Mastitis pathogen identification using polymerase chain reaction in New Zealand milk samples

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ABSTRACT

Rapid identification of the pathogen responsible for an intramammary infection in a dairy cow can support mastitis management decisions. Polymerase chain reaction (PCR) has become available to identify mastitis pathogens in milk, offering a rapid and sensitive test. The performance of a commercial, real-time PCR assay (PathoProof Complete-12 Mastitis PCR assay; Thermo Fisher Scientific Ltd., Vantaa, Finland) was compared with traditional bacterial culture for the identification of the most frequent pathogens in New Zealand, *Streptococcus uberis* and *Staphylococcus aureus*, during three stages of lactation. Aseptically collected quarter milk samples were analysed by culture and a subset (n=343) selected for PCR analysis based on infection status in culture. Using culture as the reference test, PCR had a relative sensitivity and specificity of 86.8%, and 87.7% (kappa=0.74) for detecting *S. uberis* and 96.4% and 99.7% (kappa = 0.96) for detecting *S. aureus*. Relative sensitivity for detecting *S. uberis* was similar throughout lactation whereas relative specificity was lower at the first milking post-calving (64%) and higher in mid-late lactation (97.7%). Initial validation of the PCR assay identified issues in *S. uberis* detection, particularly when milk samples were from freshly calved cows or from cows whose milk contained clots indicating clinical mastitis. Dilution of some colostrum and some clinical samples was required for detection of bacteria by PCR, due to the presence of PCR inhibitors in the milk. The PCR assay used in this study is not recommended for mastitis pathogen identification in early lactation as the majority of infections caused by *S. uberis* occur in the first month of lactation. PCR testing offers a number of opportunities and advantages to improve udder health and milk quality but for uptake in New Zealand, development is required to better suit colostrum samples. Greater clarity is required regarding the interpretation of PCR results and the use of information from such tests for decision-making.

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THESIS STRUCTURE

This thesis follows a traditional structure, beginning with an introduction to mastitis pathogen identification and a review of the literature in mastitis diagnostics, in particular the use of polymerase chain reaction (PCR) as an alternative to traditional bacterial culture to identify mastitis-causing pathogens. This develops the justification for the study objectives and hypothesis. The materials and methods section is split into two components, firstly considering milk sample collection, bacterial culture, and PCR testing. The second methods chapter comprises the procedures involved in the development of the PCR assay for use on New Zealand milk samples. Finally, the results are presented, discussed, and analysed.

1 INTRODUCTION

Mastitis is inflammation of the mammary gland, predominantly occurring in response to invasion of that gland by pathogenic bacteria. Mastitis is the most important disease of dairy cattle worldwide due to its high incidence in all dairy systems, and its effects on animal welfare, milk quality, and total milk produced and processed (Barkema et al., 2009). It is also the most costly disease affecting dairy cattle, with the cost to the New Zealand dairy industry alone estimated at NZ$280 million per year (NMAC, 2010). Costs associated with mastitis include:

- antibiotics and other veterinary treatments;
- milk discarded during the milk withholding period;
- additional labour for managing infected cows;
- penalties for exceeding the regulatory bulk milk somatic cell count (BMSCC) limit;
- premature culling and cull cow replacement;
- temporary or permanent loss in milk production (representing more than two thirds of the total losses incurred by mastitis (Akers and Nickerson, 2011)).

Mastitis can occur in clinical and subclinical forms. Clinical mastitis is identified by visible changes to the udder, such as swelling and redness, increased temperature of the infected gland, and abnormal appearance of milk, such as signs of clots and discolouration within, and often beyond, the first three streams of milk (SmartSAMM, 2012). Conversely, subclinical mastitis is not visibly detectable, but involves inflammation as indicated by an elevated somatic cell count (SCC) in the milk. Subclinical mastitis requires detection by indirect methods such as the California mastitis test (CMT; also known in New Zealand as the Rapid Mastitis Test), electrical conductivity, presence of enzymes associated with tissue damage in milk (lactate dehydrogenase (LDH) and *N*-acetyl-B-D-glucosaminidase (NAGase) (Kitchen, 1981)), and individual cow SCC. Intramammary infection (IMI) and subclinical mastitis are terms that are often used interchangeably (Barkema et al., 1997a). IMI infers the presence of an infectious organism but not necessarily an inflammatory response (Berry and Meaney, 2006), whereas subclinical mastitis indicates an inflammatory state in the mammary gland, but not necessarily an infectious pathogen (Griffin et al., 1987a). However, in most cases, the inflammatory state is caused by the colonisation of pathogenic microorganisms. Whether or not disease develops depends on the interactions between the host, the pathogen and environmental factors (Bradley et al., 2007).

A wide array of microorganisms can cause mastitis; more than 130 different species have been identified as the pathogen responsible for causing IMI (Watts, 1988). However most mastitis cases are caused by one primary pathogen (Watts and Yancey, 1994), with approximately ten bacterial species or groups of species accounting for more than 95% of mastitis infections (Makovec and Ruegg, 2003; Tenhagen et al., 2006; Bradley et al., 2007; Koivula et al., 2007). Mastitis-causing pathogens are usually defined as being either contagious or environmental in origin, depending on their primary reservoir and how they are contracted and transferred between animals (Bramley et al., 1996). Different countries tend to have different patterns of pathogen prevalence (Table 1). As the name suggests, contagious pathogens are spread between cows and in most cases originate within an infected mammary gland. The most common contagious pathogens causing mastitis worldwide are *Staphylococcus aureus*, *Streptococcus agalactiae* and Mycoplasma species. *S. aureus* is commonly isolated from milk of New Zealand dairy cows, whereas mastitis caused by *Mycoplasma bovis* is very rare in New Zealand (McDonald et al., 2009), and the prevalence of *S. agalactiae* is very low (Petrovski et al., 2011b). Environmental pathogens survive in the cows' habitat, such as soil, plant material, manure, bedding, stand-off pads, races, and contaminated water and on body sites of the cow other than the mammary gland (Bramley et al., 1996). Many species of environmental bacteria can cause mastitis, but those frequently responsible include several species of streptococci, such as *Streptococcus uberis* and *Streptococcus dysgalactiae*, and Gram-negative bacteria such as *Escherichia coli a*nd *Klebsiella pneumoniae*. The pathogen most commonly isolated from clinical mastitis in New Zealand is *S. uberis*, with the greatest incidence occurring in the first month of lactation (McDougall, 1999). Further classifications of mastitis-causing pathogens include minor pathogens, such as coagulase-negative staphylococci (CNS) and *Corynebacterium bovis*. The greatest increase in the SCC of mastitic milk occurs during infection by major pathogens *S. aureus*, *S. agalactiae*, and coliforms such as *E. coli*, whereas minor pathogens (e.g. CNS) only cause a moderate increase in SCC, compared with uninfected animals (Oliver and Calvinho, 1995).

Mastitis control interventions are intended to reduce the incidence and duration of IMI, improve the health of the cow, and resume production of high quality milk (Barlow et al., 2013). The prevention of mastitis is the best practical outcome on farm, but response strategies are necessary for when disease occurs. Early detection of mastitis can enable earlier intervention and potentially a better response to treatment (Milner et al., 1997), reducing the severity of disease and the risk of infection spreading to other

cows in the herd. The decision to treat is often made without knowledge of the quarter's bacteriological status, but different species vary in their susceptibility to different classes of antibiotics, with some strains or species having resistance to certain types. Therefore, identifying the specific bacteria causing mastitis can assist in management decisions such as selecting the most appropriate antibiotic for lactating and dry cow therapy and selecting cows to cull.

Table 1. Proportions of common mastitis pathogens isolated from samples collected in various studies in New Zealand and overseas. (AU = *S. aureus*; AG = *S. agalactiae*; UB = *S. uberis*; DY = *S. dysgalactiae*; CNS = coagulase-negative staphylococci; NG = no growth; CM = clinical mastitis; LAB = samples submitted to laboratory).

		% of samples							
Study Country Type	**N**	**AU**	**AG**	**UB**	**DY**	**CNS**	**Coli-form**	**Other***	**NG**
McDougall et al., 2007 NZ CM	1462	16.5	-	32.0	6.1	5.5	-	3.6	23.4
Petrovski et al 2011b NZ LAB	25288	23.4	0.3	23.6 (2.7)**	6.2	7.2	3.7	10.0	9.8
Olde Riekerink et al., 2008 Canada LAB	3033	10.3	0.1	6.3 (10.6)**	4.0	5.1	12.7	7.4	43.9
Koivula et al., 2007 Finland LAB	77051	15.9	0.2	11.9	8.3	21.1	4.4	5.6	28.6

*Other includes, *Actinomyces* spp., *Arcanobacterium* spp., *Bacillus* spp., Corynebacteria, *Pasteurellaceae*, Pseudomonads and other (Petrovski et al., 2011b).
**Environmental streptococci (excluding *S. uberis*) in parenthesis.

The most frequent use of antibiotics in dairy cattle is for the treatment or prevention of mastitis (Sundlof et al., 1995; Mitchell et al., 1998). For example, a Wisconsin-based survey of 20 conventional dairies found that of the total antibiotic use, 80% was

attributable to mastitis (Pol and Ruegg, 2007). The drive to reduce antibiotic usage reflects two concerns: 1) the potential for antibiotic-resistant pathogens to be transferred through the food chain, ultimately risking the transfer of antibiotic-resistant genes from animal to human pathogens; and 2) the reduction in efficacy of antibiotic therapy for the treatment of infections caused by resistant bacteria (van Werven, 2013). There is well documented evidence that strains of resistant bacteria exist (Owens et al., 1997; Sol et al., 2000; Makovec and Ruegg, 2003; Pol and Ruegg, 2007); however the development of resistance to commonly used antimicrobial products for dairy cattle appears to be static (Petrovski et al., 2011a; Lago et al., 2014). Further benefits of minimising antibiotic use include the cost saving from treatments and milk discard, and the reduced requirements for labour and management of high risk cows.

In New Zealand, standard treatments take into account the antimicrobial resistance patterns (Laven and Holmes, 2008). Mastitis treatment failure is usually a result of the cow or bug, not the treatment. Antimicrobial sensitivity testing can provide some insight to determine in vitro susceptibility of pathogens to classes of antibiotics, however there are poor criteria for the commonly used disc diffusion method, and can be a mismatch between in vitro and in vivo results (Constable and Morin, 2003). The benefits of pathogen identification were demonstrated in a study by Lago et al. (2011). Selective treatment based on on-farm culture systems provided significant reductions in discarded milk and half the amount of antibiotics used compared with the ‘treat all with clinical signs’ approach (Lago et al., 2011). Basic treatment advice for the selective group was to treat Gram-positive infections, and leave Gram-negative and ‘no growth’ infections. Even though there was a delay in treating cows in the selective group (1-2 days), there was no significant difference in cure rates between the two groups. However, animal welfare must be considered when no treatment is given. Antimicrobial treatment of subclinical mastitis during lactation is not cost effective, particularly when caused by CNS (Swinkels et al., 2005; Steeneveld et al., 2007). Dry cow therapy also presents an area where large reductions in antibiotic use can be made. More than 45% of antibiotic use for mastitis in dairy cattle was attributed to dry cow treatment in the Netherlands (van Werven, 2013). In 2012, regulatory changes concerning blanket dry cow therapy were made with the aim of minimising the total use of antibiotics (van Werven, 2013). Cows without an IMI, as defined by the absence of bacterial pathogen in milk, no longer receive dry cow therapy at dry off. Therefore, pathogen identification prior to drying off is a legal requirement in the Netherlands, and it is possible that other countries may follow suit. In New Zealand, current estimates are that 80-90% of farms are using dry cow therapy, with about 70% of the national herd

receiving antibiotics at drying off (J. E. Hillerton, pers. comm.; S. McDougall, pers. comm.).

Determining the type of pathogen that predominates in the herd can provide the basis for mastitis investigations, whether the trigger point is an outbreak or increased incidence of CM, rising BMSCC, or for milk quality regulations. Pathogen identification can allow the clinician or farmer to understand the mechanisms of spread within the herd, answering questions such as how the cow was exposed to bacteria, what the risk of spread might be and what management adjustments can be made to prevent further infections. When contagious pathogens predominate, this generally points to areas for improvement in the milking process, whereas a high prevalence of environmental bacteria suggests a hygiene issue in the surroundings. At the individual cow level, identification of infections as being caused by specific bacteria, e.g. *S. aureus*, may assist farmers with culling decisions, due to the poor cure rates of such infections (Barlow et al., 2013). At the farm level, animal health, welfare, efficiency and product quality are breached by mastitis, therefore biosecurity and biocontainment measures must be implemented to prevent the introduction and spread within the herd (Barkema et al., 2009). Although it is difficult to gather accurate prevalence data on a national basis, an indication of the likely prevalence is important when considering the development of mastitis control programmes and research aimed at reducing the incidence and impacts of mastitis in the industry.

There is a fine line between implementing good mastitis control and maintaining milk quality with the requirement to reduce potentially unnecessary antibiotic usage, and finding the balance between the two may be difficult. A mastitis control programme must balance the welfare of the animals, the economics of treatment and disease, and the level of antibiotic use (Hogeveen et al., 2014).

2 LITERATURE REVIEW: THE USE OF POLYMERASE CHAIN REACTION AND BACTERIAL CULTURE FOR THE IDENTIFICATION OF MASTITIS PATHOGENS

1.1 Tests for pathogen identification

The identification of a bacterial pathogen from an aseptically collected milk sample is considered to be the definitive diagnosis of mastitis, first proposed by the International Dairy Federation (IDF) in 1975 (IDF, 2011). The traditional diagnostic test for identifying pathogens in milk is bacterial culture, with the presence of bacteria indicated by their growth on an appropriate growth medium following a period of incubation (Hogan et al., 1999). Bacterial species are identified based on phenotypic characteristics including colony morphology, serotyping and analysis of enzymatic profiles (Hogan et al., 1999; Oliver et al., 2004). Bacterial culture is currently regarded as the gold standard for identifying mastitis pathogens (Hogan et al., 1999).

An alternative test for pathogen identification is the molecular technology, polymerase chain reaction (PCR). PCR detects DNA sequences that are unique to a specific species or group of bacteria, thus confirming the presence or absence of bacterial DNA, from both viable and non-viable organisms. Modern diagnostics and laboratory automation have paved the way for molecular technologies to be applied in mastitis pathogen identification. The need for a rapid and accurate test led to the development of PCR assays to detect mastitis-causing pathogens (Riffon et al., 2001; Gillespie and Oliver, 2005). Initially, PCR-based mastitis tests targeted a single pathogen (Riffon et al., 2001), a technique known as simplex PCR. The number of bacterial targets simultaneously identified by PCR has increased through the use of multiplex PCR (Gillespie and Oliver, 2005; Koskinen et al., 2009). The accuracy of the test has improved, and the cost and time involved have declined (Koskinen et al., 2010). The first commercialised mastitis PCR assay (PathoProof, Thermo Fisher Scientific Ltd., Vantaa, Finland) was introduced in 2008. Recently, another kit has become commercially available (Mastit 4, DNA Diagnostic, Risskov, Denmark). These commercial kits use real-time PCR, which enables the quantification of the original amount of bacterial DNA present in a sample, automation of results, and shorter throughput times compared with standard PCR (Koskinen et al., 2008).

1.2 Bacterial culture as the gold standard test for mastitis pathogen identification

When comparing diagnostic tests, it is important to use the best and most accurate gold standard as the reference test. However, there is no perfect method that can definitively determine the 'true' bacteriological status of a mammary gland (Dohoo et al., 2011a). A 'true' gold standard would correctly classify infected and non-infected quarters 100% of the time (Dohoo et al., 2011a). Diagnosing mastitis to this level would require invasive monitoring procedures that would not be practical. Culture procedures are generally accepted as the only method to reliably detect the cause of an IMI (Dohoo et al., 2011a) and are the current gold standard for identifying mastitis pathogens (Oliver et al., 2004). The use of an imperfect test as the reference when evaluating a new test may bias the results, as sensitivity and specificity may be underestimated (Cederlöf et al., 2012). Thus, the limitations of using culture as the reference test must be recognised.

1.3 Characteristics of bacterial culture and PCR as diagnostic tests

According to Holdaway (1990), a diagnostic test should be:

1. Sensitive
2. Specific
3. Repeatable
4. Rapid
5. Inexpensive

The following sections will provide a comparative evaluation of bacterial culture and real-time PCR, in terms of their ability to identify mastitis pathogens, based on these criteria.

1.3.1 Test sensitivity and specificity

Sensitivity and specificity are general terms used to describe the quality of a testing method for a specific pathogen (Saah and Hoover, 1997). The analytical sensitivity of a test is the lowest concentration of a particular organism in a sample that can be accurately measured by an assay. Analytical specificity is the ability of an assay to detect one particular organism rather than others in a sample (Saah and Hoover, 1997). In diagnostic terms, a test's sensitivity represents its ability to correctly identify samples in which a particular organism is truly present. Diagnostic specificity is the ability of the test of interest to correctly identify samples that do not contain a particular organism. These parameters describe the performance of a test for a given population,

under field conditions. When evaluating a new test or method, performance measures are made relative to the reference test, or gold standard, which provides the best estimate of the true infection status of a sample. False negative results occur when the result of an individual test is negative but the disease or condition is truly present (IDF, 2011). False positives occur when the result of an individual test is positive but the disease or condition is truly not present in an individual (IDF, 2011). In both a clinical and research setting, confirmation of a pathogen requires a test with optimised specificity to reduce the proportion of false positives. When ruling out the presence of a pathogen, maximised sensitivity is desired, to limit the number of false negatives (Dohoo et al., 2009). Sensitivity and specificity should be considered together as a change in test characteristics will generally be reflected by an increase in one and reduction in the other, and finding the right balance often depends on the objectives of the sampling.

