Using polymerase chain reaction to identify *Streptococcus uberis* in bovine milk: how does it compare with bacterial culture?

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Abstract

Rapid identification of the pathogen responsible for an intramammary infection in a dairy cow can support herd 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 was compared with traditional bacterial culture for the identification of the most-frequent pathogen in New Zealand, *Streptococcus uberis*, during three stages of lactation. Aseptically collected quarter-milk samples (n=6,848) were analysed by culture, and a subset (n=315) selected for PCR analysis based on *S. uberis* infection status. Using culture as the reference test, PCR had a relative sensitivity of 89.0%, and a specificity of 64.3% (kappa=0.49) for detecting *S. uberis* in milk collected at the first milking post-calving. The relative sensitivity and specificity for samples collected in mid-late lactation were 89.3% and 97.7%, respectively, indicating a greater agreement between the two tests later in lactation (kappa=0.88). Accurate diagnostic tests are necessary for mastitis management. In early lactation, when *S. uberis* prevalence is high, bacterial culture should be used. The PCR assay tested in this study is most useful after the first month of lactation.

Keywords: bovine mastitis; pathogen identification; polymerase chain reaction; *Streptococcus uberis*

Introduction

Mastitis is an inflammatory condition of the mammary gland, predominantly caused by bacterial infection. It is the most-costly disease of dairy cattle worldwide; the annual cost estimated to be more than $280 million for New Zealand alone (SmartSAMM 2013). Identification of the specific bacteria causing mastitis is an important part of mastitis control, assisting in herd-management decisions, such as selecting the most appropriate antibiotic for lactating- and dry-cow therapy, and culling. The accepted 'gold-standard' test for laboratory-based pathogen identification is bacterial culture (Hogan et al. 1999). Polymerase chain reaction (PCR) has recently become an alternative option for testing milk for the presence of specific mastitis pathogens in diagnostic laboratories in North America, Europe and Australia. Compared with culture, the main advantages of PCR include faster diagnosis, objective bacterial identification, and the ability to detect lower concentrations of bacteria through the amplification of DNA from both viable and non-viable microorganisms (Koskinen et al. 2009; Taponen et al. 2009). However, despite these advantages, it is not known if the information provided by PCR is relevant to the clinician and farmer (Penry et al. 2014). The ability of PCR to detect mastitis-causing pathogens depends on the efficiency of the DNA extraction, the design of the PCR assay, the sample history and the pathogen being targeted (Penry et al. 2014). PCR will only identify the bacterial species that the assay has been specifically designed to detect, meaning rare pathogens may be missed.

Many international studies have focussed on identifying the contagious mastitis pathogens *Streptococcus agalactiae*, *Mycoplasma bovis* and *Staphylococcus aureus* (e.g. Koskinen et al. 2010; Mahmmod et al. 2013). The main cause of mastitis in New Zealand is *Streptococcus uberis*, an environmental pathogen responsible for up to 75% of clinical mastitis cases in early lactation (McDougall 1998). Relatively few studies have compared the performance of PCR and culture for detecting *S. uberis* (Gillespie & Oliver 2005; Koskinen et al. 2010). Research is required to determine if PCR is a suitable diagnostic tool for identifying mastitis pathogens in New Zealand, particularly *S. uberis*. The aim of the study reported here was to compare the performance of a commercial mastitis PCR assay with traditional bacterial culture for the detection of *S. uberis* in milk samples collected throughout lactation.

Materials and methods

Sample collection

During the 2013/14 production season, milk samples (n=6,848) were collected from individual quarters of spring-calving mixed-age dairy cows on two Waikato-based DairyNZ research farms in early, mid and late lactation. Milk samples were collected aseptically following SmartSAMM (Smart Approach to Minimising Mastitis) guidelines (2013). In early lactation, all functional quarters of 480 cows were sampled at the first and tenth milking after calving. Quarters identified as culture-positive for any bacterial pathogen were sampled again 30-37 days into lactation. In mid and late lactation, 330 cows were sampled, and on both occasions, all culture-positive quarters were resampled seven days later. Additionally, any quarter diagnosed with clinical mastitis was sampled before administering treatments. All sampling was approved by the Ruakura Animal Ethics Committee (No. 12948).

Bacteriological analysis

Samples were kept chilled at 4°C until bacterial culture, which occurred within 24 hours of sample collection. Briefly, 10 µL of milk was streaked onto one quadrant of an aesculin blood agar plate (Fort Richard Laboratories Ltd, Otahuhu, Auckland, NZ). Plates were incubated aerobically at 37°C for 48 hours. A quarter was

identified as infected when at least one colony of a bacterial species was isolated. Bacterial identification was confirmed following the National Mastitis Council guidelines (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). Samples were then stored at -20°C. Bacteriology was repeated on samples selected for PCR testing, and colony counts were completed to obtain a quantitative measure of bacterial numbers (colony forming units (CFU)/mL). Additional biochemical testing using API 20 Strep strips (bioMerieux, Lyon, France) was conducted for a subset of samples for confirmation of bacterial identification.

