The use of PCR to diagnose the cause of intramammary infections in dairy cattle

Created: January 2019

Revision due: January 2024

Authors: Richard Olde Riekerink (Bayer), David Kelton (University of Guelph), Abhijit Gurjar (Zoetis), John Middleton (University of Missouri)

Background

Quick and correct identification of the bacteria which cause mastitis is useful in making decisions about treatment and crucial in management of this economically important disease. Traditionally, bacteria have been identified using routine bacterial culture techniques. Recommendations and guidelines to identify mastitis causing bacteria are described in the NMC Laboratory Handbook on Bovine Mastitis (2017). Polymerase chain reaction (PCR) diagnostic techniques for the detection of pathogen DNA sequences were developed in the 1980s primarily for use in research applications. However, in the last few years, diagnosis of bacterial intramammary infection (IMI) using PCR techniques has become commercially available. Consequently, questions about the accuracy and utility of PCR for mastitis diagnosis are frequently asked.

It is important to understand that the question(s) that PCR diagnostics answer may differ from those that culture-based techniques answer. Culturing a milk sample determines if viable bacteria are present above a certain concentration threshold and, if present, some level of bacterial identification is achieved. With PCR, depending on which primer sets are used, the presence of genetic material from certain pathogen(s) in milk can be determined. While the interpretation of the result of the 2 approaches may differ, both may provide useful information and have advantages and disadvantages.

The advantages of PCR compared with traditional culture methods are: PCR is quick, produces fewer false negative responses and can be carried out on preserved milk samples so that cooled transport is not needed. The disadvantages of PCR are that it only identifies pathogens for which the primers are included in the test, it is currently more expensive than bacteriological culture, and it does not currently distinguish between live and dead organisms.

This NMC fact-sheet aims to give the reader some guidance in deciding whether or not to use or offer PCR techniques for the identification of bacterial pathogens causing mastitis, and the limitations of sample type in the application of this technology.

What is PCR?

PCR indicates whether DNA sequences from that specific pathogen(s) are present in the biological sample. PCR tests require an enzyme-based DNA amplification reaction. The term ‘chain reaction’ refers to several cycles of copying a specified stretch of DNA, in this case from the genome of the microorganism in question. PCR detection of DNA can be done using conventional or real-time (also termed quantitative or qPCR) methods. In conventional PCR, the DNA is amplified and detected using gel electrophoresis. In

contrast, most commercial assays use qPCR systems which use special reagents and equipment that allow quantification of the number of DNA amplification cycles required for detection in real time. Based on the number of amplification cycles needed to detect DNA and using a predetermined cycle threshold ("ct") for a particular bacterial pathogen, a diagnosis of positive or negative is determined. Thus, the sensitivity/specificity of the qPCR based system can be changed by changing the number of cycles at which a positive/negative decision is made; similar to changing the volume of milk that is cultured and the number of colonies required to define a sample as positive. Culture based isolation and identification of pathogens in milk have limitations in terms of time and sensitivity. Molecular methods, particularly PCR analysis of milk samples, improve upon these limitations. However, PCR based systems may be limited by the DNA extraction method used. DNA isolation from a food matrix such as milk can present a host of difficulties due to the presence of inhibitory substances, including Ca^{2+}, proteinases, fats and milk proteins, any of which may interact directly with the DNA polymerase to block enzyme activity during the PCR. Blocked polymerase activity poses a risk of complete reaction failure and false negative results, or may reduce sensitivity of detection. For this reason, it is necessary to choose a DNA extraction method that can significantly reduce these problems.

PCR assays present a powerful tool to detect pathogen DNA in aseptically collected diagnostic milk samples from cows with clinical and sub-clinical mastitis. Furthermore, application of PCR to routine milk-recording composite cow-milk samples has been compared to bacteriological culture of aseptically collected quarter and composite samples for several contagious pathogens. The latter comparisons suggest that PCR applied to milk recording samples can have a greater sensitivity and specificity for detection of the contagious pathogens, *Staphylococcus aureus* and *Streptococcus agalactiae*. However, data suggests that, in some cases, carry over from previously milked cows may cause false positive test results when using milk recording samples collected using an in-line sampler.