1.3.1.1 Sample quality

The likelihood of detecting a pathogen if present in milk not only depends on the analytical accuracy of the test but also on factors relating to the sample and sampling process. Proper aseptic sampling technique is essential when identifying mastitis pathogens to avoid sample contamination. Many mastitis-causing pathogens can also exist in the environment and on teat skin, so the risk of microorganisms not residing within the udder becoming a contaminant of milk samples is high when proper techniques are not followed (Hogan et al., 1999). Samples collected aseptically from an individual mammary gland can provide the most reliable information about an infection because the level of confidence that the pathogen isolated did originate within the mammary gland is much higher compared with samples not collected aseptically. A sample is considered to be contaminated if three or more dissimilar colony types are present in culture (Hogan et al., 1999). There is no guideline for defining contaminated samples using PCR as the diagnostic test; whether or not the same definition as for culture should apply is unclear (Pyorala and Katholm, 2014). Contaminated samples affect both the sensitivity and specificity of a test. Samples that are truly negative may either be incorrectly classified as infected in culture if there are fewer than two different species isolated, or classified as contaminated, giving no result as the sample cannot be interpreted, limiting the specificity. Any sample that was truly infected but was masked by the contaminating pathogens will not be detected, reducing the sensitivity. The quality of samples collected will impact on the test performance characteristics of both culture and PCR.

The method of delivery of samples to the laboratory may also have an effect on sample quality as a lack of adequate refrigeration can allow bacteria, including any contaminants, to multiply and misrepresent the bacteriological state of the mammary gland. A study in the USA analysed contamination rates for different sample delivery methods where posted samples had a 26% contamination rate, compared with 7% for samples that were delivered directly to the laboratory after collection (Dinsmore et al., 1990). Posted samples were kept on ice packs, but the length of time to delivery would have prevented these being chilled for the entire duration as recommended. Conversely, hand delivered samples arrived in the laboratory faster (Dinsmore et al., 1990). Laboratories in New Zealand report that a significant proportion of samples arrive to the laboratory at room temperature (K. Cooper and Y. MacPherson, pers. comm.), reducing sample quality which may affect the accuracy of diagnosis in standard culture. If the criterion for contamination in culture is not applied for PCR testing, the pathogen present in the highest concentration may be assumed to be the cause of IMI. If common contaminants of milk are not targeted by the PCR test, they will not be detected and will not impact other pathogens in the sample. Alternatively, bacterial growth can be inhibited during sample transportation by adding a preservative, such as bronopol which is used in herd test samples. Pathogens from these samples are not culturable due to the inhibitory effect of the preservative; however, bacterial DNA can be detected by PCR, which may provide comprehendible results for samples and a more convenient option for farmers and clinicians.

Samples are commonly stored frozen after collection on farms, which may affect the viability of some species of bacteria. The quality of freezers on farms may also be poor, subjecting the samples to repeat cycles of freezing and thawing, similarly affecting pathogen viability (Storper et al., 1982, reported by Biddle et al., 2004). Most studies have shown that freezing has no effect on the recovery rate of *S. aureus* (Schukken et al., 1989, Murdough et al., 1996, Godden et al., 2002; Artursson et al., 2010). There are inconsistencies in the recovery of *Streptococcus* spp. and CNS following freezing. Murdough et al. (1996) found no difference in in *S. uberis* and *S. dysgalactiae* detection after 6 weeks of frozen storage. Conversely, Storper et al. (1982) reported a 36% loss in non-*agalactiae* streptococci after samples were stored frozen for 4 weeks. Schukken et al. (1989) showed greater CNS recovery after freezing, but Murdough et al. (1996) reported no difference for this group. Recovery of Gram-negative bacteria and Mycoplasma from clinical mastitis samples is reduced after any length of frozen storage (Schukken et al., 1989; Biddle et al., 2004; Boonyayatra et al., 2010). Gradual thawing was suggested to reduce the loss in bacteria (Biddle et al., 2004). Freezing

samples is recommended to farmers for convenience, to slow the autolytic components of the immune system present in milk, and reduce overgrowth of contaminants (Barkema et al., 1997b). The sensitivity of detecting some pathogens in culture is reduced by freezing samples, but the main mastitis pathogens in New Zealand are *S. aureus* and *S. uberis*, so the effects of freezing may not significantly reduce the sensitivity of detecting bacteria. More research is required to fully understand the effects of freezing and sample age on pathogen detection by PCR, but the detection of DNA from non-viable bacteria should mean the sensitivity is not affected by freezing, provided that the target region of DNA remains intact (Paradis et al., 2012).

Milk samples sent for analysis are often samples from cases of clinical mastitis which consist of altered physical and biochemical characteristics compared with non-mastitic milk. Components of the immune system such as somatic cells and immunoglobulins, and the proportion of mammary epithelial cells are elevated in mastitic milk, which may remain elevated for some time following bacteriological clearance of the infection (Oliver and Calvinho, 1995). Immune components do not usually affect pathogen detection in culture when storage conditions are appropriate, but can affect the efficiency of DNA extraction and subsequently the success of the PCR reaction (Gillespie and Oliver, 2005; Cremonesi et al., 2006; Cressier and Bissonnette, 2011). The DNA extraction is designed to remove impurities and PCR inhibitors from the milk, preparing DNA for amplification by PCR. A range of DNA extraction methods can be used to purify DNA (see Dibbern et al., 2015). The spin-column-based extraction method has been used in validation studies of the commercial PathoProof PCR assay in Europe (Koskinen et al., 2010; Hiitio et al., 2015) but this method has been reported to be incompatible for some clotted mastitic milk samples (Cressier and Bissonnette, 2011). Molecular methods must be robust when used routinely with clinical samples (Chakravorty and Tyagi, 2001; Keane et al., 2013) as inefficient DNA extraction can lead to poor recovery of DNA and therefore limited sensitivity of PCR.

1.3.1.2 Sample frequency

Using an enhanced gold standard of three consecutive samples in culture,Dohoo et al. (2011a) evaluated if the ‘true’ infection status of a mammary gland could be determined based on a single sample. The results showed a lack of sensitivity for all species involved (<90%, and in many cases <50%), except for *S. aureus* (90.4%). In contrast, single samples appear to be adequate for detecting some pathogens using PCR (Cremonesi et al., 2006; Studer et al., 2008; Botaro et al., 2013), depending on the PCR assay and gold standard used to measure sensitivity and specificity.

Collection of duplicate samples, at the same sampling occasion, or consecutive samples, at least one day apart, increased the likelihood of detecting some pathogens in culture and reduced the chance of incorrectly identifying a contaminant as the causative pathogen (Sears et al., 1990; Erskine et al., 1988; Dohoo et al., 2011b). For *S. aureus*, culture of duplicate samples from cows had a high level of agreement (92-94%), indicating little benefit from analysing a second sample collected at the same time (Jasper et al., 1974; Erskine et al., 1988). In contrast, consecutive samples were shown to increase sensitivity of *S. aureus* detection from 75% for single samples to 94% and 97-98% when a second (Sears et al., 1990) or third consecutive sample was collected, respectively (Sears et al., 1990; Buelow et al., 1996). Failing to identify cows infected with contagious pathogens can lead to a more severe disease and greater exposure of microorganisms to other cows in the herd (Ruegg, 2003).

The level of agreement between duplicate samples collected from cows infected with coliform bacteria and other environmental pathogens was less than 80% (Erskine et al., 1988). Duplicate samples interpreted in series (both had to be positive) increased sensitivity compared with a single sample, but coincided with a loss in specificity (Dohoo et al., 2011b). On the other hand, consecutive samples, also interpreted in series, had a sensitivity that was intermediate between that of single and duplicate samples, but had less of a decline in specificity. While collecting consecutive samples optimises sensitivity and specificity, this strategy is time-consuming, costly and impractical on a routine basis (Ruegg, 2003). There is a trade-off between the costs of collecting multiple samples from one quarter and the total number of cows that can be sampled. For herd investigations into a mastitis problem, increasing the number of cows can provide more information about pathogen prevalence in the herd in a clinical situation, and maximise the number of quarters enrolled in a research study (Dohoo et al., 2011b). The frequency of sampling therefore depends on the objectives of the investigation.

1.3.1.3 Sample type

Samples may be collected from an individual quarter, as a cow composite sample (e.g. routine herd test sample), as a pooled sample from groups of cows, or from the bulk tank. Quarter samples provide the most reliable information of predominant pathogen types in a herd when samples are collected from a range of both clinical and subclinical cases. Sensitivity and specificity are maximised in aseptically collected quarter samples for all pathogen types, compared with samples collected at any higher level. Prior information about the cow and specific quarters such as CMT score and SCC can direct the farmer to select the most appropriate quarters to sample.

Composite milk samples usually have lower sensitivity for detecting mastitis pathogens because of the dilution effect from other uninfected quarters but as the number of infected quarters increased, sensitivity was improved (Reyher and Dohoo, 2011). Collecting a single composite sample from subclinically infected cows may only identify 58 to 63% of *S. aureus* infections (Franken et al., 1995; Lam et al., 1996). Collecting two or three consecutive composite samples over five days increased sensitivity to 86% and 95% when just one quarter was infected with *S. aureus* (Lam et al., 1996). Composite samples were comparable with quarter samples for detecting *S. agalactiae* (sensitivity = 95-100%; Dinsmore et al., 1991). Herd test samples are collected via a milk meter and contain a preservative, so reliable pathogen detection in culture is not possible. Using PCR, contagious mastitis pathogens can be detected at similar sensitivity to quarter samples (Penry et al., 2014) and interpreted in terms of their role in causing IMI, but interpretation of environmental mastitis-causing bacteria is not reliable as these could have originated from the environment, teat skin or milk meter, and not necessarily the udder (Kelton and Godkin, 2014).

Like composite samples, pooled and bulk tank milk samples have less sensitivity than quarter samples. For pooled samples, if cows are selected on the basis of suspected infection, the dilution effect of uninfected quarters may be minimised. A recent study suggested that one moderate to high level infection caused by *S. agalactiae* or *M. bovis* may be detected by PCR when up to 1,000 cows are in the group (Penry et al., 2014). Sampling bulk tank milk enables screening and surveillance at the herd level. The positive predictive value for the presence of at least one infected cow when the pathogen was found in bulk milk culture was estimated for *S. agalactiae* (98%), *S. aureus* (97%), and *Mycoplasma* spp. (80%; Wilson et al., 1997). The sensitivity for detecting *Mycoplasma* spp. was low (range 33-59%) for a single bulk tank sample because of dilution, latent infections or intermittent shedding (Gonzalez and Wilson, 2003) but multiple samples collected over time can provide more reliable information (Hogan et al., 1999). PCR has been shown to be more sensitive than culture for detecting *S. agalactiae* (Katholm et al., 2012). A positive PCR test for *S. agalactiae* or *M. bovis* usually indicates infection in the herd. A positive bulk tank milk test for *S. aureus* must be interpreted with caution, as *S. aureus*, although a contagious pathogen, has also been known to colonise teat skin (Haveri et al., 2008). A repeat test and further investigation of individual suspect cows might be necessary if the BMSCC continues to rise. Any environmental pathogens detected by PCR in bulk tank samples should be ignored when considering cause of IMI; however the presence of these bacteria could point towards hygiene issues in the environment and/or milking practices

that could contribute to poor milk quality and udder health (Katholm et al., 2012). Importantly, a negative result for any contagious pathogen cannot be reliably interpreted as freedom from infection caused by these pathogens, hence repeat testing offers greater confidence.

1.3.1.4 Target of test

To be useful in any mastitis testing programme, a diagnostic test must identify as many potential infectious agents as possible to reduce the likelihood of an incorrect diagnosis. In standard bacterial culture (Hogan et al., 1999), mastitis-causing pathogens are detected by growth of viable bacteria on a quadrant of blood agar media, when 0.01 mL of milk is spread on a plate and incubated aerobically at 35-37 °C for at least 48 hours. These standard conditions are suitable for most mastitis-causing pathogens, but special culture conditions can be used to enhance the sensitivity of detecting certain pathogens (Table 2).

In comparison to culture, the target of the PCR test is much more limited. PCR only detects the bacteria that are targeted by the primers included in the assay and is not dependent on the viability or biological functionality of bacteria (Cressier and Bissonnette, 2011). To develop a PCR assay, oligonucleotide sequences unique to the target species of interest must be identified, and primers designed to match these sequences (Bustin, 2004). This allows amplification and quantification of the target DNA only. This can be difficult as many species associated with bovine mastitis are closely related, thereby increasing the risk of cross-reaction with any non-target DNA present in the sample, which would produce a false positive result. But, as strains originating from a single species may have diverged genetically, the target sequence must also be common to all strains of a species to limit false negatives (Gillespie and Oliver, 2005). For example, the sequence of the clumping factor gene associated with *S. aureus* is not identical in all strains, but the design procedures and validation of a kit would be expected to test a vast number of strains from various geographic locations, enabling the design of a primer that can detect related strains through the similarity and homology of the targeted gene sequences (Koskinen et al., 2009). Thus, the target sequence must be highly conserved within a species, but variable between species (Phuektes et al., 2001). Continuous validation is necessary to ensure a commercial kit remains current with changes in genetic variation and mutations within a species.

The type of PCR used determines the number of pathogens that can be detected in a test. Simplex PCR identifies one target per PCR reaction, whereas most mastitis PCR assays now use multiplex PCR, enabling the simultaneous detection of multiple

pathogens in a single assay (Gillespie and Oliver, 2005; Koskinen et al., 2009). Multiplex PCR requires several primer sets in each reaction, each designed to amplify DNA associated with one species or species group (Gillespie and Oliver, 2005). Multiplex PCR may be linked with a reduction in analytical sensitivity (10-100 fold lower) when compared with simplex PCR, using the same primers for each target (Phuektes et al., 2001). This could be a result of competition between individual reactions for reagents (Madico et al., 2000). However, multiplex PCR enables reduction in cost per sample as more pathogens can be detected within one reaction. Using multiplex real-time PCR, only four different bacterial targets can be amplified within one reaction, as each target is represented by a different dye. Considering the large number of pathogens that have been identified as a cause of IMI (137; reported by Watts, 1988), it is very possible for PCR to miss pathogens when not specifically targeted in the assay used. Current PCR assays can detect up to 15 of the most common mastitis-causing bacteria or bacterial groups, along with the gene for penicillin resistance (PathoProof Complete 16 Mastitis PCR Assay; Thermo Fisher Scientific Ltd., Vantaa, Finland). Even so, the PCR assay must be suited to the local major mastitis pathogens, and testing with culture might be necessary to rule out rare pathogens not detected by the assay.

PCR detects DNA from both viable and non-viable organisms increasing the apparent sensitivity of the test. Culture only reveals bacteria capable of growing under standard conditions (Hogan et al., 1999). Poor sample storage and handling, or antibacterial activity of milk components can lead to death or impaired growth of bacteria after sampling. However, using PCR, detection is possible provided that the target sequence of DNA remains intact. DNA is a stable molecule and may exist for a period of weeks following death of an organism (Keer and Birch, 2003). It would be useful to know the proportions of viable and non-viable organisms present in the milk but the current PCR assays are not capable of making this distinction (Schukken et al., 2010), although the technology does exist (Keer and Birch, 2003) but has not yet been applied in the mastitis field. The clinical importance of detecting non-viable organisms is yet to be evaluated (Schukken et al., 2010), although it is thought that incorporating dead bacteria confounds interpretation (Olde Riekerink et al., 2014).

1.3.1.5 Test characteristics

The PCR amplification process involves the replication of target DNA, essentially doubling the number of copies of the target sequence during successive cycles of the PCR reaction (Bustin, 2004). Thus, only a small amount of target DNA is required as the starting material, enabling a relatively low level of detection using PCR, compared

with culture. Additionally, the increased sensitivity of PCR may partially be explained by the increased sample volume used for DNA extraction (0.35-0.40 mL compared with 0.01 mL for standard culture). Koskinen et al. (2009) demonstrated 100% analytical sensitivity for a commercial PCR assay (PathoProof) based on cultured isolates, not directly on milk samples. This 100% sensitivity suggests that samples yielding PCR-positive results do indeed contain bacterial DNA from the targeted species (Taponen et al., 2009). In a large scale field comparison, Koskinen et al. (2010) reported that the sensitivity of PCR was as good as culture for most pathogens with a notable exception that the sensitivity for detecting *S. aureus* was shown to be much higher than culture, while Wellenberg et al. (2010) showed that the average relative sensitivity of PCR was higher than culture, even after pre-enrichment of samples for culture. The limit of detection in culture is 100 CFU/mL when 0.01 mL is spread, which is 1 CFU/plate. The PathoProof PCR assay can detect as few as 5.6 gene copies per PCR reaction and up to 50 copies, depending on the pathogen (Koskinen et al. 2008). Detection limits measured as number of copies per PCR reaction have little relevance in mastitis studies due to the high and often variable amounts of PCR inhibitors between samples from different cows or stages of lactation (Chakravorty and Tyagi, 2001; Botaro et al., 2013). Using milk from PCR-negative clinical mastitis cases, the limit of detection for various pathogens was between 200 and 810 CFU/mL (Koskinen et al., 2008). For other PCR assays, detection limits have been reported as low as 1 CFU/mL (when samples were enriched with trypticase soy broth and incubated overnight; Gillespie and Oliver, 2005) and up to 1,150 CFU/mL (Graber et al., 2007) and anywhere in between (Koskinen et al., 2008; Silvennoinen et al., 2010).

Negative results from culturing samples from cows with clinical and subclinical signs of infection are reported to occur in approximately 30% of samples submitted to commercial laboratories (Bradley et al., 2007; Olde Riekerink et al., 2008). PCR was demonstrated to detect at least one pathogen in 43% and up to 79% of samples when no pathogen was isolated in culture (Taponen et al., 2009; Bexiga et al., 2011; Keane et al. 2013). In some cases, major pathogens were detected that were missed in culture, but often these were the common minor pathogens, CNS and *C. bovis*. Compared with culture, PCR often detects more bacterial species within a sample, explained by a combination of lower detection levels, increased sample volumes, and the detection of non-viable organisms (Keane et al., 2013). However, identifying minor pathogens alongside major pathogens may not add much value to the investigation.

There are a number of techniques that can be used to increase the sensitivity of detection of different pathogens in culture (Table 2); however these are generally associated with reduced specificity, and increased time, labour and costs.

