# Molecular tests for milk – performance and application in Australia

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Molecular test technologies, such as polymerase chain reaction (PCR), are not new in either human or veterinary medicine diagnostics. However, the application of these technologies to the assessment of milk for the presence of mastitis pathogens is relatively recent.

In the mid 2000s, the University of Melbourne Veterinary Science Faculty developed a PCR for *Streptococcus agalactiae*; this test had a limited commercial life. Subsequently, the Livestock Teaching Unit, University of Sydney, developed a separate molecular test for *Strep. agalactiae* and *Mycoplasma spp.* using loop-mediated isothermal amplification (LAMP). This test is in the research and validation phase.

In early 2011, a new milk molecular test, the PathoProof™ PCR, became commercially available in Australia, through Dairy Technical Services.

Because the validity of various possible applications of molecular testing in milk were undefined, in July 2011, Countdown Downunder, through the generous support of the Geoffrey Gardiner Dairy Foundation, commenced a research project to assess the performance, application and interpretation of these tests. This research will continue until September 2012. This paper presents interim findings to May 2012. Further communication on the overall results of this research will be available to farmers and advisers during 2012/13 through Countdown. The partner in this research has been Dairy Technical Services with valued project assistance from the University of Sydney, Fonterra, Murray Goulburn Co-op, Pfizer and Gribbles Pathology.

This paper focuses largely on the PathoProof™ PCR milk molecular test, as it has been the primary testing subject for this research project. However, it is highly likely that the principles of test application determined through this work will apply to other milk pathogen molecular tests (for example, LAMP) if they become commercially available.

## Finding the conceptual fit for this test

Countdown has developed the Herd Mastitis Dynamics Chart and used this in adviser and farmer training. This chart was an attempt to represent the movement of animals between non-infected and infected groups in any herd, regardless of size, calving system, management and location. In this chart, at any point in time, each animal has only one of two possible mastitis states – they are either non-infected, where no mastitis pathogens are present in any quarter, or they are infected with one or more mastitis pathogens in at least one quarter. Where an animal is infected, the individual cow cell count will generally be greater than 250,000 cells/mL.

This chart (Figure 1) can be used to describe some of the factors which can lead to new infections such as poor performance in teat disinfection, milking machine function, milking management and environmental control. The factors which determine 'cures' or animals going from the infected to the clean group are largely limited to lactation treatments and dry cow treatments. In general, there are far more factors that can effectively influence or drive the new infection rate than there are factors that increase rate of cures. Identifying major factors influencing spread in the herd is vital for tailored, herd-specific, mastitis control.

Countdown Technote 13 (extract in Figure 2) outlines the steps an advisory team should undertake when investigating a mastitis problem herd**.** The extract in Figure 2 details the start of this process. When describing the presenting problem, a number of tests can be applied to provide information about the nature and extent of new and more chronic infections. These include: a) bulk milk cell count (BMCC) data; b) individual cow cell count (ICCC) data; and c) standard milk culture of samples from individual cows. Some advisers also employ other ancillary tests such as the Rapid Mastitis Test, milk electrical conductivity and data from other in-line mastitis sensing technologies to assess infection status. Identifying the predominant mastitis pathogens is a key step in this, as this knowledge assists in determining the mechanisms of spread,

