Contract session: Evaluation of the performance of real time PCR for detection of *Staphylococcus aureus* in dairy cows, using herd test milk samples

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Introduction

Mastitis in dairy cattle is of considerable consequence in NZ, with estimates of over $200 million per annum loss of potential earnings caused by reduction in milk quality and decreased production (Blackwell & Lacy-Hulbert 2012).

Somatic cell count (SCC) is used by farmers for udder health monitoring–with an increase in cell count being an indicator of infection. On-farm, monitoring the bulk milk (BM) SCC is used to gauge sub-clinical mastitis infection in the herd. An increase in or a consistently high BM SCC can indicate infection. Herd Testing (HT) provides the individual animal SCC. The Smart SAMM guidelines Seasonal Approach to Managing Mastitis 2011 plan suggests a target for national average bulk-milk somatic cell count of less than 150,000 cells/ml (Woolford et al. 1995), BMSCC exceeding 400,000 cells/ml are subject to dairy company penalties. Individual animal SCCs of over 150,000 cells/ml, or 120,000 cells/ml for heifers, are indicative of an infection.

The epidemiology of mastitis in New Zealand differs from that in other countries due to the pasture-based and seasonal management systems. Infections are either of environmental origin, with *Streptococcus uberis* being the primary cause in NZ, or spread as contagious pathogens, primarily *Staphylococcus aureus* (*S. aureus*) and *Streptococcus dysgalactiae* (McDougall 2002). Although high resolution rates can be achieved for environmental infections, *S. aureus* cure rates are significantly lower - particularly in older cows with an infection history. *S. aureus* may become an intracellular infection with abscess formation, which makes clearance and killing of the bacteria difficult and culling may be advisable to limit spread of bacteria in the herd. A diagnosis of *S. aureus* in ongoing mastitis, cases is therefore, helpful for treat-versus-cull decisions.

Bacterial culture has been the gold standard for diagnosis and must be done on milk samples collected in a strictly sterile fashion (Hogan 1999). More recent technologies for diagnostics include MALDI-TOF mass spectroscopy (reviewed by Singhal et al. 2015) which requires fresh bacterial cultures, and PCR which specifically identifies bacterial DNA (Koskinen et al. 2009; Graber et al. 2007). The real-time PCR technology, as used in this study, is a more quantitative PCR method with a numerical read-out, and hence best-suited for diagnostic purposes.

This study had two objectives, the first being to evaluate whether real-time PCR can detect a range of NZ-derived *S. aureus* bacteria in DNA extracts from foremilk samples, with acceptable sensitivity and specificity. The second was to assess whether HT-derived milk samples could be used for reliable detection of *S. aureus*, by comparing the PCR results from HT milk with bacteriology and PCR on aseptically collected foremilk samples from the same animal.

HT milk is a convenient and easy way to sub-sample milk from a large number of animals. As *S.aureus* bacteria can be present on skin surfaces, it could be present in HT milk as a carryover contaminant rather than true infection. An experiment to test whether the contamination of herd test milk would prevent a clear distinction being made between a true infection and a background.

Materials and methods

A total of 602 cows from 17 herds across the Waikato region of New Zealand were identified for the study. The first group of 291 animals had SCC >500,000 cells/ml or a history of raised SCC (high SCC cohort). The second group of 311 animals had a history of SCC less than 100,000 cells/ml (low SCC control group). Data was analysed only where animals had both a valid HT and sterile milk result. The study was carried out between February and May 2014, under the AEC guidelines (AgResearch Ruakura Committee).

HT milk samples were collected as a proportional sample of the whole milking according to normal LIC procedures. Typically morning and afternoon samples were collected and pooled. Some farms were on once-a-day milking so only am milk samples were collected. After completion of HT analysis, sample pots were transferred to the LIC Diagnostics Animal Health laboratory for sub-sampling and testing.

On-farm quarter foremilk collection was typically carried out late morning or before the afternoon milking, usually 5-10 days after the HT. The standard aseptic collection method described in Livestock Improvement 'Managing Mastitis Guide' was used.

Microbiology was completed according to National Mastitis Council (NMC) guidelines (Hogan et al. 1999). Ten µL of milk was streaked per quadrant onto Dexcel esculin blood agar plates (Fort Richard Laboratories Ltd, Auckland, NZ), which were incubated at 37°C for 48 hours. A haemolysis reaction was noted and positive colonies were further tested for ability to coagulate sheep serum (Fort Richard Laboratories Ltd, Auckland, NZ). A sample was contaminated if more than three colony types

were present in culture. A quarter was recorded as infected if more than one colony was noted.

Foremilk subsamples of each quarter were taken and equal volumes pooled before DNA extraction and PCR. This was to simulate a composite sample, though in practice, an infected quarter may have had lower production, so the ratio at milking was not necessarily 1:3 dilution. PCR was performed on individual quarter samples, if required, for confirmation purposes.

The commercial kit chosen for the proof of principle trial was PathoProof Major 3 or Complete 12 Kits (Thermo Scientific,Vantaa, Finland), which is suitable for detection of *S.aureus* and other mastitis targets (Koskinen et al. 2009). The DNA extraction and PCR were carried out according to the manufacturer's instructions, except that the DNA extraction was carried out using 1.5 mL sterile Eppendorf tubes, rather than those provided with the kit. The starting volume for DNA extraction was 400 µL of milk. DNA was purified using the magnetic beads-based method and elution was automated using the Kingfisher 96 (Thermo Electron, Vantaa, Finland). The PCR was run on the 7500 Fast Real Time PCR system (Applied Biosystems, California, USA).

Results and discussion

S. aureus cases were identified by microbiology culture. The diagnostic sensitivity was, therefore, defined as the proportion of 'true' positive results (according to culture) that were correctly identified by PCR and the relative specificity as the proportion of 'true' negative results correctly identified by PCR.

The number of *S. aureus* cases detected in the high SCC cohort on each farm varied between 0 and 86.7%. Notably, the farm with no cases had a history of culling all *S. aureus* positive animals.

S. aureus was identified in 7% of cases where SCC was less than 250,000 cells/ml, 19.5% of cases when SCC was 250-500,000 cells/ml, 39.1% of cases when 500,000-1 million cells/ml and 34.4% of cases when SCC greater than 1 million cells/ml. There was very good agreement between the PCR and culture on sterile milk samples with sensitivity of 99.1% and specificity of 99.0%.

With herd-test milk samples, the specificity remained the same at 99%, whereas milk sensitivity was 70%. Possible contributing factors to the lower sensitivity are the concentration of bacteria in the composite samples; production from infected quarters may have been less, hence there is dilution of bacteria. Daily shedding of *S. aureus* can be variable, dependent on genotype (Walker et al. 2011), so the different sample dates may also contribute. We also compared the levels of *S. aureus* bacteria in foremilk versus hindmilk, and found foremilk is typically enriched in *S. aureus* bacteria, so PCR on this sample type would give a higher test sensitivity.

Due to LIC's high through-put product requirements, the manual handling required during DNA extraction, made this commercial kit unsuitable for our purposes. During 2014-2016, an alternative magnetic bead-based DNA extraction and PCR process was evaluated and comparable sensitivity and specificity was achieved. A reporting process with a suspect category was introduced; this captured likely positive cases but recommended a re-test confirmation. Case studies were carried out across several farms, in conjunction with vets, to see how the test performed in this format, and to better understand the usefulness of the results for management decisions, and on dry-cow antibiotic usage. As a consequence, we have released this test product via vet clinics, to a limited number of North Island herds in the 2016/17 season.

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