Table 2. Techniques to assist standard bacterial culture for the enhanced detection of mastitis pathogens in milk

Technique	Advantage	Implication	Reference
Media			
Aesculin blood agar	Assist differentiation of streptococci	May mask haemolysis and colony pigmentation as a result of black discolouration of media around aesculin-positive organisms	Hogan et al., 1999
Various selective media	Useful for selective growth of Gram-negative bacteria, *S. aureus*, streptococci and *Mycoplasma* spp., (e.g. in order; MacConkey agar, Vogel Johnson, Modified Edwards media and modified Hayflick agar)	More expensive, may have shorter shelf-life	Hogan et al., 1999
Bi- or tri-plates	Simultaneous growth of various pathogens on selective media rather than sequential	More expensive, often shorter shelf-life	Britten , 2012
Incubation conditions			
Up to 10 days incubation	Some species are slow growing e.g. *Nocardia* spp., *Mycobacterium* spp.).	Increased use of resources, not practical, long delay to result	Hogan et al., 1999
Lower temperature e.g. 25^{o}C	Increased detection of *Serratia marcescens*	Most mastitis-causing pathogens grow well between 35-37^{o}C	Oliver et al., 2004
Anaerobic incubation	Some species require anaerobic conditions to grow, e.g. Mycoplasma	Doubles resources as need to grow in aerobic conditions for standard culture	Oliver et al., 2004
Inoculum volume			
0.1 mL plated (c.f.	Increased detection of pathogens e.g. *S. aureus*	Increased growth of contaminants, which may	Lam et al., 1996;

0.01 mL)	detection 78% to 90% (Lam et al., 1996), 89% to 96% (Walker et al., 2010)	mask causative pathogen. Specificity may decline (e.g. from 95% to 86%; Lam et al., 1996)	Walker et al., 2010
Pre-culture enrichment			
Pre-incubation	4-18 hours pre-incubation can encourage multiplication of viable pathogens for increased detection	Increase time to result, increase contaminants	Dinsmore et al., 1992; Artursson et al., 2010
Pre-freezing	Overnight freezing may enhance recovery of *S. aureus* by releasing intracellular bacteria	Reduce recovery of coliforms, increase time to result	Villanueva et al., 1991; Dinsmore et al., 1992
Enrich in broth	Increase sensitivity	Reduced specificity, encourage contaminant growth	Thurmond et al., 1989; Sol et al., 2002

1.3.1.6 Test interpretation

The sensitivity of detecting bacteria in culture can be optimised by adjusting the number of CFU/mL of milk used to indicate a true IMI (Dohoo et al., 2011a; Torres et al., 2009). A consensus was developed with contribution from a range of international mastitis researchers and practitioners that stated a quarter should be considered infected if two out of three consecutive samples were positive for the same organism or if a single sample had at least 10 colonies isolated from 0.01 mL of milk (Andersen et al., 2010). Using these criteria, the estimates of sensitivity were maximised for the detection of each pathogen investigated. However, different pathogens should have altered thresholds for positive classification (Torres et al., 2009). Isolation of at least 100 CFU/mL (or 1 CFU per 0.01 mL plated) was suggested for major contagious pathogens (*S. aureus*, *S. agalactiae* and *Mycoplasma* spp.). Specificity using this threshold remains high, because these pathogens are not often found in the environment, so when isolated in culture they generally indicate true infection (Dohoo et al., 2011a). For environmental pathogens, the rate of false positives would be high if the threshold was 100 CFU/mL. A higher percentage of agreement between duplicate samples was found when using 1,000 CFU/mL to detect environmental pathogens and minor pathogens (Torres et al., 2009). Alternatively, Griffin et al. (1987a) advised an additional measure of inflammation, such as SCC or CMT, to provide more assistance in determining the true IMI status of a quarter or cow. If identifying as many existing infections as possible is the priority, then the definition should be that a quarter is

infected if a single colony is isolated from 0.01 mL plated (Dohoo et al., 2011a). However, if false positives are undesirable, then higher thresholds for environmental and minor pathogens are recommended.

The National Mastitis Council (NMC) recommends interpretation of duplicate samples in series, i.e. the same pathogen must be isolated from both samples to confirm IMI (Oliver et al., 2004). For consecutive samples, the same pathogen should be recovered from at least two out of three samples to declare the cow as 'truly' infected. This gold standard aims to increase specificity and reduce false positives, but this is associated with a reduction in sensitivity, as sensitivity and specificity of a test are inversely related. Parallel interpretation of samples was also assessed as an option to increase the number of infections detected in culture, as only one of the two duplicates requires a positive diagnosis to define as an IMI (Dohoo et al., 2011b). As expected, however, specificity was lowest when samples were interpreted in parallel, as opposed to in series.

In real-time PCR, a cycle threshold (Ct) value is obtained for each sample based on the number of amplification cycles required to detect the bacterial targets. The presence of a targeted pathogen is indicated when the level of fluorescence emitted from the products of the PCR reaction reaches a pre-set threshold. The greater the amount of target DNA in the sample, the fewer cycles it takes to reach threshold level (Koskinen et al., 2008). The level of detection can be categorised into 'high', 'medium', and 'low', based on the Ct value. If the fluorescence signal does not exceed the threshold after a set number of cycles (often 40), the result is reported as 'not detected'. One option to increase the specificity of the test relative to culture is to reduce the threshold for determining a sample as positive (Mahmmod et al., 2013b), but this will offset the tests sensitivity.

1.3.2 Repeatability

A test should be repeatable and consistent between laboratory personnel. Multiple results from a sample should have a low coefficient of variation (Holdaway, 1990). In culture, the reproducibility of results between laboratories may vary due to human factors, variation in tests used and quality of the samples in terms of contamination. According to the NMC, there should be at least 90% agreement between diagnostic laboratories in the diagnosis of samples (Hogan et al., 1999). In the UK, 12 laboratories participated in a quality assurance scheme to assess the variation of errors in diagnosis between laboratories (Griffin et al., 1987b). Using single samples, the range of incorrect diagnosis was 2-47%; however, eight laboratories had more than 90%

agreement. Concordance improved when duplicate samples were assessed in series, with errors reduced to 0-18%, and 10 laboratories correctly diagnosing at least 95% of the samples. Proficiency testing of seven diagnostic laboratories in Finland demonstrated an overall performance from 63% to 93% for correctly identifying mastitis pathogens (Pitkälä et al., 2005). In New Zealand, culture procedures at each laboratory are based on the NMC Laboratory Handbook on Bovine Mastitis (Hogan et al., 1999) which is currently under revision. It was suggested that differentiating between *S. uberis* and *Enterococcus* spp., and *S. aureus* and CNS requires more consistent approaches between laboratories (Hawkins and Cooper, 2014). No proficiency testing has been undertaken in New Zealand to compare the repeatability of pathogen identification across different diagnostic laboratories, but it has recently been identified as an area for development (Hawkins and Cooper, 2014).

In comparison, PCR-based assays target DNA sequences, providing an objective indication as to whether or not that bacterial DNA is present in the sample (Koskinen et al., 2009). By focussing on the unique nucleic acid sequence of specific microorganisms rather than on their phenotypic expression of which are encoded by the nucleic acids, the subjectivity of phenotypic characterisation that occurs in culture is removed via the user-independent approach of PCR (Gillespie & Oliver, 2005).

1.3.3 Rapidity

1.3.3.1 Time to result

A result using real-time PCR can be determined in as little as 4 hours once the sample is received by the laboratory (Koskinen et al., 2010), offering a much more rapid diagnosis than culture, which takes a minimum of 48 hours to confirm a result (Hogan et al., 1999). PCR offers a much faster progression through the stages of investigation compared with culture, especially if Mycoplasma are the cause of the problem. A faster diagnosis can allow for the timely selection of antibiotics that are known to target the specific pathogen identified, potentially reducing the unnecessary use of broad-spectrum antimicrobials and increasing the chance of cure (Pyörälä, 2002). Although PCR allows the determination of a result much faster than culture, the test is not still ‘cow-side’, which would be necessary for guiding treatment decisions in individual clinical cases.

1.3.4 Cost

A diagnostic test must be inexpensive in terms of capital costs and running costs for widespread use (Holdaway, 1990). Real-time PCR technology is more expensive than

bacterial culture, reported as around two to three times the price of culture (Paradis et al., 2012). Capital costs of PCR are also greater than for culture, as PCR testing requires the PCR instrument and computer for analysis, and often more equipment, depending on the method of DNA extraction.

1.4 Pathogen identification testing in New Zealand

The method of pathogen identification in commercial diagnostic laboratories in New Zealand is bacterial culture. PCR is not used routinely, primarily because of the cost compared with culture, the confusion around interpretation of results and the non-existence or very low prevalence of Mycoplasma and *S. agalactiae* for causing intramammary infections (McDonald et al., 2009). In New Zealand, the typical farm system operates on a seasonal basis, with a compact calving period in the spring. The highest incidence of clinical mastitis occurs in the first month of lactation and of these clinical mastitis cases, up to 75% are caused by *S. uberis* and a further 20% are caused by *S. aureus* (McDougall, 1999). Hence mastitis control in New Zealand cows during early lactation must target these pathogens.

The main reasons for identifying mastitis pathogens in New Zealand are to identify the on-farm pathogen challenges. Use in individual treatment cases is limited due to the immediate treatment of clinical cases when detected as recommended by industry (SmartSAMM, 2012). PCR testing could offer advantages in terms of speed, accuracy and automation of results. The need for prudent use of antibiotics is being widely acknowledged across the dairy industry worldwide. This could lead to greater use of and reliance on mastitis pathogen identification tests. Currently, there is no knowledge on the performance of the commercial PathoProof PCR assay in New Zealand. There is also a lack of understanding of how to interpret PCR results, when to use PCR instead of culture, and if PCR can be considered as a practical tool for mastitis pathogen identification in New Zealand.

3 STUDY OBJECTIVES

The objective of this study was to assess the performance of the PathoProof Complete-12 Mastitis PCR assay against conventional microbiological culture methods using multiple quarter milk samples collected from cows at Lye and Scott Farms over the 2013/2014 lactation in order to provide industry guidance on the practical use of PCR technology in New Zealand. The specific hypothesis was that the PathoProof PCR assay had an equivalent sensitivity and specificity to bacterial culture for detecting the specific mastitis-causing pathogens *S. aureus* and *S. uberis*.

4 MATERIALS AND METHODS

4.1 Study design

The study was a comparative trial investigating the performance of the molecular based detection method, PCR, relative to conventional bacterial culture, using quarter milk samples collected from New Zealand dairy cows. The study was conducted between June 2013 and March 2014 on DairyNZ's Waikato-based research farms, Lye Farm and Scott Farm. This study was approved by the Ruakura Animal Ethics Committee (No. 12948).

4.2 Cows

The study was conducted on 480 spring-calving Friesian-Jersey cross mixed age cows and heifers. Cows were managed in a pasture-based system according to normal farm practice throughout the study period, unless they were involved in separate, concurrent trials, when management was under the discretion of the project leader for that trial. In most cases, the management practices associated with concurrent trials were not considered to affect milk such that would affect the comparison of the tests and, therefore, results of this study. However, cows were excluded when this was not the case.

All cows at Lye Farm (n=330) and 150 cows on Scott Farm were included in the early lactation sampling. Only Lye Farm cows were sampled at mid- and late-lactation. The cows at Scott Farm were on a specific trial that required bacteriological data in early lactation so samples were readily available for use in this study. Samples from cows that left the farm before the end of the season were kept, provided full sets from an earlier stage of lactation were available and they met the criteria for sample selection for the test comparison. Samples were also routinely collected from cows at the first milking at a research farm operated by DairyNZ in Taranaki (WTARS). Due to the limited number of *S. aureus* infections at Lye and Scott Farm, samples from six cows with at least one quarter infected with *S. aureus* were included in this study.

4.3 Sample collection

4.3.1 Sampling schedule

A series of samples were collected during three stages of lactation: early lactation (June-October 2013), mid-lactation (November 2013), and late lactation (February 2014).

In early lactation, a single milk sample was routinely collected from all quarters at the first (M1) and tenth (M10) milking following parturition. Quarters identified as culture-positive for any bacterial pathogen at either M1 or M10 were further sampled at least 30 days into lactation (D30; range 30-37), forming a set of three samples for quarters identified as being infected in early lactation. For those quarters enrolled in the study from cows at WTARS farm, the days for sample collection were not exactly the tenth milking and 30 days into lactation, but were as close as possible to these dates.

During early November, a sample was collected from all quarters of cows at Lye Farm for routine bacteriology (R1). Quarters identified as positive by culture for any bacterial pathogen at R1 were resampled 7 days later (R1+7), forming a set of two samples for quarters identified as being infected in mid-lactation. Similarly, in February, all quarters were sampled (R2) and infected quarters were re-sampled 7 days later (R2+7), forming a set of two samples representing quarters identified as being infected in late-lactation.

Additionally, any quarter that presented as clinical mastitis (CM) on either farm was sampled by milking staff and, if this occurred between June and early September, a second sample was collected from the quarter approximately 30 days later. Re-sampling of quarters that were defined as contaminated by culture occurred on a case-by-case basis. M1 samples were always afternoon milking samples (collected prior to the first milking following calving) and all other samples were collected at the morning milking.

4.3.2 Treatment of clinical cases

Quarters that displayed signs of clinical mastitis were managed according to normal farm practice throughout the trial. Milking staff always collected a pre-treatment sample and administered and recorded treatments. Samples were still collected from cows as part of the sample schedule even when cows were treated with antibiotics.

4.3.3 Milk sampling protocol

Milk samples were collected following the NMC guidelines (Hogan et al., 1999) which are outlined in the DairyNZ Standard Operating Procedure (M3) – Aseptic sampling of udder secretion for bacteriology. Briefly, sterile plastic bottles with screw tops (30 mL capacity) pre-labelled with cow number, quarter, date and herd code, were matched to the correct cow to be sampled, prior to milking. Cotton wool swabs moistened with 70% alcohol were used to remove all traces of dirt from teats and teat ends, starting with the cow's front quarters and working towards the rear quarters teat ends were scrubbed vigorously until they looked clean, using a new swab for each teat. Rear quarters were

then sampled first to minimise the risk of sample contamination. The first few streams of milk were discarded. The bottle lid was removed and held face down in the same hand as the bottle to avoid contaminants from the surroundings. Additionally, the bottle was held as horizontal as possible (i.e. <45°) to minimise the chance of contamination. A sample of 25 mL was collected for bacteriology, subsampling for storage and SCC testing. The lid was secured immediately after sample collection and after all quarters were sampled, the cow was milked by milking staff.

If at any stage of sampling a sample became obviously contaminated, or aseptic conditions were disrupted, teats were re-cleaned and a second sample was collected for analysis. This procedure aimed to minimise sample contamination and ensure confidence in bacteriological analysis. Only trained personnel approved for aseptic udder secretion sampling were involved in sample collection. Samples were kept at 4 °C until culture.

4.4 Bacteriology

Bacteriology was carried out according to the NMC Laboratory Handbook on Bovine Mastitis (Hogan et al., 1999). Bacterial culture was performed at the DairyNZ Mastitis Bacteriology Laboratory. All bacterial identifications were confirmed by an experienced laboratory technician, and this was kept consistent; therefore, the same criteria were applied to each quarter sample analysed throughout the study period. Additional biochemical testing by Strep API (bioMerieux, Lyon, France) was completed for a sub-set of samples for confirmation purposes.

4.4.1 Bacteriological culture

Culture was conducted within 24 hours of collection, on chilled samples. Initial culture entailed streaking, with a sterile plastic loop, 0.01 mL of milk from one quarter sample onto one quadrant of an aesculin blood agar plate (Fort Richard Laboratories Ltd, Otahuhu, Auckland, NZ), containing 5% whole bovine blood and 0.1% aesculin. Four quarter samples from one cow were streaked into separate quadrants on a plate. Plates were incubated aerobically at 37 °C for 48 hours, with a preliminary examination at 18-24 hours. Presumptive identification of bacteria was according to the method described by the NMC (Hogan et al., 1999). Staphylococci and streptococci were distinguished by their respective positive and negative response to the catalase test. *S. aureus* and CNS were distinguished by their coagulase activity using the tube coagulase test (Becton, Dickinson and Company, Sparks, MD, USA). Biochemical tests for distinguishing streptococci included sodium hippurate, aesculin hydrolysis, inulin hydrolysis, growth in salt (NaCl) and the CAMP test. A plate score of 1, 2 or 3 was

given based on the number of colonies of a specific species isolated (Table 3). A quarter was identified as infected with a major mastitis pathogen (i.e. *S. aureus* and *S. uberis* etc.) when one or more colonies were isolated from the 0.01 mL inoculated on the plate as the goal was to detect as many existing infections as possible (Dohoo et al., 2011a). A quarter was identified as infected with a minor mastitis pathogen (e.g. CNS, *C. bovis*) when three or more colonies were isolated (Hogan et al., 1999). A sample was considered to be contaminated if three or more dissimilar colony types were present in culture (Hogan et al., 1999).

Table 3. Bacterial culture plate scores and corresponding number of colonies of major mastitis pathogens on a plate (Hogan et al., 1999).

Plate score	No. colonies/plate
0	0
1	1-10
2	11-30
3	>30

4.4.2 Repeat bacteriology

Bacteriological procedures were repeated for samples selected for PCR following a period of storage. Samples were thawed overnight in the refrigerator and 0.01 mL was streaked on a plate quadrant as described above. In addition, so as to measure the number of bacterial colonies in each sample, colony counts (CFU/mL) were also obtained through spreading 0.1 mL on a whole plate with a sterile plastic spreader. Three dilutions were prepared for each sample (10^{-1} – 10^{-3}). This was done in duplicate to increase the accuracy of the count.

4.4.3 Confirmation of streptococci

A commercial identification system for streptococci, API 20 Strep (bioMerieux, Lyon, France), was used for 14 samples that grew in culture but were not detected by PCR testing. A pure culture was prepared by sub-culturing the sample following growth on aesculin blood agar. An API strip containing 20 biochemical tests was used for each sample, following the manufacturer's instructions. Briefly, a sterile swab was used to harvest the culture from the sub-cultured plate and to inoculate 2 mL of API Suspension Medium. Turbidity greater than 4 on the McFarland scale was sufficient to proceed. Approximately 0.1 mL of the suspension was distributed in each of the first nine wells on the strip, and the tenth well was filled with the same suspension. The remaining suspension was added to the API GP Medium ampule and mixed by

swirling. The last 10 wells were filled with this suspension and any leftover was discarded. Mineral oil was added to the 9th to 20th well, inclusive, to form a convex meniscus. Each strip was incubated at 37 °C for 4 hours. The various reagents, according to manufacturer's instructions, were added to wells 1-9 and reactions recorded after 10 minutes. The strips were returned to the incubator for a further 24 hours. Interpretation followed manufacturer instructions using the Identification Table provided with the instructions for use. A numerical value was assigned to each well according to observations, and the numbers for different groups of tests collated on the results sheet, to determine a 7-digit numeric profile which coded for a species of bacteria.