In order to ensure accurate data when performing PCR analylsis of milk samples, key criteria have to be fulfilled. For example, it is important that quality protocols include the use of appropriate control samples to confirm accuracy and reproducibility. Effective quality control systems need to be established and monitored. For example, use of quality protocols, including the use of control samples that ensure that the system is working properly and which confirm data reproducibility and quality. It is also important to ensure the quality of the milk samples that are collected in the field.

Is it reliable?

The answer depends on the question. Based on several published studies and laboratory experience, it has been argued that PCR can yield information that is as useful as bacteriological culture in certain circumstances. An overview of the varying attributes of culture and PCR based protocols is summarized in Table 1. Tables 2 and 3 compare bacterial culture and PCR based on performance measures.

Table 1. Overview of attributes of bacteriological culture and PCR.

Bacteriological culture	**PCR technique**
- Large range of bacteria can be identified - Experience required to interpret laboratory test results (also experience required for field interpretation) - 0.01 to 0.1 mL (10 - 100 µL) of milk is streaked on the culture plate(s) - Lab turn-around 24 to 48 hours, or sometimes longer for additional identification or slow-growing pathogens, e.g. *Mycoplasma* spp. - Needs cold transport and storage - Bacteria alive and present and can be used for further testing/interrogation, e.g. antimicrobial susceptibility testing, speciation, or strain-typing - Price generally cheaper, mostly depending on cost of labor - Low start-up investment - Special culture media and conditions are required for certain pathogens, e.g. *Mycoplasma* spp.	- Number of bacterial species restricted to primers in the kit - No test interpretation experience required (but still need experience for field application of the results using cow and herd history) - 0.35 mL (350 µL) of milk is used for DNA extraction and testing - Lab turn-around 4 hours - Bacteria may no longer be viable - Generally more expensive than culture - High start-up investment because of equipment needs - Antimicrobial susceptibility testing restricted to beta-lactamase presence only (and we don't know for sure which bacteria in the sample have the beta-lactamase gene) - Analytical sensitivity comparable to culture or better

Table 2. Overview of performance of bacteriological culture and a commercially available PCR kit for different milk sample types.

Milk sample	**Bacteriological culture**	**PCR technique**
Individual quarter collected aseptically	- Literature suggest approximately 20 - 45% culture-negative in clinical mastitis samples (e.g. Olde Riekerink et al., 2008; Hertl et al., 2014)	- Evidence suggests that the proportion of culture negatives are halved (Taponen et al., 2009) and more positive samples (Wellenberg et al., 2010) - Could be used on milk containing antibiotics (theoretical) - Detects dead bacteria as long as present in large enough numbers
Comingled or composite milk recording meter-collected, non-aseptic samples	- Useful for contagious bacteria such as *Staph. aureus, Strep. agalactiae,* and Mycoplasma with the reasonable assumption their origin was an infected quarter. However, presence of large amounts of contaminating bacteria can obscure identification	- Opportunity for automated diagnostics on milk recording samples (be aware of potential carry over from previous cows) - Useful for contagious bacteria such as *Staph. aureus*, *Strep. agalactiae, and Mycoplasma* with the reasonable assumption their origin was an infected quarter. Specifically looking for these and, therefore, easier to identify than cultures with potential contaminants present
Bulk milk sample	- Generally a wider range of bacteria are present - Quantification by plate counting	- Determines presence of target bacteria - Quantification hard to interpret and currently not validated - Useful for low-prevalence bacteria such as *Strep. agalactiae* (Mweu et al., 2012; Katholm et al., 2012) - Results seem to be associated with milk quality (Katholm et al., 2012); however clear interpretation criteria do not exist

Table 3. Comparison of PCR on composite individual cow milk recording samples to bacteriological culture on samples (usually quarter milk) collected aseptically for detection of various mastitis pathogens.