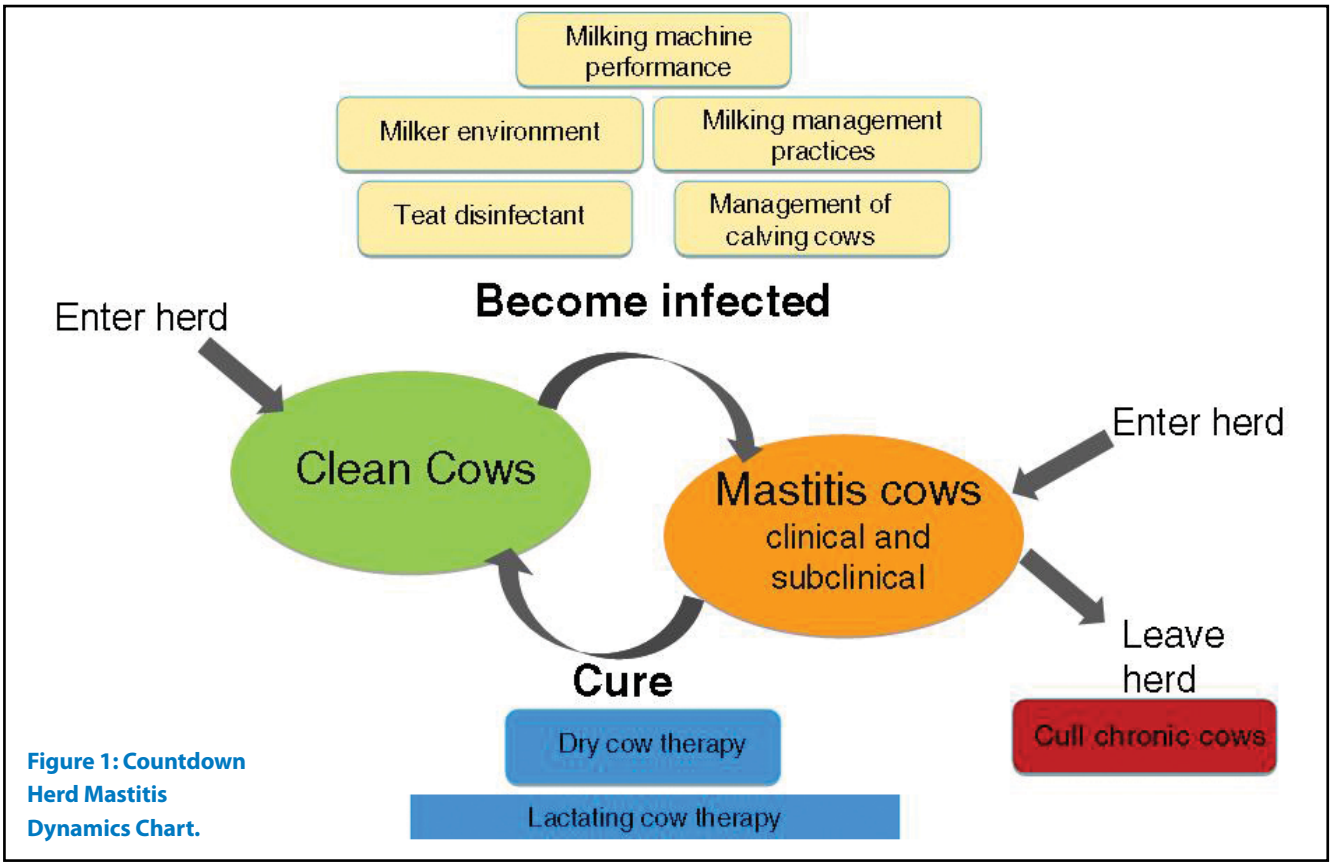


**Figure 1: Countdown Herd Mastitis Dynamics Chart.**

and hence, the major factors influencing spread. The questions this research has attempted to answer are, in the Australian context:

1. Where does milk molecular testing fit among these other tests?

2) How should such tests be interpreted both during a mastitis investigation and where mastitis risk mitigation work is being undertaken (Countdown MAX service model)?

## PCR test outline

The PCR is an example of one type of molecular test for detecting mastitis pathogens. The test differs from standard milk culture as it is designed to identify target strands of DNA uniquely associated with each organism.

Recall that DNA is double-stranded, with the strands cross-linked to each other along their entire length by links between bases (adenine linking to thymine, and guanine linking to cytosine).