4.5 SCC testing

SCC testing was completed on all samples, except M1 (colostrum), within four days of collection. Samples were sent to Livestock Improvement Corporation (LIC; Hamilton, New Zealand) for cell counting using the Fossomatic automated cell counter (Foss Electric, Hillerod, Denmark).

4.6 Sample handling and storage

During early lactation, once a culture result was obtained and recorded, all milk samples were subsampled into two aliquots in sterile 5 mL tubes. "A" and "B" duplicates were stored in separate freezers at -20 °C. The remainder of each sample was sent for SCC testing. All samples collected in early lactation were stored whereas only some of the samples collected in mid- and late-lactation were stored, to reduce the requirements for storage. Those stored in the latter stages included: all quarters from any cow with an infection in one or more quarters as identified by bacteriology; samples from quarters with a previous infection in the current lactation; and a selection of samples from cows of various ages with quarters that had an infection-free history.

Early lactation samples were stored for 3-9 months prior to further analysis. Mid- and late-lactation samples were stored for a maximum of 4 and 2 months, respectively. This length of time reflected the delays in beginning PCR analysis due to the need to develop the protocol for that analysis (Chapter 5). Samples were thawed by refrigeration and were kept chilled at 2-4 °C until bacteriology and PCR was undertaken. Samples were then returned to frozen storage for retesting if required.

4.7 Selection of samples for PCR testing

Sample sets were selected for PCR analysis on the basis that at least one sample was culture-positive for *S. uberis* in the set of three or two samples from early and mid/late

lactation, respectively. Additionally, a proportion of culture-negative samples from each stage of lactation were included in the comparison. This incorporated a range of culture-positive and culture-negative samples reflecting the changes in infection status over time. Where a set included a contaminated sample in culture, it was excluded from the analysis because the PCR method does not have clear guidelines on defining a contaminated sample (Koskinen et al., 2010).

4.8 PCR testing

The PCR testing procedure for each sample involves bacterial DNA extraction from raw milk, followed by amplification of DNA by real-time PCR and interpretation of results. PCR testing using the PathoProof Mastitis Complete 12 kit (Thermo Fisher Scientific Ltd., Vantaa, Finland) was performed at a commercial laboratory (Livestock Improvement Corporation; LIC) by the author. The PathoProof Mastitis Complete-12 Kit identifies 11 mastitis-causing bacterial species or species groups and the β-lactamase penicillin resistance gene in staphylococci. The bacteria and the β-lactamase gene are detected simultaneously in four separate multiplex real-time PCR reactions. The specific primer mixes for the four reactions can identify the following bacterial species and groups:

PathoProof Primer Mix 1

- *S. aureus*
- *Enterococcus* sp. (including *E. faecalis and E. faecium*)
- *C. bovis*

PathoProof Primer Mix 2

- Staphylococcal β-lactamase gene
- *E. coli*
- *S. dysgalactiae*

PathoProof Primer Mix 3

- *Staphylococcus* sp. (including *S. aureus* and all relevant CNS)
- *S. agalactiae*
- *S. uberis*

PathoProof Primer Mix 4

- *Klebsiella* sp. (including *K. oxytoca and K. pneumoniae*)
- *Serratia marcescens*
- *Trueperella pyogenes* and/or *Peptoniphilus indolicus*

This study focussed on two pathogens, *S. aureus* and *S. uberis*, as these are the predominant mastitis-causing pathogens in New Zealand. For this reason and consideration of the available resources, only two of the four reactions were run for each sample; Primer Mixes 1 and 3. The kit was, therefore, capable of detecting six mastitis pathogens (*S. aureus, Enterococcus* sp., *C. bovis, Staphylococcus* sp., *S. agalactiae and S. uberis*) in this study because of these restrictions. Each sample was represented in four individual wells on the PCR plate, as there were two primers for each sample, and the PCR was performed in duplicate or replicate. Samples in a set were always run together for both the DNA extraction and real time-PCR procedure.

4.8.1 Workflow of the assay

The PathoProof Mastitis Complete-12 Kit contains all the necessary reagents for bacterial DNA extraction and real-time PCR. The F-870L kit is sufficient for 384 tests, using a 96-well format for the DNA extraction and PCR plate.

4.8.1.1 DNA extraction

Samples for DNA extraction were manually prepared by the author, following the procedure outlined in Table 4. Briefly, DNA was extracted from 400 µL of milk for each sample. Lysis solution 1 enabled the enzymatic lysis of somatic cells in the milk, which was followed by centrifugation to separate bacterial cells from the lysed somatic cells and from PCR-inhibiting substances.

Table 4. Sample preparation for DNA extraction.

1.	Thaw milk sample and mix thoroughly by vortexing.
2.	Pipette 400 µL of milk into a sterile 1.5 mL Eppendorf tube, avoiding milk clots if possible.
3.	Add 400 µL Lysis Solution 1 to milk and mix.
4.	Centrifuge for 3 minutes at 1500 x g (6000 rpm).
5.	Remove the supernatant, including the top layer of fat.
6.	Resuspend the pellet in 100 µL of Lysis Solution 2. If the pellet does not resuspend well add 100-200 µL sterile water and mix by pipetting.
7.	Transfer the sample into the “Sample plate”.

DNA was extracted by the Kingfisher 96 (Thermo Electron, Vantaa, Finland), which had the ability to purify DNA from 96 samples simultaneously. DeepWell plates were prepared according to Table 5, and were loaded into the Kingfisher 96, along with a sterile tip plate. Each well corresponded to one sample. One negative control was included in each extraction (reagents only). The workflow of the extraction protocol is described in Table 6. Further lysis with lysis solution 2 disrupted the cell walls of Gram-negative and Gram-positive bacteria allowing the magnetic bead-based techniques to

purify bacterial DNA. When the extraction was completed, the elution plate containing extracted DNA in AE buffer was sealed with an Adhesive Plate Seal and stored at -20 °C unless immediately used for PCR setup and testing. The collection efficiency of particles in the sample has been stated to be ≥95% (Thermo Electron, Vantaa, Finland).

Table 5. Wash and elution plates and reagent volumes for DNA extraction.

Plate name	Reagent	Volume per well (µL)
Wash 1	Buffer AW1	800
Wash 2	Buffer AW1	500
Wash 3	Buffer AW2	500
Wash 4	Buffer AW2	500
Wash 5	Water containing 0.02% Tween 20	600
Elution*	Buffer AE	150
Sample plate	Proteinase K Sample	40 100-300**

*Prepared in Elution plate, not DeepWell.

**Volume varied according to Step 6 in Table 4.

Table 6. Workflow of the DNA Extraction.

1. Lysis 1 of sample Heating temperature: 45°C
2. Lysis 2 of sample Heating temperature: 85°C
3. Lysis 3 of sample Heating temperature: 95°C
4. Add bead mix (amounts per sample): Ethanol 200 µL Buffer RLT 200 µL MagAttract Suspension G 40 µL
5. Bind sample
6. Wash 1
7. Wash 2
8. Wash 3
9. Wash 4
10. Wash 5
11. Elution Heating temperature: 72°C

4.8.1.2 Real-time PCR

Reagents for the PCR included the MasterMix, the appropriate primer mix, and the sample (Table 7). The MasterMix was an optimised buffer at pH 8.5, including 9.0 m*M* $MgCl_2$, 0.4–0.8 m*M* deoxynucleoside triphosphates and a hot start DNA polymerase.

Each primer mix contained oligonucleotides for identification of the three specified targets as well as an Internal Amplification Control (IAC). This IAC was composed of a 93-bp fragment of lambda-DNA, where 10^6, 10^5, 10^4 and 10^3 copies of the lambda-DNA fragment were included in reactions 1, 2, 3 and 4, respectively (Figure 1; Koskinen et al., 2009). Each well, therefore, had an internal measure of reaction success, indicated by an IAC Ct value within a specified range (Table 8), and the shape of the amplification curve was characteristic of an acceptable reaction (Figure 1).

Table 7. Reagents and volumes for PCR reaction setup.

PCR reagent	Volume per well (µL)
MasterMix	10
Primer Mix (1 or 3)	5
Sample/eluate	5

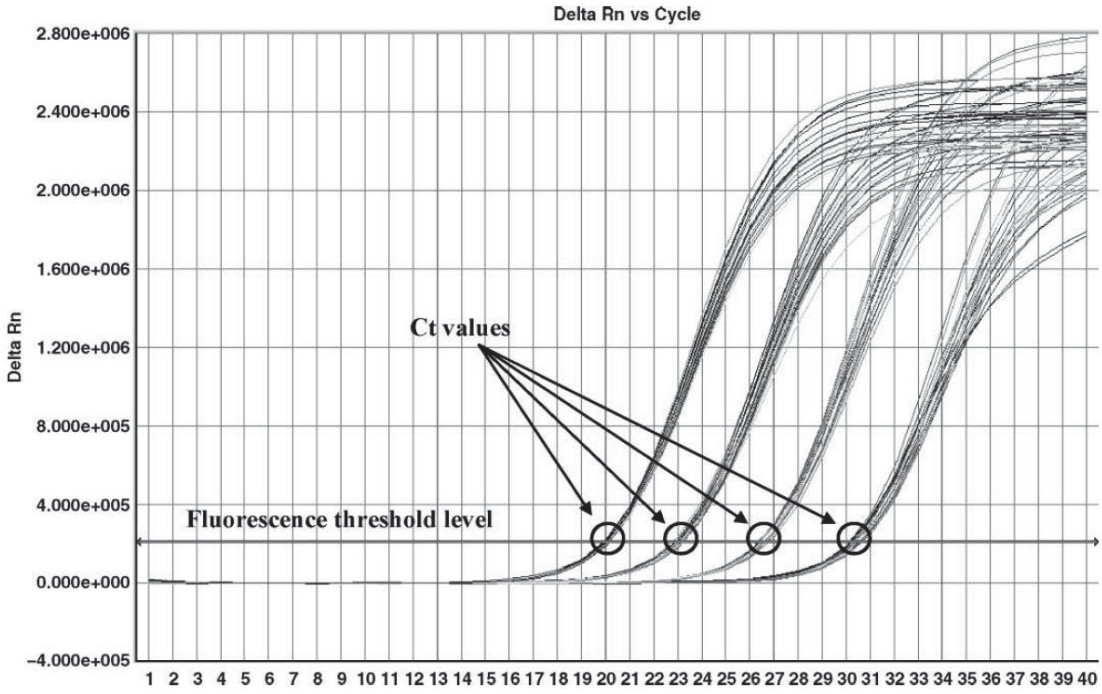

Figure 1. Internal Amplification Control curves obtained from a 93-bp fragment of lambda-DNA included in the Primer Mixes 1, 2, 3, and 4 of the PathoProof Mastitis PCR Assay. Illustrated are the IAC amplification curves obtained from the 4 real-time PCR reactions of the assay for 20 different bacterial culture samples (Koskinen et al., 2009).

An additional control was included in every PCR run; the Universal Amplification Standard. This contained control DNA for all 11 bacterial targets to 1) allow the verification of acceptable reaction conditions, and 2) provide the control curve for each target to manually set the threshold for distinguishing a sample as positive or negative.

All PCR reactions were carried out by the author using the 7500 Fast Real Time PCR system (Applied Biosystems, California, USA). PCR setup was at a physically separate working area to the DNA extraction and the PCR reactions were run in a different laboratory. PCR reagents were thawed thoroughly, before mixing and briefly centrifuging. Sufficient volumes of MasterMix and the specific Primer mix were added to a sterile Eppendorf tube and mixed; 15 µL was then dispensed into each well of a 96 well plate (12 by 8) correlating to the number of samples included in the run, with the left six columns of the plate containing reagents for Primer Mix 1 and the right side for Primer Mix 3. The extraction plate was removed from the freezer, allowing samples to thaw completely, and 5 µL of each eluate (sample) was mixed by pipetting and dispensed into the designated well on the PCR plate. One row contained 12 wells, so for example the plate layout for early lactation sets consisted of the left six wells containing two duplicates of the three samples from the same set. The six wells on the right-hand side of the plate contained the same samples, but for Primer Mix 3. Each PCR run also included the IAC as a positive control and a negative control (sterile water) to confirm that cross-contamination had not occurred in the laboratory. The positive and negative controls from the DNA extraction were also included in the PCR reaction to confirm DNA extraction success.

Once the PCR plate was prepared and contained all reagents, sample and appropriate controls, the plate was sealed with a compatible optically clear sealer. The plate was centrifuged briefly for 5-10 seconds and placed in the 7500 Fast Real Time PCR system to start the reaction. The thermal cycling protocols were consistent for each PCR run, and were as follows:

- Pre-denaturation – 10 minutes at 95°C (repeated once)
- Denaturation – 5 seconds at 95°C; 1 minute at 60°C (repeated 40 times)
- Annealing – 5 seconds at 25°C (repeated once)

4.8.1.3 Interpretation

Analysis of the PCR result for each sample was completed on the 7500 Software, version 2.0.6 (Applied Biosystems, Life Technologies Corporation). Based on technical advice from a Thermo Fisher Scientific representative, the thresholds for determining a positive and negative sample were manually set for each target at one tenth of the plateau of the Universal Amplification Standard (threshold level illustrated in Figure 1).

In real-time PCR assays, a positive reaction occurs when a fluorescent signal is detected, which is expressed as the Ct value. Ct represents the number of cycles

required for the fluorescent signal to become strong enough to be distinguished from background values. PathoProof PCR runs for 40 cycles, but a cut-off of 37 was used to score a reaction as positive (≤37) or negative (>37). This Ct cut-off value was used because obtaining a three cycle difference in Ct of the targets compared with the negative control is the generally accepted standard for reliable separation of a true positive signal from background noise or contaminants (Bustin, 2004). If the fluorescent signal did not exceed the threshold after 40 cycles, the result was 'not detected', indicating the absence of the target DNA and therefore confirming the reaction as negative for that target. Acceptable PCR conditions were confirmed for each target by checking the IAC Ct values were within a specified range for each target (Table 8) and the shape of the amplification curve was as expected. This measure was necessary before scoring a reaction as positive or negative. Additionally, the negative control was confirmed as negative.

Table 8. Cycle threshold (Ct) values of the Internal Amplification Control (IAC) to confirm acceptable reaction conditions.

Reaction	Acceptable Ct range for IAC
Primer Mix 1	28.5 – 31.5
Primer Mix 2	24.5 – 27.5
Primer Mix 3	21.5 – 24.5
Primer Mix 4	18.5 – 21.5

4.9 Comparison of the tests

The sensitivity and specificity of the PCR assay were assessed relative to bacterial culture that was described by a combination of bacteriology scores from fresh and frozen culture and plate colony counts, interpreted in parallel (Dohoo et al., 2011b). The combined definition of a positive result in culture for each pathogen was that at least one of the following conditions was met:

- Plate score of 1, 2 or 3 in fresh culture
- Plate score of 1, 2 or 3 in frozen culture
- Plate colony count of >0 CFU/mL

All samples were run by PCR as either duplicates or replicates in this study (see Chapter 5). PCR Ct values were averaged if both duplicates were <37. If one duplicate was ≥37, and one duplicate was <37, the latter value was used. If both duplicates were

≥37, the sample was considered to be negative (Koskinen et al., 2009). For M1 and CM samples, it was expected that the Ct value for the undiluted Rep1 would be less than the Ct value for the diluted 1 in 10 Rep2, and if this was the case, Rep1 was used for analysis. If Rep1≥Rep2, Rep2 was used as it was likely that the undiluted sample was inhibited in the PCR. Rep2 was also used if Rep1 was missing due to unacceptable IAC conditions for either Primer 1 (*S. aureus* target) or Primer 3 (*S. uberis* target). This was the case for 19 samples. Coefficient of variation (CV) and repeatability were estimated for samples that had two valid Ct values (<40). Samples were grouped based on Ct value <30 or ≥30 to assess if there were CV differences for samples with low and high Ct values. A Ct value of 30 was used to split groups as this value is used by the PCR kit manufacturer when reporting results semi-quantitatively as + (≥30), ++ (24-30) or +++ (<24) for *S. aureus* and + (≥31.1), ++ (21-31) or +++ (<21) for *S. uberis* (Thermo Fisher Scientific Ltd., Vantaa, Finland). A further division at 24 and 21 was not possible due to insufficient sample numbers with Ct values <24.

Relative sensitivity and specificity were calculated separately for *S. uberis* and *S. aureus* based on the results of each sample in both culture and PCR tests (Table 9). Relative sensitivity was defined as the proportion of 'true' positive results (according to culture) that were correctly identified by PCR and relative specificity as the proportion of 'true' negative results correctly identified by PCR (Equation 1 and 2; Martin, 1984). Positive predictive value (PPV) and negative predictive value (NPV) were also calculated (Equation 3 and 4; Parikh et al., 2008). PPV provides the probability that the quarter was culture positive when PCR tested positive for a specific pathogen, and NPV estimates the probability that the quarter was culture negative when the PCR tested negative for a specific pathogen (Parikh et al., 2008). Mid- and late-lactation samples were combined for analysis due to low sample numbers.

Table 9. Two-by-two representation of the possible outcomes from testing PCR against the reference test bacterial culture (adapted from Parikh et al., 2008).

	Culture-positive	Culture-negative	
PCR-positive	True positives (TP)	False positives (FP)	Total test positives: TP + FP
PCR-negative	False negative (FN)	True negatives (TN)	Total test negatives: FN + TN
	Total positives: TP + FN	Total negatives: FP + TN	Total population: TP + FP + FN + TN

Equation 1. Relative sensitivity calculation

$$\text{Sensitivity \%} = [TP / (TP + FN)] \times 100$$

Equation 2. Relative specificity calculation

$$\text{Specificity \%} = [TN / (TN + FP)] \times 100$$

Equation 3. Positive predictive value (PPV) calculation

$$\text{PPV \%} = [TP / (TP + FP)] \times 100$$

Equation 4. Negative predictive value (NPV) calculation

$$\text{NPV \%} = [TN / (TN + FN)] \times 100$$

Basic statistical analyses were performed using Excel and Minitab (version 16.2.3, Minitab Inc.). The Kappa statistic (κ) was used to determine the agreement beyond that expected by chance, between culture and PCR, and between fresh and frozen culture results (Cohen, 1960).