Selection of samples for PCR analysis

Samples were selected for PCR analysis in full sets of either three or two samples collected from individual quarters in early or mid-late lactation, respectively, on the basis that at least one sample in the set was culture-positive for *S. uberis*. A small number of culture-negative sample sets from each stage of lactation were also included. Samples collected from quarters that had been treated with intramammary antibiotics within 21 days of the scheduled sample collection were excluded from analyses (n=28), leaving 315 samples for the comparison of the PCR test with bacterial culture (Table 1).

Real-time PCR Assay

The real-time PCR assay (PathoProof Mastitis Complete 12, ThermoScientific, Vantaa, Finland) was capable of detecting 11 mastitis-causing bacterial species or species groups in four separate multiplex reactions (each reaction included 3 targets and a control), however, two reactions were excluded, as the targeted pathogens were not relevant to the study. Therefore, the assay used could identify six bacterial targets: *S. aureus*, *Enterococcus* sp. (including *E. faecalis and E. faecium*), *Corynebacterium bovis*, *Staphylococcus* sp. (including *S. aureus* and coagulase-negative staphylococci (CNS)), *S. agalactiae* and *S. uberis*. The assay included all reagents for bacterial DNA extraction and real-time PCR.

Samples were manually prepared for DNA extraction using 400 µL of milk as the starting volume, according to the manufacturer's instructions. Enzymatic lysis and centrifugation steps separated bacterial cells from somatic cells and PCR-inhibiting substances. DNA was purified using the magnetic beads-based approach and elution was automated using the Kingfisher 96 (Thermo Electron, Vantaa, Finland). PCR reactions were completed in duplicate for all samples except first milking (colostrum) and clinical mastitis samples, which were run as undiluted and 1 in 10 diluted replicates of the sample extract. This protocol was adjusted following a validation study that identified PCR reaction failure in some colostrum and clinical samples (Steele, unpublished). Each PCR run was prepared in a 96-well plate, with each well combining 5 µL of extracted sample DNA, 10 µL of Master Mix, 5 µL of the reaction specific Primer Mix, and an Internal Amplification Control (IAC). Real-time PCR was run on the 7500 Fast Real Time PCR system (Applied Biosystems, California, USA). The thermal cycling protocol involved a 10-minute pre-denaturation phase at 95°C, followed by 40 cycles of 5-second denaturation at 95°C and 60-second annealing at 60°C. A final 5-second annealing step cooled samples to 25°C. Samples were considered positive or negative for a bacterial target based on a cycle threshold (Ct) value of 37 (as described by Koskinen et al. 2009). The Ct represents the minimum number of PCR cycles required to reach a pre-determined fluorescence threshold value, with a lower Ct value indicating a greater quantity of DNA in the sample. Before scoring any reaction as positive, acceptable PCR conditions were confirmed by verifying that the Ct values and shape of the amplification curves for the IAC were acceptable (Taponen et al. 2009). Negative controls were included to rule out cross-contamination in the laboratory.

Comparison of the tests

The sensitivity and specificity of the PCR assay was assessed relative to bacterial culture. 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 (Martin, 1984). Mid and late lactation samples were combined for analysis, due to the low incidence of *S. uberis* infections. Ct values were averaged if both duplicates were <37. When both duplicates were ≥37, the sample was considered negative (see Koskinen et al. 2009). Data were analysed using Minitab (version 16.2.3, Minitab Inc.). The Kappa statistic (κ) was used to determine the agreement between culture and PCR, beyond that expected by chance (Cohen 1960).

Results

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

Sensitivity and Specificity

Bacterial culture and PCR both identified *S. uberis* in 125 samples. There were 19 samples that were PCR false-negative (6.0%), i.e., positive in culture but negative in PCR, and 21 PCR false-positive samples (6.7%), i.e., negative in culture but positive in PCR. API 20 Strep testing confirmed *S. uberis* identification in 12 of these false-negative samples. The PCR test had a relative sensitivity of 86.8% and relative specificity of 87.7% across all stages of lactation (κ=0.744; Table 1). There were large differences in the specificity of the PCR test at the different stages of lactation (range 64-98%), with the lowest specificity at the first milking sample and highest in mid-late lactation. Sensitivity was similar for all sampling points (85-89%), except for at 30-37 days in milk (57%; Table 1); however, there was a limited number of infected samples at this sampling point (n=7).