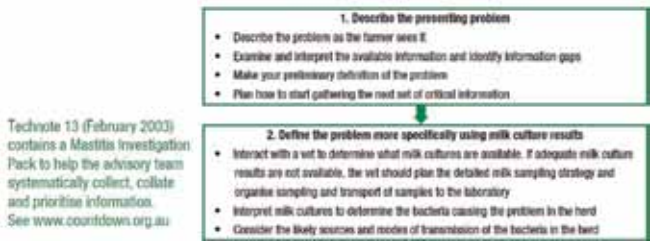


**Figure 2: Extract from Countdown Technote 13 Mastitis Investigation Flowchart.**

The polymerase chain reaction synthesises a very large number of copies of a specified sequence (or section) of the DNA (target DNA) in a sample. The target DNA sequence is defined by short sequences either side of it; 'primers' are necessary to bind to these short sequences.

A PCR reaction cycle consists of three steps:

1. Denaturation: The sample is heated, denaturing the DNA from double to single strands.
2. Annealing: The sample is cooled, allowing the primers to bind to short sequences either side of the target DNA sequence. One primer binds to each DNA strand.
3. Elongation: Using DNA polymerase, on each of the single strands, the complementary strand is partially added, commencing at the primer and continuing for a variable distance along the strand. Thus, the single strands become double strands for this distance.

This three-step cycle is repeated; the number of double stranded products doubles with each cycle. By the third cycle, some of the double-stranded products represent the target DNA only between the short sequences either side.
With further cycles, these become the predominant product in the mixture. After 30 cycles, the original DNA had been amplified a billion-fold.

The PathoProof™ PCR is a 'real time' PCR. With real time PCR assays, a positive reaction occurs when a fluorescent signal is detected. A positive result can be expressed as the

**Table 1: A comparison of the attributes of PCR and standard milk culture (adapted from Bradley et al. 2011).**

| | PCR | Standard milk culture (bacteriology) |
|---|---|---|
| Bacteria identified | - At present, PathoProof™detects 12 organisms<br>- PathoProof™can also detect the penicillin resistance gene | - Bacteriology has potential to identify a wide range of bacteria, including more exotic bugs<br>- It does not detect Mycoplasma species- a rare cause of mastitis in Australia<br>- A separate test must be carried out to identify penicillin resistance |
| Sampling | - PCR can detect dead and live bacteria<br>- PCR can be carried out on milk samples treated with preservative, allowing normal milk recording samples to be used for mastitis testing | - Bacteriology can only identify live bacteria<br>- Bacteriology using milk samples treated with preservatives is uninformative<br>- No growths can result when bacteria die between collection and plating. |
| Interpretation | - Because PCR is a new technology in Australia, there is limited knowledge around interpreting results; Countdown is currently completing research in this area<br>- Vets and farmers need to be educated in interpretation of results | - Bacteriology has been used for many years in Australia, providing extensive experience in interpretation<br>- Most farmers and vets know how to interpret results |
| Contamination | - There is currently no good measure for identifying contaminants from the udder and teat skin identified through PCR | - Bacteriology allows easier identification of contamination |
| Time | PCR testing takes less than 4 hours | Bacteriology takes 24-48 hours |
| Cost | About $45 per milk sample | About $15-$20 per milk sample |

cycle threshold (Ct). Ct is the number of cycles required for the fluorescent signal to become strong enough to be distinguishable from background values.

The PathoProof™ PCR being evaluated as part of this research is available commercially in two testing formats. The first format tests for the presence of DNA from four organisms: *Strep. agalactiae*, *Staphylococcus aureus*, *Streptococcus uberis* and *Mycoplasma bovis*. A second format tests for these organisms and another eight organisms, along with the gene that confers penicillin resistance in *Staph. aureus*.

Of the organisms targetted using this PCR, only *Strep. agalactiae* and *Mycoplasma bovis* originate only from inside infected quarters. All other organisms can replicate outside the udder, so unless aseptic milk samples are collected, they can originate from teat skin and the milking plant.

Milk samples can be from individual quarters, individual cow samples (milk pooled from all four quarters from the same cow), samples from groups of cows pooled, or bulk vat samples. The presence of milk preservative, such as bronopol, does not interfere with the test function and contamination of the sample also does not interfere with the conduct of the test.