5 DEVELOPMENT OF THE PCR ASSAY

Prior to this study, there were no known reports of the use of the PathoProof Mastitis PCR Complete-12 assay in New Zealand, so between August and December 2013 the kit was validated for use on New Zealand bovine milk samples. This enabled the author to gain experience in performing the test before processing samples for the experimental part of this study. The validation steps identified some areas where minor deviations from the PathoProof Mastitis Complete-12 assay instructions were necessary. While the Materials and Methods chapter reports the refined protocol used in the study, this chapter presents the minor deviations made to the manufacturer's instructions, including reasons for these deviations and results from validation experiments. Results will be discussed in Chapter 7. It is important to note that limited samples were used in each of these experiments, but these were considered to be sufficient to highlight any issues with the protocol and provide a satisfactory indication to proceed with the experiment after making any necessary changes.

5.1 Inadequate collection tubes for preparing sample for DNA extraction

Raw milk samples (n=20) that had been stored at -20 °C for up to two years were used in the first validation experiment (Expt 1). These samples represented different stages of lactation (early, mid and late lactation). Bacteriology was completed on fresh samples and was repeated just prior to PCR testing. DNA extraction and PCR was undertaken according to the manufacturer's instructions (Thermo Fisher Scientific, Vantaa, Finland). Samples were prepared for DNA extraction using the 2 mL collection tubes provided (Figure 2).

Results from the PCR reaction were indicative of cross-contamination between samples. Of the 20 samples, 10 were culture-positive for *S. uberis* and seven were *S. aureus* positive in culture. The PCR test identified 14 samples positive for *S. uberis* and 10 samples positive for *S. aureus* (Table 10). A further two samples had unacceptable IAC Ct values and amplification curves, indicating failure of the bacterial DNA target amplification. Based on these results, the relatively sensitivity and specificity was 80% and 25% for *S. uberis* and 100% and 72% for *S. aureus*, respectively (Table 10). Six samples were PCR false positive for *S. uberis* and three were PCR false positive for *S. aureus*, using bacterial culture as the reference test.

Figure 2. Collection tubes provided with the PathoProof PCR kit.

The 2 mL collection tubes provided with the assay (Figure 2) were difficult to use during the sample preparation part of the extraction. Tubes and lids were joined together in groups of eight in such a way that working with one sample in the group meant that adjacent samples were exposed to potential cross-contamination. A repeat experiment (Expt 2) of a selection of the same samples (n=8) used 1.5 mL sterile Eppendorf tubes, which provided less risk for carryover between tubes, as the lids were only open when working with the sample. Out of six samples with acceptable IAC characteristics (the two samples failed as before), five samples had agreement in their culture and PCR results (Table 10), and there was just one sample that was positive for *S. uberis* in culture that was not detected by PCR. The improvement in accuracy of detection of both pathogens resulted in the use of Eppendorf tubes for the DNA extraction in the experimental study.

5.2 Failure of PCR reaction in colostrum and clinical samples

Samples with unacceptable IAC Ct values and amplification curves indicate failure of the PCR reaction. The IAC is composed of a 93-bp fragment of lambda-DNA; Primer mix 1 (reaction 1) contained 10^6 copies of lambda-DNA and Primer mix 3 (reaction 3) had 10^4 copies of lambda-DNA. Each primer mix contained primers and probes for this IAC DNA as a control to confirm the success of PCR and thus successful amplification of the specific bacteria targeted in the reaction. The two samples that 'failed' in Expt 1 were repeated but the IAC continued to indicate reaction failure in Expt 2 (Figure 3). Milk samples collected from freshly calved cows were tested in Expt 3 to rule out any issues with frozen samples in the first experiment. One sample in this experiment 'failed'. A common feature of this sample and the previous 'failed' samples was that they were either thick colostrum or clotted clinical mastitis milk samples; but not all

samples with that appearance failed. A 1 in 10 dilution in sterile water was tested on the DNA extract from these samples and the PCR was repeated. IAC characteristics were acceptable in all those that had failed previously (Expt 5) and sensitivity and specificity estimates improved (Table 10). Additional dilutions including 1 in 2, 1 in 4, and 1 in 8 were tested against undiluted and 1 in 10 for colostrum, clotted and normal milk samples, with the lowest Ct values (indicating greatest bacterial load) for *S. aureus* occurring in undiluted extracts and in 1 in 10 dilutions for *S. uberis* (data not shown). While the dilution improved *S. uberis* detection for some colostrum samples, there was potential for samples with Ct values >34 for *S. uberis* to shift to >37 and therefore be undetected due to the reduced sensitivity. The protocol was then adjusted for the experimental part of the study to run colostrum (M1) and CM samples as both undiluted (Rep1) and at a 1 in 10 dilution (Rep2) routinely, with samples at other time points run undiluted as normal. This practice managed to dilute unknown PCR inhibitors in certain samples without compromising sensitivity in others.

Table 10. Number of samples in which *S. uberis* and *S. aureus* were identified in bacterial culture (BC) and/or PCR, including the relative sensitivity and specificity of the PCR test.

	N	IAC fail*	BC pos / PCR pos	BC pos / PCR neg	BC neg / PCR pos	BC neg / PCR neg	Rel. Se (%)	Rel. Sp (%)
S. uberis								
Expt 1	20	2	7	2	6	2	80	25
Expt 2	8	2	2	1	0	3	67	100
Expt 3	8	1	4	0	2	1	100	33
Expt 4	12	1	1	2	0	8	33	100
Expt 5	16	-	5	1	0	10	83	100
S. aureus								
Expt 1	20	2	6	0	3	8	100	72
Expt 2	8	2	3	0	0	3	100	100
Expt 3	8	1	3	0	0	4	100	100
Expt 4	12	1	2	0	0	9	100	100
Expt 5	16	-	8	0	1	7	100	87.5

*IAC = Internal Amplification Control; fail refers to unacceptable Ct value and/or shape of the amplification curve of the IAC DNA, included as a control in each reaction.

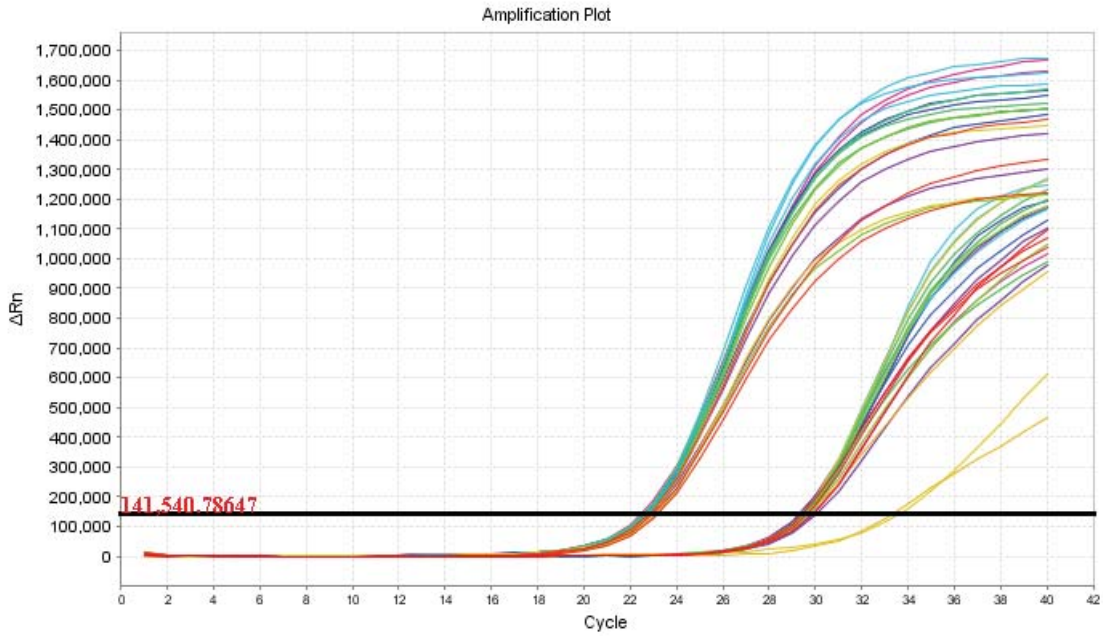

Figure 3. Amplification plot showing the IAC amplification curve for samples in Expt 1. The black horizontal line is 1/10th of the plateau of the amplification curve, representing the threshold for determining a sample as positive or negative. The samples with Ct ~23 represent Primer mix 3 and the samples with Ct ~30 represent primer mix 1. The two yellow samples are those with unacceptable IAC Ct values and amplification curves.

5.3 Limit of detection of PathoProof PCR assay for detecting *S. uberis* in milk

Earlier experiments highlighted some inaccuracies in *S. uberis* detection using the PathoProof PCR assay in culture isolates (Expt 4) and directly from milk (Expt 1, 2, 3, and 5); whereas, *S. aureus* detection appeared to be satisfactory, out of a total of 64 samples tested (some repeats; Table 10). A standard curve experiment was set up to determine the limit of detection of the PathoProof Mastitis PCR kit for detection of *S. uberis* in raw milk samples.

A PCR-negative milk sample was required as the diluent to prepare the dilution series for the standard curve. DNA was extracted from duplicate samples of four culture-negative quarters from cows in late lactation and all four PathoProof primers were used in the assay to confirm that samples were PCR-negative for the 11 mastitis pathogens targeted. Two of the culture-negative quarters showed negative results for the targeted pathogens in both duplicates. Two duplicates from the one of these culture-negative quarters were then used as the diluent. Two strains of *S. uberis* (SR115 (McDougall et al., 2004)) and a wild strain identified in pure culture of a clinical mastitis sample from the DairyNZ Lye Farm research herd) were sub-cultured to generate a pure culture. A single colony was inoculated from each strain into 5 mL of the diluent and incubated at

37 °C for 24 hours, producing a bacterial concentration of approximately 10^7 CFU/mL for each strain. A 10-fold dilution series was prepared over the range 10^0–10^9 and three aliquots from each dilution were collected for colony counting (0.3 mL), PCR testing (1 mL) and for frozen storage (1 mL). Colony counts were completed in triplicate, using spread plating of 0.1 mL. For PCR testing, samples were extracted in duplicate for each dilution, and PCR for each sample extract was also run in duplicate. Procedures for bacteriology and PCR testing were as described earlier (Chapter 4).

The limit of detection in culture using 0.1 mL as the inoculum was 4-6 CFU/mL for the two strains of *S. uberis* (Table 11). The limit of detection using PCR was at the 10^{-4} dilution, which was measured as 4,900-5,800 CFU/mL in culture. For the SR115 strain, the Ct value was >37, so would be considered as negative. A 1 in 10 dilution would be expected to increase the Ct value by ~3.3 cycles using PCR; however this was not the case in these samples. The limit of detection depends on the quality of the DNA extraction and the milk sample itself (see Discussion). More replicates and use of milk samples at various stages of lactation would have improved the quality of results from this study but were not completed due to time constraints.

Table 11. Culture results (CFU/mL and log CFU/mL; average of triplicates) and PCR Ct values (average of duplicates) for the standard curve experiment for undiluted and dilutions ranging from 10^{-1}-10^{-6} for two strains of *S. uberis* (SR115 (McDougall et al., 2004) and wild). ND = not detected.

		Culture (CFU/mL)		Culture (log CFU/mL)		PCR Ct value	
		SR115	**Wild**	**SR115**	**Wild**	**SR115**	**Wild**
Dilution	**0**	7E+07	7E+07	7.84	7.87	19.58	17.13
	10^{-1}	6E+06	7E+06	6.75	6.81	23.78	20.58
	10^{-2}	6E+05	3E+05	5.74	5.42	29.98	24.65
	10^{-3}	51681	55804	4.71	4.75	32.53	28.15
	10^{-4}	5799	4898	3.76	3.69	38.45	32.95
	10^{-5}	383	447	2.58	2.65	ND	ND
	10^{-6}	4	6	0.55	0.76	ND	ND

6 RESULTS

6.1 Descriptive data

A total of 343 quarter milk samples from 108 cows were analysed by both bacterial culture and the PathoProof mastitis PCR assay. These were selected from a larger data set of 6,848 milk samples that were collected during the 2013/14 lactation at DairyNZ's Lye and Scott Farms in the Waikato, and from a further 20 samples collected from six cows at a farm in Taranaki (WTARS).

Of the 343 samples, 270 were collected in early lactation (June - September 2013), 31 from mid-lactation (November 2013) and 42 from late lactation (February 2014). The average and range in DIM at each of the sampling points is presented in Table 12. Clinical mastitis samples (n=3) were collected from quarters presenting with clinical signs in early lactation, but on a separate day to the sampling points included in the study. Twenty-eight samples were collected from cows that had been previously treated with antibiotics in the period before scheduled sample collection (range 0-21 days post treatment). These samples were excluded from analyses (unless stated otherwise), leaving 315 samples for the comparison.

Table 12. Average and range of days in milk (DIM) of cows at the time of sampling for each sampling point.

	Average DIM (days)	Range
M1	0.1	0-2
M10	5.6	3-14
D30	33.8	15-44
CM	11.3	8-15
R1	99.3	51-128
R1+7	122.3	104-141
R2	205.9	149-223
R2+7	216.7	188-232

Bacterial culture isolated *S. uberis* as the predominant pathogen in 143 (46%) samples for comparison. No pathogen was isolated from 133 (42%) samples. *S. aureus* (n=28), CNS (n=7) and other (n=4) were the predominant pathogen cultured from the remaining samples (Table 13).

Table 13. Number of samples that had no growth in culture (OO) or where *S. uberis* (SU), *S. aureus* (SA), coagulase-negative Staphylococci (CNS) or other pathogens were identified in culture as the predominant pathogen, excluding post-treatment samples, at each sampling point.

	OO	SU	SA	CNS	Other*	Total
M1	7	72	4	2	2	87
M10	33	34	4	2	0	73
D30	69	7	5	1	0	82
CM	0	2	0	0	0	2
R1	13	5	3	1	0	22
R1+7	1	5	2	0	0	8
R2	9	10	5	1	1	26
R2+7	1	8	5	0	1	15
Total	**133**	**143**	**28**	**7**	**4**	**315**

*Other includes *E. coli*, streptococci (other than *S. agalactiae*, *S. dysgalactiae* and *S. uberis*), yeast, and enterococci.

6.2 Strep API testing

Strep API testing was completed on 14 samples that were culture-positive and PCR-negative for *S. uberis*. In 12 samples, *S. uberis* was confirmed as the pathogen of interest, agreeing with the identification in culture. In two samples, Strep API testing identified the pathogen as *Enterococcus* spp., indicating misidentification of *S. uberis* in culture. A follow-up observation in culture confirmed *Enterococcus* spp. as the pathogen of interest. Therefore, the bacterial culture results were corrected for in the analysis to reflect better the sensitivity of the PCR test.

6.3 Sensitivity and specificity estimates of the PCR test

6.3.1 Across lactation and within the different stages of lactation

Any sample that returned a positive diagnosis for a pathogen in at least one of the three bacteriological results was considered to be infected. Using the combined bacteriological results for the 315 samples collected across the season, both culture and PCR identified *S. uberis* in 125 samples (Table 14). PCR detected *S. uberis* in just two more samples than culture (146 vs. 144); however there were 40 conflicting results between culture and PCR (12.7%); 19 samples were PCR false negative i.e. positive in culture but negative using PCR, and 21 samples were PCR false positive i.e. negative in culture but positive using PCR. Of these 40 conflicting results, 36 were early

lactation samples (M1, M10, D30; Table 15). Across all samples, the PCR test had a relative sensitivity of 86.8% and relative specificity of 87.7% for detecting *S. uberis* using the parallel interpretation of bacteriology, and relative sensitivity and specificity of 91% and 82.3%, respectively, using the series interpretation for culture (Table 14). Using the parallel interpretation of bacteriology, the PPV was 85.6% and the NPV was 88.8%. The agreement between methods for *S. uberis* detection was κ = 0.744 (Table 16).

Table 14. Number of samples positive and negative for *S. uberis* in culture (interpreted in parallel and in series) and PCR (excluding post-treatment samples) and relative sensitivity and specificity of the PCR test.

		Culture (parallel)		Culture (series)		
		Pos	**Neg**	**Pos**	**Neg**	**Total**
PCR	**Pos**	125	21	112	34	146
	Neg	19	150	11	158	169
	Total	144	171	123	192	**315**
Relative sensitivity		86.8%		91.1%		
Relative specificity			87.7%		82.3%	

Table 15. Cross-classification of bacterial culture (BC) and PCR results for the detection of *S. uberis* and *S. aureus* at each sampling point, excluding post-treatment and clinical mastitis samples (M1=first milking, M10=tenth milking, D30=30 days in milk, R1+R2=mid- and late-lactation combined).

Sample stage	N	BC pos / PCR pos	BC pos / PCR neg	BC neg / PCR pos	BC neg / PCR neg
S. uberis					
M1	87	65	8	5	9
M10	73	29	5	11	28
D30	82	4	3	4	71
R1 + R2	71	25	3	1	42
S. aureus					
M1	87	3	1	0	83
M10	73	4	0	0	69
D30	82	5	0	1	76
R1 + R2	71	15	0	0	56

To assess the PCR test performance at different stages of lactation, relative sensitivity and specificity were estimated for the different sampling points included in the study (Table 16). Sensitivity was similar for all sampling points (85-89%), with the exception of D30 (57%). However, there were insufficient numbers of infected samples (7/82) at this sampling point to accurately determine sensitivity. There were large differences in the specificity of the PCR test at the different stages of lactation, ranging from 64% for M1 (colostrum) to 98% for mid-late lactation (R1-R2).

Table 16. Relative sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV) of the PCR test with 95% confidence intervals in parentheses, and associated kappa statistics for *S. uberis* and *S. aureus* detection at each sampling point (M1=first milking, M10=tenth milking, D30=30 days in milk, R1+R2=mid- and late-lactation combined).