Table 1 Relative sensitivity and specificity of the PCR test and associated kappa statistics for *S. uberis* detection from bovine milk (using bacterial culture as the gold-standard) for all samples and split into the different sampling points across the season.

	N	Relative Sensitivity (%)	Relative Specificity (%)	Kappa Statistic (κ)
All samples	315	86.8	87.7	0.744
First milking	87	89.0	64.3	0.491
Tenth milking	73	85.3	71.8	0.565
30 days in milk	82	57.1	94.7	0.487
Mid-late lactation	71	89.3	97.7	0.881

Discussion

To be suitable as an alternative to culture for detecting *S. uberis* in bovine milk in New Zealand, the relative sensitivity and specificity of the PCR test should be equivalent to culture (i.e. 100%) throughout the full lactation. However, there were 40 samples (12.7%) for which culture and PCR results conflicted. Of the 21 PCR false-positive samples, 13 had Ct values ≥33, indicating a low concentration of bacteria in the sample. According to PCR, the remaining eight samples had sufficient bacterial concentrations (Ct <33), but may not have been detected in culture if bacteria were dead or growth-inhibited. Post-treatment samples were removed from analysis as they would have exaggerated the number of apparent 'false-positive' results due to the inhibitory effects of antibiotics on growth in culture. All but one of the PCR false-positive samples were collected during the first month of lactation, limiting the relative specificity of the PCR test to 64-72% in early lactation.

PCR did not detect *S. uberis* in 19 samples that were positive in culture (PCR false-negatives). The bacterial count in these samples ranged from 0 to 3,600 CFU/mL, with eight samples having low bacterial concentrations (<100 CFU/mL). The PathoProof PCR assay can reportedly detect as few as 16.7 gene copies per mL (Koskinen et al. 2008), and if one gene copy was assumed to be equivalent to one CFU, bacteria should have been detected in at least four of these samples. However, analytical sensitivity should not be considered to equal diagnostic sensitivity under field conditions (Koskinen et al. 2010), as supported by the inconsistent detection of *S. uberis* in this study. Milk contains several substances which can affect the efficiency of both DNA extraction and the PCR reaction (Koskinen et al. 2008). Of the 19 PCR false-negatives, nine were from the first milking, and five from the tenth milking. The large proportion of antibodies, somatic cells and tissue debris in colostrum could inhibit the PCR test for these samples to a greater extent than for milk samples later in lactation.

Two samples collected in late lactation from the same quarter, one week apart, were culture-positive for *S. uberis* (1,800-3,600 CFU/mL), and confirmed by API Strep testing. However, these were not identified by PCR. Although no sequence data was available, the bacterial strain may have differed from the strains targeted in the PCR kit, since the kit was developed in Finland using a range of isolates (n=29) from six countries in North America and Europe (Koskinen et al. 2009). The exclusion of Australasian isolates in the development of this assay could limit the range of strains that can be identified by the test.

This is the first study assessing the performance of the PathoProof Mastitis PCR assay on milk samples in New Zealand. Compared with our results, 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). 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 & Oliver (2005) reported 100% sensitivity and specificity; however, PCR was used on bacterial isolates after culture, rather than directly on raw milk samples (Gillespie & Oliver 2005).

Determining the true specificity of PCR is difficult because there is no accurate gold-standard test to determine the infection status of a quarter. Bacterial culture was used as the gold-standard in this analysis but is not without limitations, in particular if the PCR test correctly identified bacteria that culture had missed, then it will be penalised when compared with culture. However, questions remain around the clinical relevance of detecting dead and low levels of bacteria from a quarter that appears otherwise healthy (Penry et al. 2014). It is likely that in the future, the application of new technologies to distinguish between live and dead bacteria will improve the value of PCR as a mastitis diagnostic tool. However, the data from this study suggest that PCR should not be used on its own to make decisions regarding treatment. Additional strategies (e.g. combining PCR results with clinical history of the cow and somatic cell count data) are required to improve overall confidence about the presence or absence of particular organisms and their impact on mammary health.

Conclusion

The PCR assay used to detect *S. uberis* is not recommended for use with New Zealand milk samples at the beginning of lactation, but shows more promise later in lactation. Sensitivity was moderately high in early, mid and late lactation, but the relative specificity, compared to culture, was low in early lactation compared with mid and late 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.

Acknowledgements

We acknowledge the farm and technical staff at Lye and Scott Farm for assistance with sample collection, and Barbara Kuhn-Sherlock for the statistical support. We also thank Livestock Improvement Corporation for the use of laboratory facilities. Funding for this project was provided by Ministry of Business, Innovation and Employment (MBIE) and by New Zealand dairy farmers through DairyNZ Inc.

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