Pathogen	**Bacteriological culture on quarter milk samples**	**PCR on milk recording composite metered milk samples**
Staph. aureus in lactating cow; threshold for determining IMI: • Culture ≥ 2 CFU/0.01ml • CT-value cut-off ≤ 37 (Mahmmod et al., 2013a)	Sensitivity* = 53% Specificity** = 89%	Sensitivity = 91% Specificity = 99%
Staph. aureus in lactating cow; threshold for determining IMI: • Culture ≥ 1 CFU/0.01ml • CT-value cut-off ≤ 37 (Nyman et al., 2016)	Sensitivity = 64% Specificity = 98%	Sensitivity = 86% Specificity = 98%
Staph. aureus at dry off; threshold for determining IMI: • Culture ≥ 1 CFU/0.01ml • CT-value cut-off ≤ 37 (Cederlof et al., 2012)	Sensitivity = 83% Specificity = 97%	Sensitivity = 93% Specificity = 95%
CNS in lactating cow; threshold for determining IMI: • Culture ≥ 3 CFU/0.01ml • CT-value cut-off ≤ 37 (Nyman et al., 2016)	Sensitivity = 21% Specificity = 93%	Sensitivity = 90% Specificity = 76%
Strep. dysgalactiae in lactating cow; threshold for determining IMI: • Culture ≥ 3 CFU/0.01ml • CT-value cut-off ≤ 37 (Nyman et al., 2016)	Sensitivity = 65% Specificity = 99%	Sensitivity = 84% Specificity = 93%
Strep. uberis in lactating cow; threshold for determining IMI: • Culture ≥ 3 CFU/0.01ml • CT-value cut-off ≤ 37 (Nyman et al., 2016)	Sensitivity = 63% Specificity = 99%	Sensitivity = 81% Specificity = 98%

*Sensitivity: ability of a test to correctly identify a true positive milk sample
*Specificity: ability of a test to correctly identify a true negative milk sample

Recommendations:

PCR techniques can be a viable alternative to classical bacterial culture in the diagnosis of intramammary infections causing clinical and subclinical mastitis when using aseptically collected quarter or composite samples. PCR may be more sensitive, faster, and does not necessarily require refrigerated transport of samples.

Using PCR for composite milk samples, typically collected by herd recording organizations can be useful for identifying cows with intramammary infections caused by contagious bacteria such as *Staph. aureus, Strep. agalactiae* or Mycoplasma. It is important to note that a positive PCR result only tells the interpreter that DNA from that organism was present in the milk sample, not the source (e.g., milk from the current cows or residual milk from the previous cow) or viability of the organism. As with bacterial culture, test results must be interpreted in conjunction with the clinical history of the cow and herd, which may include SCC data and observations of milking routine and milking equipment function. Hence, data interpretation is best performed in consultation with the herd veterinarian or milk quality specialist in the context of the sample source and how it was collected

Pre-milking udder preparation is important for reducing the contamination of milk recording metered samples and should include cleaning of teats and udder, pre-stripping to remove fore-milk, and teat end disinfection with 70% alcohol. These practices have been shown to improve the quality of the milk recording sample for diagnosis of intramammary infection with *Staph. aureus* (Mahmmod et al., 2013c).

Due to the high risk of contamination milk recording meter samples, correct interpretation of presence of environmental bacteria is difficult and is NOT recommended.

No interpretation criteria exist for using PCR in bulk tank milk samples with the exception of the presence of *Strep. agalactiae*, for which the PCR is more sensitive than culture. In addition, little research has currently been conducted to determine which sampling strategy and method is the most cost-effective to identify cows infected with contagious pathogens. Murai et al. (2014) showed that qPCR testing ranked 2nd after culture in this situation.

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