	Se (%)	Sp (%)	PPV (%)	NPV (%)	Kappa
S. uberis					
All samples	86.8 (80.2, 91.9)	87.7 (81.8, 92.2)	85.6 (78.9, 90.9)	88.8 (83.0, 93.1)	0.744
M1	89.0 (79.5, 95.1)	64.3 (35.1, 87.2)	92.9 (84.1, 97.6)	52.9 (27.8, 77.0)	0.491
M10	85.3 (68.9, 95.0)	71.8 (55.1, 85.0)	72.5 (56.1, 85.4)	84.8 (68.1, 94.9)	0.565
D30	57.1 (18.4, 90.1)	94.7 (86.9, 98.5)	50.0 (15.7, 84.3)	95.9 (88.6, 99.2)	0.487
R1 + R2	89.3 (71.8, 97.7)	97.7 (87.7, 99.9)	96.2 (80.4, 99.9)	93.3 (81.7, 98.6)	0.881
S. aureus					
All samples	96.4 (81.7, 99.9)	99.7 (98.1, 99.9)	96.4 (81.7, 99.9)	99.7 (98.1, 99.9)	0.961
M1	75 (19.4, 99.4)	100 (96.5, -)	100 (36.8, -)	98.8 (93.5, 99.9)	0.851
M10	100 (47.3, -)	100 (95.8, -)	100 (47.3, -)	100 (95.8, -)	1
D30	100 (54.9, -)	98.7 (93.0, 99.9)	83.3 (35.9, 99.6)	100 (96.1, -)	0.903
R1 + R2	100 (81.9, -)	100 (94.8, -)	100 (81.9, -)	100 (94.8, -)	1

S. aureus was identified by both culture and PCR in 27 samples, and 286 samples were concordantly identified as *S. aureus*-negative (Table 17). Across all 315 samples,

the relative sensitivity for *S. aureus* detection was 96.4% and specificity was 99.7% (Table 16). PPV and NPV were 96.4% and 99.7%, respectively. The agreement between methods for *S. aureus* detection was κ = 0.961. While the proportion of samples identified as infected with *S. aureus* in the samples used for the comparison was low (9%), the majority of samples were correctly diagnosed as either positive or negative across the stages of lactation, with just one false negative at M1 and one false positive at D30 (Table 15). At the other sampling points, M10 and mid-late lactation, the relative sensitivity and specificity of detecting *S. aureus* were both 100% (Table 16).

Table 17. Number of samples positive and negative for *S. aureus* in culture and PCR, excluding post-treatment samples.

		Culture (n samples)		
		Pos	**Neg**	**Total**
PCR (n samples)	**Pos**	27	1	28
	Neg	1	286	287
	Total	28	287	**315**

6.3.2 Differences within bacterial culture results

Bacteriological results were obtained from culturing (0.01 mL of milk) within two days of sample collection, or after a period of frozen storage, prior to PCR testing. Plate colony counts were also completed for all samples at the time of frozen culture and PCR testing, using 0.1 mL of milk. The time lapse between sample collection and PCR testing for all samples was an average of 200 days (Table 18).

Table 18. Period of time (days; average and range) between sample collection and PCR testing for samples collected in early, mid- and late-lactation.

	Average (days)	**Range**
Early	206	113-256
Mid	257	127-441
Late	115	33-350
All samples	200	33-441

Absolute numbers of *S. uberis* positive (score 1-3) and negative (score 0) samples remained the same for culture of fresh and frozen samples (n=131 and 212, respectively; Table 19). However, the bacterial culture score in the fresh and frozen

culture was different for 49 samples (14.3%). There were 15 samples that had greater bacterial recovery after frozen storage (indicated by the right-hand side of the shaded diagonal line in Table 19). Seven of these samples were culture-negative as fresh samples, but returned a positive diagnosis for *S. uberis* from samples after frozen storage. Fewer bacteria were isolated in culture after frozen storage in 34 samples, reducing the plate score between the two cultures. Seven of these samples were no longer positive for *S. uberis* after frozen storage. In nine samples, the culture of fresh and frozen samples were both negative, but *S. uberis* was isolated using plate colony counts (range 10-80 CFU/mL). Two samples were positive in culture initially, but had no bacteria isolated in both culture and plate colony counting after the period of frozen storage.

The relative sensitivity and specificity estimates for the PCR test varied slightly when individual culture results were used as the reference test. Colony counting produced more samples that were *S. uberis* positive, compared with culture of fresh and frozen samples (141 vs. 130; Table 19), increasing the relative specificity by almost 4% to 87.4%. The relative sensitivity was similar across culture from fresh and frozen samples and colony counting, ranging from 87.9% to 89.2%.

Table 19. Cross-classification of samples with bacterial culture scores of 0, 1, 2 and 3 for *S. uberis* isolation in culture of fresh samples (rows) and frozen culture (columns), including post-treatment samples. Shaded cells represent those that had no change in score following the frozen storage. κ = 0.740.

		FROZEN				
		0	**1**	**2**	**3**	**Total**
FRESH	**0**	205	5	0	2	212
	1	4	12	5	1	22
	2	2	14	4	2	22
	3	1	5	8	73	87
	Total	212	36	17	78	343

In fresh sample culture, 30 samples were positive for *S. aureus* (score 1-3; Table 21). After frozen storage, 29 samples remained positive. All samples that were negative culture initially were also negative in culture after frozen storage. No samples had more bacteria recovered after frozen storage. The left side of the shaded cells represents the number of samples that had fewer bacteria recovered (n=5; Table 21). One of these samples had a score of 1 in culture of the fresh sample, but had no bacteria recovered

in culture after the period of frozen storage (score 0). This sample was a post-treatment sample, collected two days after antibiotic treatment commenced for that quarter. All samples that were *S. aureus* positive in culture initially were also positive in colony counting; the one culture-negative result for frozen sample had a bacterial concentration of 10 CFU/mL for plate colony counting. Thus, the sensitivity and specificity estimates for *S. aureus* detection in fresh sample, frozen sample and colony counts were the same (Table 20), as post-treatment samples were excluded from these calculations.

Table 20. Cross-classification of results of fresh culture, frozen culture and colony counts (PCC) in bacterial culture (BC) and PCR for the detection of *S. uberis* and *S. aureus* in 315 quarter milk samples, including relative sensitivity and specificity of the PCR test and associated kappa statistics.

	BC pos / PCR pos	BC pos / PCR neg	BC neg / PCR pos	BC neg / PCR neg	Se (%)	Sp (%)	Kappa
S. uberis							
FRESH	115	15	31	154	88.5	83.2	0.704
FROZEN	116	14	30	155	89.2	83.8	0.717
PCC	124	17	22	152	87.9	87.4	0.750
S. aureus							
FRESH	27	1	1	286	96.4	99.7	0.961
FROZEN	27	1	1	286	96.4	99.7	0.961
PCC	27	1	1	286	96.4	99.7	0.961

Table 21. Cross-classification of samples with bacterial culture scores of 0, 1, 2 and 3 for *S. aureus* isolation in fresh culture (rows) and frozen culture (columns), including post-treatment samples. Shaded cells represent those that had no change in score following the frozen storage. κ = 0.909.

		FROZEN				
		0	1	2	3	Total
FRESH	**0**	313	0	0	0	313
	1	1	3	0	0	4
	2	0	1	1	0	2
	3	0	2	1	21	24
	Total	314	6	2	21	343

6.4 Sensitivity and specificity using different cycle threshold cut-offs

The cut-off Ct value for determining positive and negative results as recommended by the manufacturer was 37 and was used in results presented so far. Sensitivity and specificity of a test can be adjusted by changing the threshold Ct value used to determine a sample as positive or negative. Due to limited numbers of samples containing *S. aureus*, only data for *S. uberis* were used for this analysis.

Applying a lower cut-off (e.g. Ct 32; Mahmmod et al., 2013b) for samples in this study maximised the specificity, but reduced the sensitivity for detecting *S. uberis* in milk (Figure 4). At higher cut-offs (e.g. Ct 37 or 39), the opposite was true. For M1 samples, specificity rapidly declined from 92% to 50% as the Ct cut-off increased from 32 to 39 (Table 22). A similar trend was seen for M10 samples, but over a smaller range (88% to 67%). Sensitivity was highest at a cut-off of 37 for M1 samples (89%), and 39 for M10 samples (91%). Increasing the Ct cut-off from 32 to 37 did not change the specificity of the PCR test for mid-late lactation samples; however the sensitivity was increased from 71% to 89%. There was no benefit in using a Ct cut-off of 39 for mid-late lactation samples. D30 samples were excluded from this analysis due to the limited number of samples infected with *S. uberis* at this sample point.

Table 22. Relative sensitivity (Se) and specificity (Sp) (95% confidence intervals in parentheses) of the PCR test for identifying *S. uberis* at different sampling points (M1=first milking, M10=tenth milking, R1+R2=mid- and late-lactation combined), using PCR cycle threshold (Ct) cut-offs of ≤32, ≤34, ≤37, and ≤39 (same cut-offs as used by Mahmmod et al., 2013b).

	M1		M10		R1 + R2	
Ct cut-off	**Se**	**Sp**	**Se**	**Sp**	**Se**	**Sp**
Ct ≤ 32	61.6 (49.5, 72.8)	92.9 (66.1, 99.8)	67.6 (49.5, 82.6)	89.7 (75.8, 97.1)	71.4 (51.3, 86.8)	97.7 (87.7, 99.9)
Ct ≤ 34	72.6 (60.9, 82.4)	85.7 (57.2, 98.2)	73.5 (55.6, 87.1)	87.2 (72.6, 95.7)	82.1 (63.1, 93.9)	97.7 (87.7, 99.9)
Ct ≤ 37	89.0 (79.5, 95.1)	64.3 (35.1, 87.2)	85.3 (68.9, 95.0)	71.8 (55.1, 85.0)	89.3 (71.8, 97.7)	97.7 (87.7, 99.9)
Ct ≤ 39	89.0 (79.5, 95.1)	50.0 (23.0, 77.0)	91.2 (76.3, 98.1)	66.7 (49.8, 80.9)	89.3 (71.8, 97.7)	95.3 (84.2, 99.4)

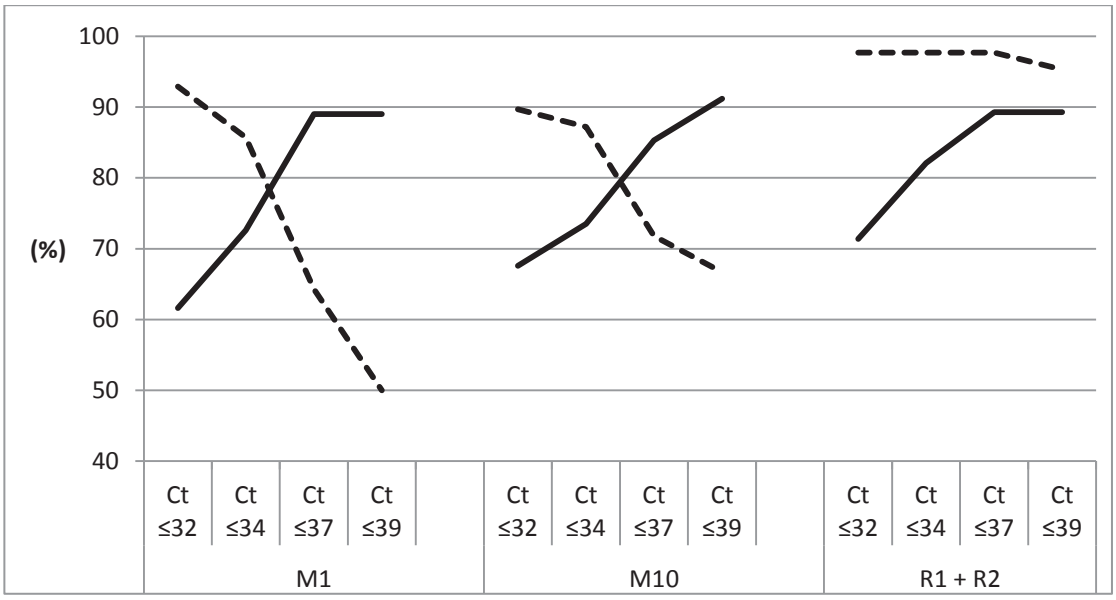

Figure 4. Relative sensitivity (–) and specificity (--) of the PCR test using different PCR cycle threshold (Ct) cut-offs of ≤32, ≤34, ≤37, and ≤39 for identifying *S. uberis* in quarter milk samples collected at different sampling points (M1=first milking, M10=tenth milking, R1+R2=mid- and late-lactation combined).

6.5 Intra-assay repeatability

Coefficients of variation (CV) of up to 3% were considered acceptable for duplicates (Bustin, 2004). Replicates (M1 and CM) were not included, as the difference between Rep1 and Rep2 was expected to be 3.3. For duplicates that had two valid PCR results, the CV and repeatability measures were lower for samples with Ct values <30 compared with those with Ct values ≥30, an effect seen for both *S. uberis* and *S. aureus* identification by PCR (Table 23). CV were within the acceptable range.

Table 23. Number, mean, standard deviation (SD), coefficient of variation (CV) and repeatability of PCR Ct values for sample duplicates with Ct values <30 or ≥30 for the detection of *S. uberis* and *S. aureus* in quarter milk samples.

	N	Mean	SD	CV	Repeatability
S. uberis					
Ct <30	41	27.4	0.36	1.3%	0.99
Ct ≥30	46	33.7	0.67	2.0%	1.87
S. aureus					
Ct <30	13	28.4	0.28	1.0%	0.80
Ct ≥30	14	32.4	0.70	2.2%	1.97

6.6 Association between PCR Ct values and plate colony count

There was a negative association between PCR Ct values and log CFU/mL for the 125 samples in which *S. uberis* was identified using both tests (Figure 5). However the regression model only explains 45% of the variation in Ct values. For any given concentration of bacteria in culture (log CFU/mL), the predicted Ct value has a very large range. For example, a sample with a *S. uberis* concentration of 5 log CFU/mL (i.e. 100,000 CFU/mL), the predicted Ct value would be in the range of 21.2 and 33.2, 95% of the time.

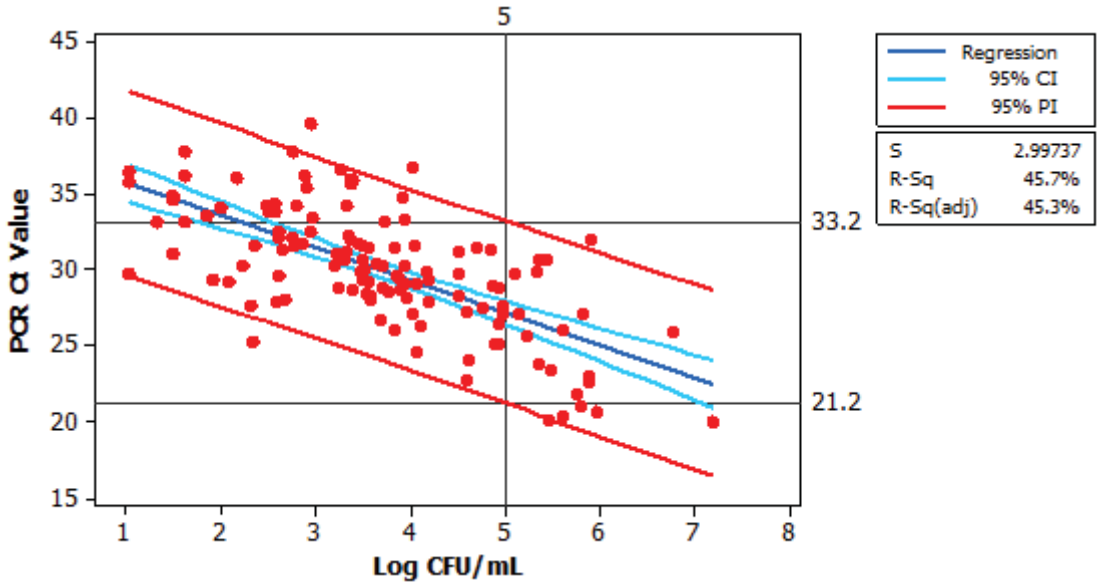

Figure 5. Regression analysis between PCR Ct values and log CFU/mL for *S. uberis* detection in quarter milk samples with 95% confidence intervals and 95% prediction intervals. The model shows a log CFU/mL value of 5 predicts a Ct value within the range of 21.2 and 33.2. R^2 = 45.7% (P<0.001).

6.7 Rapidity of PCR test

In this study, the total time taken to obtain a result from PCR ranged from 4 hours 30 minutes to 5 hours 50 minutes (Table 24). Sample preparation for DNA extraction was the most time consuming component, taking up to 3 hours when more than 60 samples were included in the DNA extraction. DNA extraction and PCR times were constant regardless of the number of samples being processed.

The minimum time taken for culture results to be confirmed was 48 hours, ranging up to 72 hours if further confirmatory tests were necessary.

Table 24. Approximate time taken for each step for the preparation of DNA extraction and real-time PCR (rt-PCR) when the numbers of samples processed simultaneously were 0-30, 30-60 or 60-96 samples.

	N Samples		
Time taken for:	**0-30**	**30-60**	**60-96**
Preparation for DNA extraction	120 min	150 min	180 min
DNA extraction		45 min	
Preparation for rt-PCR	30 min	40 min	50 min
rt-PCR		75 min	
Total	**270 min**	**310 min**	**350 min**

6.8 Results summary

- The relative sensitivity and specificity for detecting *S. uberis* across lactation was 86.8% and 87.7% respectively. In early lactation, specificity was lower (64%) compared with later in lactation (98%).
- The relative sensitivity and specificity for detecting *S. aureus* across lactation was 96.4% and 99.7% respectively, with too few samples to reflect differences in stage of lactation.
- There was greater variation in duplicates when Ct >30. Using different Ct cut-offs for determining a sample as positive can alter the performance of the PCR test.
- There was a weak association between PCR Ct values and plate colony count. Predictions of *S. uberis* concentration in milk are highly variable using the regression analysis.
- The time taken to obtain a result using PCR ranged from 4 hours 30 minutes to just under 6 hours whereas culture results took a minimum of 48 hours.