The key differences between PCR and standard milk culture are summarised in Table 1.

The Countdown research project includes the following components:

- A review of literature describing the diagnostic validity of PCRs for detecting mastitis pathogens in milk from dairy cows
- Assessment of no growth standard culture samples
- Dilution studies and other methodologies to assess PCR performance in bulk milk
- Research to estimate prevalences of pathogens
- Using pooled herd test-sampled milks

## A review of the literature

The purpose of the review of the literature describing the diagnostic validity of PCRs for detecting mastitis pathogens in milk from dairy cows was to summarise and critically evaluate scientific evidence about the diagnostic validity of polymerase chain reaction (PCR) assay results for detecting mastitis pathogens in milk from dairy cows. The review particularly focused on the PathoProof™ PCR because this assay is being offered commercially in Australia, but results for other PCRs were also reviewed for comparison. Lower limits for diagnostic sensitivity and specificity that are tolerable under various scenarios were also explored.

More than 40 papers were assessed and much of this work had been undertaken in Europe. Of the seven publications from which diagnostic sensitivities and specificities for the PathoProof™ PCR were summarised (see References), only three had been peer-reviewed. Of the five publications from which diagnostic sensitivities and specificities results for other PCRs were summarised, four had been peer-reviewed

- Disregarding results known to be based on small numbers of samples, relative diagnostic sensitivities of the PathoProof™ PCR were: *Staph. aureus* 87% and 94% (2 results); *Strep. agalactiae* 90% (1 result); *Strep. dysgalactiae* 89% and 100% (2 results); *Strep. uberis* 88% and 100% (2 results); *Corynebacterium bovis* 77% and 80% (2 results); coagulase negative *Staphylococcus* spp. 80% (1 result). In one study, diagnostic sensitivities of the PathoProof™ PCR for *Strep agalactiae* (from latent class models) were estimated as 96%, 92%, 87% and 74%, respectively, at PCR Ct value cutoffs of 39, 37, 34, and 32. No results allowed calculation of relative diagnostic sensitivity for the PathoProof™ PCR for *Mycoplasma bovis*.
- Disregarding results known to be based on small numbers of samples, relative diagnostic specificities of the

PathoProof™ PCR were: *Staph. aureus* 92% to 99% (5 results); *Strep. agalactiae* 97% to 100% (5 results); *Strep. dysgalactiae* 90% to 99% (4 results); *Strep. uberis* 80% to 97% (4 results); *C. bovis* 69% to 92% (4 results); coagulase negative *Staphylococcus* spp. 62% to 86% (3 results). Diagnostic specificities of the PathoProof™ PCR for *Strep. agalactiae* (from latent class models) were estimated as 97%, lower than estimates for culture from the same study. No results allowed calculation of relative diagnostic specificity for the PathoProof™ PCR for *Mycoplasma bovis*.

Interim conclusions from the review are:

- Diagnostic sensitivity must be above 99% when false negative test results are expensive and the particular organism is quite likely to be present in the milk sample, but lower sensitivities are tolerable when the particular organism is less likely to be present in the milk sample. High to very high diagnostic specificities are required (99% to 99.99%) when false positive test results are expensive.
- Diagnostic sensitivities of the PathoProof™ PCR for *Staph. aureus*, *Strep. agalactiae, Strep. dysgalactiae* and *Strep. uberis* are probably at least moderately high at high Ct cut-offs. However, diagnostic sensitivities of this PCR should not be assumed to be 100% and negative results should not be considered to be unequivocal proof that the organism was not present. When false negative test results are expensive and the particular organism is quite likely to be present in the milk sample, additional strategies to improve overall confidence about the absence of particular organisms are required. (Such strategies are probably also required if culture were used instead of the PathoProof™ PCR.)
- Diagnostic specificities of the PathoProof™ PCR for *Staph. aureus*, *Strep. agalactiae*, *Strep. dysgalactiae* and *Strep. uberis* are probably moderately high. However, diagnostic specificities of this PCR should not be assumed to be 100% and positive results should not be considered to be unequivocal proof that the organism was present. If the prior probability that the particular organism is present in the milk sample is low, a substantial proportion of PCR positives are likely to be false positives.
- Further studies assessing the diagnostic sensitivities and specificities of the PathoProof™ PCR with improved study designs are required.
- Studies to assess the diagnostic sensitivity and specificity of the PathoProof™ PCR for *Mycoplasma bovis* are required.
- In the studies reviewed, PCRs were almost always assessed at quarter and cow levels yet potentially, practical applications under Australian conditions may be more likely at group (i.e. pools of cows) or vat (i.e. herd) level. There is a need for information about the interrelationships in diagnostic sensitivities and specificities at these latter levels.

