiology	USA	1	111
Cheong et al.	2012	Theriogenology	USA	28	563
Deguillaume et al.	2012	Journal of Dairy Science	France	3	168
Ghasemi et al.	2012	Theriogenology	Canada	6	30
Leutert et al.	2012	Journal of Dairy Science	Germany	1	386
Machado et al.	2012	Journal of Dairy Science	USA	3	1742
Meira et al.	2012	Journal of Dairy Science	Brazil	1	76
Senosy et al.	2012	Reproduction in Domestic Animals	Japan	1	70
Werner et al.	2012	Journal of Dairy Science	Germany	1	269
Cheong et al.	2011	Journal of Dairy Science	USA	38	812
Gorzecka et al.	2011	Reproduction in Domestic Animals	Denmark	1	126
Lukaszewicz et al.	2011	Bulletin of The Veterinary Institute in Pulawy	Poland	1	67
McDougall et al.	2011	Theriogenology	New Zealand	1	303
Peter et al.	2011	Clinical Theriogenology	New Zealand	11	122
Plöntzke et al.	2011	Reproduction in Domestic Animals	Argentina	3	243
Senosy et al.	2011	Reproduction in Domestic Animals	Japan	1	58
Bacha and Regassa	2010	Tropical Animal Health and Production	Ethiopia	30	59
Dubuc et al.	2010a	Journal of Dairy Science	Canada & USA	6	1044
Dubuc et al.	2010b	Journal of Dairy Science	Canada & USA	3	1363
Gautam et al.	2010	Theriogenology	Japan	7	291
Li et al.	2010	Veterinary Research Communications	China	1	138
Plöntzke et al.	2010	Animal Reproduction Science	Argentina	3	201
Tek et al.	2010	Turkish Journal of Veterinary and Animal Sciences	Turkey	NA ²	40
Westermann et al.	2010	Theriogenology	Germany	2	230
Galvao et al.	2009	Journal of Dairy Science	USA	5	445
Gautam et al.	2009	Animal Reproduction Science	Japan	2	135
Green et al.	2009	Proceedings of the New Zealand Society of Animal Production	New Zealand	1	47
Runciman et al.	2009	Australian Veterinary Journal	Australia	6	404
Senosy et al.	2009	Theriogenology	Japan	1	45
Yavari et al.	2009	International Journal of Dairy Science	Iran	13	89

Barlund et al.	2008	Theriogenology	Canada	8	221
McDougall et al.	2007	Animal Reproduction Science	New Zealand	7	191
Hammon et al.	2006	Veterinary Immunology and Immunopathology	USA	1	83
Hendricks et al.	2006	Theriogenology	USA	1	228
Gilbert et al.	2005	Theriogenology	USA	5	141
Kasimanickam et al.	2005	Canadian Veterinary Journal	USA	1	35
Kim et al.	2005	Journal of Reproduction and Development	Korea	2	30
Williams et al.	2005	Theriogenology	UK	1	200
Ishikawa et al.	2004	Journal of Veterinary Medical Science	Japan	1	27
Kasimanickam et al.	2004	Theriogenology	Canada	2	228
LeBlanc et al.	2002	Journal of Dairy Science	Canada	27	1951
Mateus et al.	2002	Reproduction in Domestic Animals	Portugal	1	26
Seals et al.	2002	Journal of Animal Science	Honduras	1	31
Knutti et al.	2000	Journal of Veterinary Medicine Series A - Physiology Pathology Clinical Medicine	Switzerland	85	3276
Archbald et al.	1998	Theriogenology	USA	1	274
Bonnett et al.	1991	Canadian Journal of Veterinary Research	Canada	1	97
Steffan et al.	1984	American Journal of Veterinary Research	France	4	153
Studer and Morrow	1978	Journal of The American Veterinary Medical Association	USA	1	106
Hartigan et al.	1972	Irish Veterinary Journal	Ireland	80	80

¹References of papers not listed in references section of the review manuscript can be requested from the corresponding author

²NA: Not described in paper

Appendix 4

Statements of contribution to doctoral thesis containing publications (DRC 16 form) for Chapter 2 (Systematic review of diagnostic tests for reproductive-tract infection and inflammation in dairy cows) and Chapter 5 (Detection of bovine herpesvirus type 4 antibodies and bovine lymphotropic herpesvirus in New Zealand dairy cows).