7 DISCUSSION

This is the first study assessing the performance of the PathoProof Mastitis PCR assay on quarter milk samples in New Zealand. To be a suitable alternative to culture for detecting major mastitis pathogens in New Zealand, PCR would need to be comparable to bacterial culture in terms of relative sensitivity and specificity. However, this study has shown that although the results of the PCR for *S. aureus* were sufficiently in agreement with those of bacteriology for it to be used as an alternative to culture, for *S. uberis*, the most important cause of mastitis under New Zealand conditions, the relative sensitivity and specificity were not high enough to recommend the use of PCR as an alternative to bacterial culture.

This conclusion is based on the comparison between PCR and culture; it assumes that bacterial culture is a 'gold' standard test. However, for identifying IMI there is no perfect test, thus the present study has estimated the sensitivity and specificity of PCR relative to culture, rather than determining the true sensitivity and specificity of the PCR test. Using an imperfect reference test, such as bacterial culture can bias the estimates of sensitivity and specificity; for example, if PCR truly identified bacteria-positive samples better than culture, but was as good as identifying truly negative samples, then its relative specificity and sensitivity compared to culture would be an underestimate of its true sensitivity and specificity. The direct comparison of PCR and bacterial culture is further complicated by the differences between the two tests. The primary function of culture is to detect the presence of viable organisms in the milk, whereas PCR detects specific DNA sequences which may be from bacteria that are not viable (Koskinen et al., 2009). Thus, these two methods are essentially answering different questions.

In this study, despite its limitations, bacterial culture was used as the gold standard as it is the current industry standard for which PCR is being proposed as an alternative. To overcome these limitations, this study used a combination of bacteriological results for each sample; i.e. fresh culture within 2 days of sample collection and repeat culture after frozen storage, both using an inoculum volume of 0.01 mL. Additionally, plate colony counting using 0.1 mL of milk was also undertaken to improve the sensitivity of pathogen detection. Interpretation of these three bacteriological results in parallel, with at least one result being positive for the pathogen of interest, maximised the number of *S. uberis* infections detected in culture (n=146). This maximised the sensitivity of culture, and thereby reduced the chance that truly infected samples that were detected by the PCR test would be recorded as a false positive because of incorrectly recording the sample as 'not infected'. Interpretation of bacteriology in series, with all three

bacteriological results being positive, reduced the number of *S. uberis* infections detected in culture (n=123); thereby increasing the chance of false positive results according to the PCR test, relative to culture. Strep API testing on PCR false negatives for *S. uberis* provided greater confidence in culture-positive results when supported by the Strep API result.

The threshold for defining a sample as culture-positive for either *S. uberis* or *S. aureus* was 100 CFU/mL (1 CFU/0.01 mL plated) in this study. For *S. uberis* in particular, this was lower than that used in other studies (1000 CFU/mL; Zadoks et al., 2001; Torres et al., 2009), and recommended in the NMC guidelines (300 CFU/mL; Oliver et al., 2004). Repeated culture from multiple milk samples was suggested as an enhanced gold standard to establish an improved definition of IMI (Postle, 1976; Dohoo et al., 2011b). This study involved collection of consecutive samples; three samples over four weeks in early lactation and two samples each in mid and late lactation. Due to the dynamic nature of infections, particularly in early lactation, consecutive samples were not analysed in series. Instead, aligning consecutive samples from individual quarters assisted interpretation of conflicting PCR and culture results, to provide some indication of infection status at the time of sampling, compared with previous or subsequent samples. It was common for a quarter to have a current IMI at the first milking, which was not detectable by the tenth milking. The purpose of the gold standard in this study was to provide the best possible indication of presence or absence of a particular pathogen at the time of sampling. Since PCR is reported to have a high analytical sensitivity (Koskinen et al., 2009), the definition of infection in culture should use the lowest possible threshold for defining a sample as positive to detect as many infections as possible. Use of a higher threshold may have biased the sensitivity and specificity estimates of the PCR test by missing samples that were truly positive, but below the threshold limit in culture.

An alternative method for evaluating diagnostic tests when there is no perfect gold standard is Bayesian latent class modelling, which accepts that the accuracy of both the reference test and test of interest are unknown (Dohoo et al., 2009). This approach is based on several important assumptions: 1) that the two tests being compared are biologically independent; 2) that data used to evaluate tests are available from multiple populations with different prevalence; and 3) that the sensitivity and specificity of the tests are constant across those populations (Enøe et al., 2000). The Bayesian approach requires the specification of prior distributions in which results can depend on the quality of prior information used in the model (Paradis et al., 2012). While some studies have used this approach for comparing bacterial culture and PCR as mastitis

diagnostic tests (Paradis et al., 2012; Cederlof et al., 2012; Mahmmod et al., 2013b, c), latent class modelling was not used in this study. The combined definition for bacteriology was considered to be an acceptable gold standard, particularly in regard to its specificity (Dohoo et al., 2011b; Paradis et al., 2012). Analysis using this modelling could provide the next step for this research to compare and contrast estimates of sensitivity and specificity using the different analytical methods.

7.1 Sensitivity, specificity and repeatability of the PCR test for detecting *S. uberis*

7.1.1 False negatives

In this study, PCR did not detect *S. uberis* in 19 samples that were positive in culture (PCR false negatives), leading to a sensitivity of 87% across all stages of lactation. Possible reasons for false negative results can include:

- Inefficient DNA extraction affecting the recovery and detection of bacterial DNA from the sample;
- Bacterial concentration below the limit of detection of the PCR assay in milk;
- PCR primers not detecting *S. uberis* due to strain variation;
- Incorrect diagnosis or recording in culture.

Six of the 19 PCR false negative samples contained *S. uberis* at concentrations between 10 and 100 CFU/mL in colony counting and a further 11 had >100 CFU/mL, but were not detected by PCR. Two false negative samples had 0 CFU/mL but were positive in culture for either the fresh or frozen sample. Of the 19 PCR false negatives, 16 were from early lactation (eight M1's, five M10's and three D30's); however this simply reflects the fact that the majority of samples were collected in early lactation and the sensitivity of the PCR test for detecting *S. uberis* was similar throughout lactation (85-89%) except for D30 (57.1%). However, the low number of infected samples on D30 (7/82) meant that the 95% confidence interval on that sampling point were very wide (18.4%-90.1%).

Twelve of the false negative PCR samples were confirmed as *S. uberis* by Strep API testing; four could not be tested as they did not grow in culture at the time of Strep API testing, which occurred approximately 3 months after PCR testing. The remaining three samples were not tested due to samples having at least one duplicate with a PCR Ct value between 37 and 40 for *S. uberis*, which were later considered as PCR-negative because a three cycle difference is the accepted standard to indicate reliable

separation of a true positive result (<37) from potential contaminants or background noise (Bustin, 2004).

A bacterial concentration of 100 CFU/mL is considered to be the limit of detection in culture when 0.01 mL of milk is used as the inoculum volume (Hogan et al., 1999). Using a larger inoculum volume of 0.1 mL of milk in colony counting, the limit of detection was reduced to 10 CFU/mL. Since PCR is supposedly a highly sensitive technique, it would be expected to detect bacteria at a lower concentration than culture, as just a single piece of DNA is required for amplification. The PathoProof PCR assay has been reported to have an analytical sensitivity of 100% and detect as few as 16.7 gene copies per PCR reaction (Koskinen et al. 2008). According to Taponen et al. (2009), one bacterial cell can correspond to 1 CFU; however it is unlikely that all bacterial cells in a sample would grow in culture. Additionally, staphylococci and streptococci exist as aggregates or chains, which group together to form one colony (Taponen et al., 2009). Considering this, the number of bacterial cells in a sample is likely to be much higher than the number of CFU recorded in culture.

Koskinen et al. (2008) reported that the detection limit of the PCR assay in milk ranged from 200 to 810 CFU/mL (individual results were not specified for each bacterial target). In the validation study reported in Chapter 3, the limit of detection of the PathoProof mastitis PCR assay for detecting *S. uberis* was estimated to be 1,000 CFU/mL of milk; however this was based on just two replicates using milk from late-lactation cows without any trace of bacteria in both culture and PCR. In the experimental study, PCR detected *S. uberis* in samples where the bacterial concentration was as low as 10 CFU/mL, but this was very inconsistent as the bacterial concentration in the false negative samples ranged from 0 to 3,600 CFU/mL. Analytical sensitivity should not be considered to equal diagnostic sensitivity under field conditions (Koskinen et al., 2010), a concept supported by the inconsistent detection of *S. uberis* in this study. The ability of PCR to detect bacterial DNA in milk depends not only on the concentration of bacteria in the sample, but also sample factors which can affect the efficiency of the DNA extraction.

While the system used for DNA extraction in this study (Kingfisher 96, Thermo Electron, Vantaa, Finland) specified a 95% collection efficiency of DNA, suboptimal DNA extraction could have limited the recovery of bacterial DNA or limited the availability of target DNA sequences for amplification by PCR. Milk is a difficult matrix, containing many substances that can affect the PCR reaction. Components such as calcium ions, proteinases, fats and milk proteins can inhibit PCR by blocking DNA

polymerase activity (Wilson, 1997). Bacterial cell walls of Gram-positive bacteria such as *S. uberis* are robust and require enzymes and centrifugation to lyse cells and expose DNA, without causing any damage to the targeted DNA sequences (Cremonesi et al., 2006). Detection of *S. uberis* may also be made difficult as *S. uberis* has the ability to survive inside mammary epithelial cells, as a possible mechanism to evade the host immune response (Tamilselvam et al., 2006). The DNA extraction protocol should remove any impurities and potential PCR inhibitors from the sample, and lyse bacterial cells and any cells containing bacteria, for the efficient amplification of targeted DNA by PCR and accurate quantification of bacteria. The PathoProof assay has been optimised for detecting bacteria from raw milk from cows in Finland and The Netherlands (Koskinen et al., 2009; 2010). Differences in milk composition between geographical regions and stage of lactation should also be considered as a factor that can potentially affect the detection of bacterial DNA in milk. New Zealand cows produce milk with a much higher average fat content compared with cows in Europe and the USA (~5% vs. ~4%, respectively). It is possible that the current protocol for extracting DNA may not be suited to higher fat New Zealand milk samples, leading to some cases where *S. uberis* is present in the sample but not detected.

Colostrum, produced during the first milkings following parturition, is a highly viscous matrix containing a large proportion of antibodies, somatic cells and tissue debris (Pakkanen and Aalto, 1997). When present in significant amounts, removal of these substances during DNA extraction may be more difficult. *S. uberis* was not detected in eight M1 samples by PCR. A further 10 samples would have been falsely negative without the dilution of colostrum samples and some M10 repeats; an issue identified by the validation experiments in this study. This would have reduced sensitivity from 87% to 80% overall. Diluting the extracted DNA can be sufficient to reduce the effects of the PCR inhibitors (Gillespie and Oliver, 2005; Graber et al., 2007). By the tenth milking, colostrum is no longer produced (Solomons, 2002), so problems with 'colostrum' does not explain five false negative PCR results that occurred at M10. No other studies have considered the effects of seasonal milk composition on the PCR assay's performance for detecting specific pathogens. This is an important concept in New Zealand due to the compact calving period on seasonally calving dairy farms, and the subsequent high risk of mastitis during early lactation, especially in cows producing colostrum. Any PCR assay used on New Zealand farms needs to be able to accurately identify bacteria at all stages of lactation, particularly in early lactation.

Genetic diversity and mutation lead to the development of new strains within a species. *S. uberis* is a highly recombinant organism, with 15 to 18% of the genome differing

between strains (Lang et al., 2009). The analytical sensitivity of the PathoProof assay was reported as 100%, based on a collection of 643 bacterial isolates from 83 different species or subspecies of bovine, human or companion animal origin (Koskinen et al., 2009). While a broad geographical area was included as sources of isolates (Canada, Finland, Norway, Italy, Portugal, United Kingdom), there were no strains of *S. uberis* from Australasia and the USA included in the development of the assay. Although strain typing was not completed in this study, a possible explanation for some of the false negatives using PCR could be that the bacterial strain in these samples may have differed from the strains targeted by the PCR kit used. In the current study, two samples collected in early lactation from the same quarter five days apart were culture-positive for *S. uberis* (60 and 270 CFU/mL) but were consistently undetected by PCR. Similarly, two consecutive samples collected in late lactation (1,800 and 3,600 CFU *S. uberis*/mL) were not detected. Six other samples from different quarters had *S. uberis* isolated in culture in just one sample of the set of consecutive samples. The primers used in the PathoProof kit to target specific bacteria are not disclosed so it is difficult to know if strain differences driven by deviations in the nucleotide bases that code for the primer target regions are the reasons behind these false negatives. *S. uberis* isolates in New Zealand contained some differences in global clonal complexes compared with those from the UK, but some were the same (Pullinger et al., 2006). The exclusion of Australasian isolates in the development of this assay could limit the range of strains that were identified by this PCR test.

Misdiagnosis in culture is another potential reason for false negative results using PCR. False identification of mastitis-causing pathogens is possible in laboratories due to variation in tests used and the importance of subjective observation in the identification of bacteria in culture. This was illustrated by a proficiency study across 40 Finnish laboratories, where incidence of correct bacterial identification ranged from just 63% up to 91% across laboratories (Pitkälä et al., 2005). In New Zealand, the overall consistency between laboratories was considered to be high, as procedures were based on the NMC guidelines (Hawkins and Cooper, 2014). However, it was identified that a consistent approach for the routine diagnosis and speciation of streptococci and enterococci was required, as current NMC guidelines are less comprehensive for identifying *S. uberis* (Hawkins and Cooper, 2014). Incorrect diagnosis of *S. uberis* as enterococci was estimated to occur in 5-15% of samples (Salmon et al., 1998), with a suggested figure of 7% currently in New Zealand laboratories (Hawkins and Cooper, 2014). In the current study, two samples were initially diagnosed as *S. uberis* in culture, but later confirmed by Strep API testing as *Enterococcus* spp. For the calculation of

sensitivity of PCR, culture results were corrected so that these were not *S. uberis* positive, providing the best possible gold standard to compare against. Correct identification of *S. uberis* and enterococci are important in terms of antimicrobial therapy, as susceptibility profiles differ between the two, which may have implications for the prudent use of antibiotics when incorrectly diagnosed (Hawkins and Cooper, 2014). PCR may be able to remove the variation and subjective observation of streptococci seen in culture, and possibly allow standardisation between laboratories if the same commercial PCR assay is used (Pitkälä et al., 2005).

7.1.2 False positives

False positive results reduce the relative specificity of the PCR test. In this study, PCR detected *S. uberis* in 21 samples that were negative in culture (PCR false positives), leading to a specificity of 88% across all stages of lactation. Possible reasons for false positive results can include:

- PCR was detecting bacteria at lower levels than culture (culture was false negative);
- PCR was detecting DNA from non-viable bacteria;
- Cross-contamination between samples;
- Non-target DNA binding with primers resulting in cross-reaction.

A proportion of the PCR false positives may in fact be truly positive if PCR is better able to detect pathogens at lower concentrations than bacterial culture. Thus if these samples are considered as false positive based on culture, the performance of the PCR test will be penalised (Keane et al., 2013). This study used enhanced culture procedures including larger inoculum volume and repeat sampling to increase the chance of detecting the pathogen in culture.

Approximately 30% of clinical mastitis samples sent to laboratories were estimated to be culture-negative (Bradley et al., 2007; Olde Riekerink et al., 2008). A recent New Zealand study showed 20% of samples collected from cows with clinical mastitis had no pathogen isolated (Petrovski et al., 2011b). Bacteria may not grow in culture for a number of reasons. The concentration of bacteria may be at a lower level than the detection limit in culture (Sears et al., 1990). This is possible as PCR requires just one strand of the target DNA sequence, and the volume of milk used for the DNA extraction was four times that used for colony counting, and 40 times that of standard culture. Some bacteria may be growth-inhibited but culture conditions were suitable for growth of *S. uberis* (Hogan et al., 1999). However, four of the false positive samples displayed

relatively heavy growth of other pathogens in culture (*S. aureus* (n=2), 1 each of *E. coli* and yeast; ranging from 13,000 to 89,000 CFU/mL), potentially masking the growth of *S. uberis* if it was present (Cressier and Bissonnette, 2011). *S. uberis* may have been detected in culture if selective media were used; however this would not occur under routine circumstances if a pathogen had already been cultured from the sample. The PCR test is better able to simultaneously detect multiple species in a sample through the action of primers targeting pathogen-specific DNA sequences. However, low concentrations of a secondary species may limit detection if the primary pathogen is present in high concentrations due to the competition for PCR reagents in a multiplex reaction (Phuektes et al., 2001). Most mastitis infections are caused by an individual pathogen (Watts and Yancey, 1994) so the detection of an environmental pathogen such as *S. uberis* as a secondary pathogen may or may not be relevant, depending on the primary pathogen.

Another reason for a culture-negative result is that bacteria may be dead and hence not capable of growth in culture. Bacteria may have been alive at the time of collection and died during storage, or alternatively, dead bacteria may remain in the mammary gland following a previous infection. Milk contains antibacterial substances such as lactoferrin, lysozyme, lactoperoxidase, complement and immunoglobulins (Rainard and Riollet, 2006), which can contribute to the death of bacteria, along with immune components. Unfavourable sample storage and handling conditions can also lead to death or impaired growth of bacteria after sampling (Dinsmore et al., 1992) but detection may be possible using PCR provided that the target sequence of DNA remains intact (Koskinen et al., 2010). DNA is stable and therefore may exist for a period of time following death of an organism (Keer and Birch, 2003). DNA from live or dead bacteria is not differentiated in the PCR assays that are commercially available at present. Thirteen of the 21 PCR false positives in this study were from samples where the sample preceding the culture-negative, PCR-positive sample had been culture-positive for *S. uberis*. This could indicate that either bacteria were there but dead, or that bacterial concentration was too low to be detected in culture. Additionally, there were three samples that were PCR-positive and culture-negative and then positive for both methods at the subsequent sample. Possibly, PCR may have been detecting the infection earlier than culture, which may provide some advantage in terms of treatment and prognosis, depending on the pathogen. Nevertheless, microbiome studies (Oikonomou et al., 2012; Kuehn et al., 2013) have illustrated the potential for culture-independent methods to provide false positive results with almost all samples tested, even when the SCC was <10,000 cells/mL, having detectable staphylococci and

streptococci DNA (Oikonomou et al., 2012). The detection of a single DNA copy by PCR may, therefore, not provide sufficient evidence to confirm a current infection (Hadgu et al., 2005), and may not constitute an indication for treatment (Hadgu, 1999). Future improvements of PCR technology applied in mastitis diagnostics may enable the differentiation of DNA from live and dead micro-organisms providing for greater benefits of using PCR without confusing the interpretation of results.