## Assessment of no growth standard culture samples

As standard culture does not identify *Mycoplasma bovis,* it is possible that some clinical mastitis cases where there is no growth on standard culture are due to *Mycoplasma bovis*. The aim of this component was to estimate the prevalence of PCR-positives for *Mycoplasma bovis* in milk samples from clinical mastitis cases where there was no growth on standard culture.

Fifty-five milk samples from clinical mastitis cases where there was no growth on standard culture were tested using the PCR. No PCR-positives for *Mycoplasma bovis* were identified. Assuming no prior knowledge about this question, this result indicates that we can be 95% sure that the true proportion of such cases that are *Mycoplasma bovis* PCR positive is no more than 6%.

There were also no *Strep agalactiae* positives on PCR.

## Dilution studies and other methodologies to assess performance in bulk milk

As previously indicated, much of the peer-reviewed research on the diagnostic validity of the PCR was undertaken quarter samples or individual cow samples. The Countdown research group identified the need for further information about potential test performance where samples from groups of cows pooled, or bulk vat samples were used.

Dilution studies were performed using individual cow samples positive for *Strep agalactiae* and *Mycoplasma bovis* on the PathoProof™ PCR Six individual cow samples positive to either bacteria on standard or selective culture were diluted down with milk negative on the PathoProof™ PCR for these pathogens at dilution rates of 1/10, 1/100, 1/500 and 1/1000. These pathogens were chosen as it seems most likely that bulk vat testing would be most useful for these, based on the ecology of the bacteria as described previously.

For both pathogens, all individual cow samples were positive on PCR when tested undiluted and then at all serial dilutions that followed. These results suggest that PCR on bulk vat samples may have reasonable diagnostic sensitivity for detecting these pathogens in herds with low prevalences of these infections. This is the first step in building a picture of test performance on bulk vat samples.

Diagnostic sensitivity (the performance of the test in infected animals) and specificity (the performance of the test in non-infected animals) of the PCR for detecting *Strep agalactiae* in bulk vat test performance will also be estimated using PCR and BMCC data from bulk vats two herd data sets: 240 herds selected at random across the three dairying regions of Victoria and 219 herds selected because they had elevated BMCCs. The PCR test was used only once for each herd. The results of this analysis were not completed at the time of writing.

## Research to estimate prevalences of pathogens

Utilising these same 240 and 219 herd bulk vat samples, prevalences of both *Strep. agalactiae* and *Mycoplasma bovis* will be estimated at the herd level. Apparent prevalences of *Strep. agalactiae* and *Mycoplasma bovis* in the 240 samples selected at random were 14% and 0.8%, respectively. Apparent prevalence of *Strep. agalactiae* in the elevated BMCC herds was higher than that seen in the randomly selected herd set as would be expected. Based on the literature review, the specificity of the *Strep. agalactiae* PCR is probably not 100%. So it is possible that some of these *Strep. agalactiae* PCR-positive results are false positives and further work will be conducted to estimate the true herd-level prevalence of *Strep. agalactiae*. The results for *Mycoplasma bovis* suggest that the diagnostic specificity of the *Mycoplasma bovis* PCR at vat level is high.