Bacterial growth can also be inhibited in culture through the presence of antibiotics or residues from teat disinfectants in the milk. In this study, post treatment samples (within 21 days of antibiotic treatment) were removed from analysis (n=28). Had these not been excluded, apparently false positive PCR results would have occurred in 15 samples. A further 28 samples were also collected post-treatment, but exceeded the 21 day criterion (range 22-33 days post-treatment). From these samples, only one sample was recorded as a false positive. This sample was collected 26 days after treatment was initiated and remained in the analysis for calculating specificity.

Of the 21 PCR false positive samples, 16 had Ct values between 31.1 and 37, which suggests a low concentration of bacteria in the sample. A high Ct may be generated through cross-contamination, by nonspecific amplification of background DNA or through the detection of non-viable bacteria (Burns and Valdivia, 2008). Cross-contamination was minimised as the collection tubes for preparing samples for DNA extraction were changed in the validation study. Samples were also prepared for DNA extraction and PCR on separate benches, and PCR was completed in a separate laboratory. Amplification with non-target DNA can occur when the specific primer that binds with the region of target DNA unique to a species, also matches with DNA from other, often similar, species. While the analytical specificity of the PathoProof assay was 100% considering bovine isolates of *S. uberis*, there were six isolates of human origin (*Streptococcus pyogenes* (n=4), and one each of *Streptococcus sanguis* and *Streptococcus salivarius*) that reacted with the PathoProof primers for *S. uberis* (Koskinen et al., 2009). It cannot be concluded if there was any non-target binding in this study as details of the targeted genes are commercially confidential; however, one *S. uberis* positive sample according to PCR was diagnosed in culture as 'other streptococci' but unfortunately was not tested by API Strep as part of this study.

The variation between duplicates (CV) was larger for samples with Ct values ≥30 compared with samples with Ct values <30, indicating more robust results when Ct values are lower (i.e. a higher bacterial load in the sample). A Ct value of ≥31.1 for *S. uberis* corresponds to a semi-quantitative result of '+' in the Norden Lab Mastitis

software. This software was not used in the current study due to machine compatibility issues and considerations of cost and future use. Instead, thresholds for determining samples as positive or negative were manually adjusted using 10% of the plateau of the IAC curve, as recommended by the manufacturer. The manual adjustment was considered to be adequate; however there was very little difference between positive and negative samples (e.g. samples with a Ct value of 36.9 and 37.1, respectively). Duplicate PCR results assisted in interpretation of whether results were truly positive or falsely positive using PCR; however for one sample, one duplicate was positive (<37) and the other was negative (≥37). In this study, these were reported as positive to provide a more accurate reflection of how the PCR test may perform in practice, where in the interest of time and costs, it is extremely unlikely that the test would be run in duplicate for each sample.

7.1.3 Sensitivity and specificity estimates

Across lactation, the sensitivity and specificity of *S. uberis* detection were 86.8% and 87.7%, respectively. Compared with these results, the sensitivity for detecting *S. uberis* was lower (68%) and specificity higher (90%) in a study across Finland and the Netherlands where the majority of samples (94%) were from cows with clinical mastitis (n=826; Koskinen et al., 2010). Lower sensitivity may reflect inefficient extraction of DNA from some clinical mastitis samples, as was seen in the present study's validation experiment where dilution of some samples was necessary. In addition, the method of DNA extraction in that study used spin-columns (Koskinen et al., 2010), which was reported to have poorer performance with clotted milk samples (Cressier and Bissonnette, 2011). Specificity for *S. uberis* detection was 87% and 97% in two studies where culture-negative results were obtained from clinical mastitis samples (Taponen et al., 2009; Bexiga et al., 2011). Using an 'in-house' PCR assay, Gillespie and Oliver (2005) reported 95.5% sensitivity and 100% specificity when detecting *S. uberis* from milk after enrichment. While sensitivity was similar throughout lactation in the present study (excluding D30 samples), the specificity of detecting *S. uberis* was lower for M1 (64%) and M10 (72%) than for later in lactation (98%) and the agreement between methods as described by the kappa statistic was only moderate in early lactation samples (0.49-0.57) compared to the very good agreement later in lactation (0.88; Altman , 1991). Relative sensitivity and specificity estimates for early lactation samples were similar (88% and 57%) in the only other study comparing culture and PCR of quarter samples collected at a specific stage of lactation (Azizoglu et al., 2011); however no pathogen-specific measures were provided.

The calculated relative sensitivity for detecting *S. uberis* in this study was considered to be similar to actual diagnostic sensitivity, as false positive results in culture (combined with Strep API results) were unlikely. The diagnostic specificity of culture has been estimated to be >99% (Paradis et al., 2012), providing some evidence that false positives in culture are not common. Conversely, it is likely that some culture results were incorrectly diagnosed as negative for *S. uberis*, particularly when bacterial concentrations in milk were low. An estimate of diagnostic sensitivity for detecting *S. uberis* in culture using latent class analysis was 73% (Paradis et al., 2012). Considering this, the relative specificity calculated in this study may be underestimating the performance of the PCR test. It cannot be ascertained what proportion of false positive results according to culture are truly positive for *S. uberis* due to the detection of bacteria at lower concentrations (and possibly an IMI) than that achieved in culture, and what proportion of the false positives are representative of dead bacteria (not a current IMI).

In practice, the tolerable lower limits for sensitivity and specificity of a diagnostic test depend on the context for completing such tests (Penry et al., 2014). The sensitivity must be >99% when the prevalence of the particular pathogen is high and a false negative result will lead to an expensive error. A lower sensitivity may be acceptable when the prevalence of infection is low, but for *S. uberis* this is uncommon. Diagnostic specificity must be >99% when false positive test results are expensive (Penry et al., 2014). Even when the estimated specificity is high (i.e. 99%), a positive result should not be considered to be full proof that the organism was present (Penry et al., 2014). When the prevalence of infection is low (e.g. 1%), and the diagnostic specificity of the PCR test is high (e.g. 99%), the proportion of positive PCR results that are false positive can still be as high as 50% in the example (Penry et al., 2014). If the level of performance of a test is not acceptable, the test should not be used, or should be combined with other tests to increase the confidence in the result (Penry et al., 2014). Such strategies may include repeat testing of samples and/or using both culture and PCR in combination; however, these strategies are never going to be cost or time effective in routine pathogen identification.

Sensitivity and specificity estimates can be altered by changing the cut-off Ct value for defining a sample as positive using PCR. As expected, a lower Ct cut-off (e.g. 32) increased specificity of *S. uberis* detection but reduced sensitivity, as the two are inversely related. The combination of sensitivity and specificity was optimised (~80%) at lower Ct values in early lactation (~Ct 34), but later in lactation these were optimised nearer to the recommended threshold of 37, with higher estimates of sensitivity and

specificity. To reduce the likelihood of false positives, particularly in early lactation, a lower Ct cut-off could be applied, but a reduction in sensitivity would be expected. Based on the estimates from the PCR assay used in this study, ~20% of infections caused by *S. uberis* could be missed. Lower cut-offs have been suggested as a method of reducing the number of samples that are falsely positive for some pathogens in the PCR, depending on sample type and the objectives of the sampling (Koskinen et al., 2010). A lower Ct value cut-off could be applied for some environmental pathogens to reduce the number of 'contaminated' samples if the same definition of contaminated (three or more different species from a quarter sample) is used for both culture and PCR (Hogan et al., 1999). Whether or not the same criterion should be applied to PCR needs evaluation (Pyorala and Katholm, 2014); however this was not considered in the current study as not all possible pathogens were detected using the PCR assay in this study as restrictions meant only two out of four of the reactions were included.

When detecting contagious pathogens, a reduction in sensitivity would not be acceptable if the goal was to detect as many infections as possible. Recent studies have considered lower Ct cut-offs when detecting *S. agalactiae* and *S. aureus* in herd test samples to reduce the likelihood of false positives due to carry over contamination from residual milk from other samples (Mahmmod et al.,2013a, b). A high Ct value can indicate a low bacterial load, and may not truly reflect an IMI (Mahmmod et al. 2013a). At high cuts offs, the relative sensitivity was at least moderately high, but specificity was markedly less (Mahmmod et al. 2013c). The number of false positives could be reduced by using an alternative test on those samples that test positive using PCR at a high Ct cut-off. The present study did not have enough *S. aureus* positive samples to consider the effects of changing the Ct value on the estimates of sensitivity and specificity.

7.2 Sensitivity, specificity and repeatability of the PCR test for detecting *S. aureus*

7.2.1 False negatives

There was just one PCR false negative result for *S. aureus* in this study, which was for a M1 sample, infected with both *S. aureus* (700 CFU/mL) and *S. uberis* (3,600 CFU/mL), according to culture. Initially, the PCR Ct value for *S. aureus* was between 37 and 40 for the diluted replicate and negative for the undiluted Rep1, but when repeated the sample was consistently negative. *S. uberis* was identified by PCR (Ct 31.4) in this sample. In the subsequent M10 sample, *S. aureus* was still isolated in culture and *S. uberis* was not, whereas the sample was PCR-positive for both *S.*

aureus and *S. uberis* with average Ct values of 24.8 and 25.7, respectively, indicating a '++' result for both pathogens.

The limit of detection of *S. aureus* detection in milk using the PathoProof PCR assay was between 200 and 810 CFU/mL as for *S. uberis*, but in gene copies this was slightly lower than *S. uberis* (6 copies per PCR reaction; Koskinen et al., 2008). It is not known why PCR failed to detect *S. aureus* in the M1 sample, but there was some indication of PCR inhibition as the shape of the amplification curve was slightly low in all repeats but the Ct value of the IAC was acceptable. The presence of other pathogens in the sample can affect the amplification efficiency of the PCR reaction through the depletion of reagents, particularly in the late cycles of PCR (Koskinen et al., 2008). However, detection of secondary pathogens is better using PCR compared with culture as growth of pathogens can be masked by the dominant organism (Cressier and Bissonnette, 2011).

7.2.2 False positives

A D30 sample was the only PCR false positive sample according to culture, and had a Ct value of 34.0. The previous sample (M10) from this quarter indicated a dual infection with *S. uberis* and CNS, detected by both PCR and culture. The presence of CNS was confirmed in culture and PCR at D30. Differentiation of *S. aureus* and CNS was also identified by Hawkins and Cooper (2014) as an issue in culture. A plausible explanation could be that the diagnosis of CNS in culture was incorrect, and the pathogen was actually *S. aureus*. However, this potential CNS appeared in culture without a zone of haemolysis, and did not show a positive reaction to the coagulase test. The DNase and coagulase reactions for differentiating *S. aureus* and CNS are not perfect and may lead to false negative and false positive results (Fantelli and Stephan, 2003). It is important to correctly identify these pathogens as the two differ in terms of mastitis epidemiology and prognosis. An alternative explanation for this result may be that the pathogen was a genetic variant of *S. aureus* with similarities in the target region of the primer targeting CNS; hence the positive result could be due to cross-reaction with the DNA. Further investigation would be required to determine if this PCR result was accurate or falsely positive.

7.2.3 Sensitivity and specificity estimates

The sensitivity of the PCR test for detecting *S. aureus* was high for all samples collected across lactation (96.4%) with just one sample defined as PCR false negative. The small number of samples containing *S. aureus* limit the usefulness of this estimate (95% CI range 81.7, 99.9), especially when split into stage of lactation. Previously, the

sensitivity of detecting *S. aureus* has been reported as 87% in clinical mastitis cases, in the only study using the PathoProof PCR on quarter milk samples where there were sufficient numbers of infected samples (n=84; Koskinen et al., 2010). Using PCR other than PathoProof, sensitivity has been reported to be as high as 99% (Studer et al., 2008; Gillespie and Oliver, 2005). The relative specificity of detecting *S. aureus* was high in this study (99.7%; CI range 98.1-99.9%). Other studies have estimated specificity to be in the range of 92-99% (Koskinen et al., 2010; Taponen et al., 2009; Friendship et al., 2010; Bexiga et al., 2011) when the PathoProof PCR assay was used in comparison to culture. While this appears high, the cost of a false negative *S. aureus* result is likely to be higher than that of *S. uberis* as cows identified as having a *S. aureus* infection may be culled to prevent the spread of infection. Just like in culture, using one-off results is not recommended, and decisions should only be made after repeat testing or consideration of other tests such as culture.

7.3 Effects of frozen storage on bacterial culture results

Milk samples were stored frozen at -20 °C for a period of up to 441 days before PCR testing, with an average storage length of 200 days. Only 22 samples exceeded 260 days in storage, all of which were negative for *S. uberis* and *S. aureus* in fresh and frozen culture. The length of storage was longer than desired, but could not be avoided due to the delays in importation and validation of the PCR kit for use in the study, and because PCR testing could only be undertaken when laboratory facilities were available.

Freezing can affect the viability and recovery of bacteria in milk cultures, but varies between species. In this study, *S. uberis* was recovered from seven samples in culture after frozen storage that initially had no growth in fresh culture, and, no bacteria were recovered from seven samples that were initially *S. uberis* positive in fresh culture. Greater bacterial recovery occurred in eight samples whereas fewer bacteria were recovered in 27 samples, with the positive bacteriological diagnosis remaining the same for these samples. Inconsistent bacterial recovery has been reported for *S. uberis* following short-term frozen storage at -20 °C; Storper et al. (1982) reported a 36% loss in non-*agalactiae* streptococci after samples were stored frozen for 4 weeks whereas Murdough et al. (1996) detected no significant difference in the number of samples positive for *S. uberis* before and after freezing for 6 weeks, based on just five samples per pathogen. When frozen storage exceeded 100 days, the recovery rate of *S. uberis* was lower (K. Reyher, unpublished data; reported by Paradis et al., 2012) or not different (Schukken et al., 1989; Petzer et al., 2011). No studies have reported an

increase in detection of *S. uberis* after freezing, which occurred in 15 samples (4.4%) in the present study. However, most studies rarely include samples culture-negative prior to freezing. Storing samples at -80°C can yield greater survival of pathogens compared with -20 °C (Farrant, 1980); however this was not considered in the current study due to limited storage area, and because the study's focus was on the performance of the PCR test which has the ability to detect non-viable pathogens. Because PCR testing was not undertaken on samples before freezing, the effects of frozen storage on the PCR result could not be assessed in this study, but is assumed to have minor impacts (Paradis et al., 2012).

Using a higher inoculum volume increased the sensitivity of *S. uberis* detection. Plate colony counting identified a further nine samples as *S. uberis* positive but did not identify any additional *S. aureus* positive samples above that detected in fresh and frozen culture. Recovery of *S. aureus* was much less variable than *S. uberis* in this study, with just one sample initially positive, but negative for *S. uberis* after frozen storage. The same culture plate score for FRESH and FROZEN culture occurred in 98.5% of samples. Most studies agree that freezing has no effect on the recovery rate of *S. aureus* (Schukken et al., 1989, Murdough et al., 1996, Godden et al., 2002; Artursson et al., 2010). However, one study found a 50% increase in the frequency of *S. aureus* isolation in samples that had been frozen for 23 days (Villanueva et al., 1991), but this was not supported by the current study.

7.4 Speed of the PCR test

The reduced time to a result is a clear advantage of real-time PCR testing over bacterial culture. In this study, the total time for DNA extraction and PCR reactions varied from 4.5 to 6 hours, depending on the number of samples being processed. This is slightly longer than times reported by other studies and commercial laboratories, where a minimum throughput time of 3-4 hours is usually reported (Koskinen et al., 2009; 2010). Sample processing was more time consuming in this study as the laboratory where the experiments were undertaken was not set up for high throughput. Nevertheless, results of PCR testing were available much sooner compared with bacterial culture, which takes at least 2 days and often longer to confirm a diagnosis (Hogan et al., 1999). Using PCR, a result may be available to a farmer within 1 day, depending on the location of the farm relative to the laboratory and method of delivery of the samples. Milner et al. (1997) reported a faster recovery in SCC and milk yields for infections caused by *S. uberis* after early antibiotic intervention. Prompt identification of the pathogen causing infection could assist the farmer in making herd

treatment decisions and potentially increase the chances of cure, reduce the time the milk is discarded, and limit the unnecessary use of antibiotics (Pyörälä , 2002; Barkema et al., 2006).

8 CONCLUSIONS

Molecular methods such as PCR can allow for the faster identification of mastitis pathogens *S. uberis* and *S. aureus* directly from milk compared with traditional bacterial culture. While PCR is becoming increasingly available as a diagnostic tool for mastitis pathogens, traditional bacterial culture remains as the gold standard test. The PathoProof PCR assay had a relative sensitivity and specificity of 86.8% and 87.7% for detecting *S. uberis* and 96.4% and 99.7% for detecting *S. aureus*, respectively. Most of the conflicting results between the two methods for detecting *S. uberis* occurred in the first month of lactation. While some of these conflicts could be because PCR was better able to detect low concentrations of bacteria, it is likely some of the false positives were detecting DNA from non-viable bacteria. Questions remain around the clinical relevance of detecting dead and low levels of bacteria from a quarter that appears otherwise healthy. There were not sufficient *S. aureus* infections in the study population to draw clear conclusions but indications suggest the PCR assay had near equivalent performance to conventional bacterial culture. Dilution of some samples was essential to enable the detection of pathogens by PCR, as the method of DNA extraction was not always successful in removing PCR inhibitors and impurities from the sample. While recognising the limitations of the gold standard used, this PCR assay is not recommended for use with New Zealand milk samples at the beginning of lactation, but showed more promise later in lactation. This is clinically important because, under New Zealand conditions, the majority of infections caused by *S. uberis* occur in the first month of lactation. For uptake in New Zealand, the PCR assay will require development to better suit colostrum samples and enhance the detection of *S. uberis* in milk. Further advances in technology may enable the differentiation of live and dead bacteria. Combining additional cow and herd-based information such as inflammatory indicators and clinical mastitis history with PCR results can support interpretation and herd level decision making. PCR can be a useful tool to support the identification of mastitis pathogens in milk, but is unlikely to be practical for use in quarter milk samples in New Zealand.

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