## Using pooled herd test-sampled milks

Milk molecular tests can be performed using milk containing preservative as used when samples for ICCCs are taken during herd testing. The research group was interested in exploring the use of the PCR test with pooled milk samples from high ICCC cows using samples obtained as the milk samples from herd testing were being run through the cell counter. A testing protocol was derived which involved pooling two lots of ten high ICCC cows (> 400,000 cells/mL) along with one pool of five low ICCC cows from 20 herds. The herd test sample derived pools were tested with the PCR and milk samples were then collected aseptically from the same cows. The aseptic samples were then tested with both standard culture and PCR.

Analyses of this large data set across 20 herds will be completed in August 2012. If it is shown that pooled herd test derived samples play a role in assessing high ICCC cows in a useful way, this will create an added test option for advisers and herd managers.

## Next steps

Based on the research work undertaken by Countdown thus far, it appears there is a role for the milk molecular tests, and the PathoProof™ PCR more specifically. While the results of all the research components have yet to be finalised across all individual work areas, evidence is emerging that the test can be useful for the detection of *Strep. agalactiae* and *Mycoplasma bovis* at the individual cow, pooled cow sample and bulk vat level. This would be both as a surveillance screening test and as a test to be employed as a result of an investigation.

In July and August, all research components will be finalised and the Countdown team will develop a decision-making flowchart for the use of PCR on milk samples of all types and under typical scenarios. This will be made available to advisers later in 2012.

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## References

Anonymous (2012) Wisconsin Veterinary Diagnostic Laboratory. http://www.wvdl.wisc.edu/DiagnosticAids.asp; viewed 19th June 2012.

Bexiga R, Koskinen MT, Holopainen J, Carneiro C, Pereira H, Ellis KA, Vilela CL (2011) Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing. *Journal of Dairy Research* 78: 49-55.

Bradley A, Bartlett B, Biggs A, (2011). Mastitis testing : PCR or bacteriology, *UK Farmers Weekly*.

Brightling PB, Mein GA, Hope AF, Malmo J, Ryan DP. Using ICCCs. In: *Countdown Downunder Technotes for Mastitis Control,* Dairy Research and Development Corporation, Melbourne , Australia, 2000; including Technote Update Pack, February 2003.

Friendship C, Kelton D, van de Water D, Slavic D, Koskinen M (2010) Field evaluation of the PathoProof mastitis PCR assay for the detection of Staphylococcus aureus infected cows using DHI samples. *NMC Annual Meeting Proceedings (2010)*, pp 226-227.

Hames D, Hooper N (2005) *Biochemistry, BIOS Instant Notes*, Taylor & Francis Group, New York, pp 289-291.

Katholm J, Bennedsgaard TW (2011) Survey of bulk tank milk from all Danish dairy herds in 2009 and 2010 with real-time PCR. *NMC Annual Meeting Proceedings (2011).*

Koskinen MT, Wellenberg GJ, Sampimon OC, Holopainen J, Rothkamp A, Salmikivi L, van Haeringen WA, Lam TJGM, Pyörälä S (2010) Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. *Journal of Dairy Science* 93: 5707-5715.

Mahmmod Saadeldien Y, Klaas Christine I, Toft N, Katholm J (2012) Estimation the diagnostic performance of PathoProof™ Mastitis PCR and bacterial culturing for the detection of Streptococcus agalactiae in milk of Danish dairy cows using latent class models. *Poster presented at the 27th Congress of the World Association for Buiatrics, Lisbon, Portugal; 4th to 8th June, 2012.*

Taponen S, Salmikivi L, Simojoki H, Koskinen MT, Pyörälä S (2009) Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *Journal of Dairy Science* 92: 2610-2617.

Wellenberg GJ, Sampimon OC, Rothkamp A, van Haeringen WA, Lam TJGM (2010) Detection of mastitis pathogens by real-time PCR in clinical and subclinical mastitis samples. *Mastitis Research into Practice: Proceedings of the 5th IDF Mastitis Conference 2010*, pp 539-544.