



TITLE: Increased Detection of Mastitis Pathogens by Real-Time PCR compared to Bacterial Culture

AUTHORS: Orla M. Keane, Kathleen E. Budd, James Flynn and Finola McCoy

This article is provided by the author(s) and Teagasc T-Stór in accordance with publisher policies.

Please cite the published version.

The correct citation is available in the T-Stór record for this article.

NOTICE: This is the author's version of a work that was accepted for publication in *Veterinary Record*. Changes resulting from the publishing process may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in *Veterinary Record*, 2013;173:268 doi:10.1136/vr.101598

This item is made available to you under the Creative Commons Attribution-Non commercial-No Derivatives 3.0 License.



# **Increased Detection of Mastitis Pathogens by Real-Time Polymerase Chain Reaction compared to Bacterial Culture**

Orla M. Keane<sup>1\*</sup> Kathleen E. Budd<sup>1</sup> James Flynn<sup>2</sup> and Finola McCoy<sup>3</sup>.

<sup>1</sup> Animal & Bioscience Research Department, Animal & Grassland Research & Innovation Centre, Teagasc, Grange, Dunsany, Co. Meath

<sup>2</sup> Moorepark Dairy Production Research Centre, Moorepark, Fermoy, Co. Cork

<sup>3</sup> Animal & Bioscience Research Department, Animal & Grassland Research & Innovation Centre, Teagasc Moorepark, Fermoy, Co. Cork

\* Corresponding Author: Orla M. Keane

Animal & Bioscience Research Department,  
Animal & Grassland Research & Innovation Centre,  
Teagasc Grange,  
Dunsany,  
Co. Meath

E-mail: [orla.keane@teagasc.ie](mailto:orla.keane@teagasc.ie)

Phone: +353 46 9026716

Key words: mastitis, bacterial isolates, cows, PCR.

O.M. Keane: BA(mod), PGradDip, PhD.

K.E. Budd: BSc

F. McCoy: MVB, MSc

## **Abstract**

Rapid and accurate identification of mastitis pathogens is important for disease control. Bacterial culture and isolate identification is considered the gold-standard in mastitis diagnosis but is time-consuming and results in many culture-negative samples. Identification of mastitis pathogens by PCR has been proposed as a fast and sensitive alternative to bacterial culture. The results of bacterial culture and PCR for the identification of the aetiological agent of clinical mastitis were compared. The pathogen identified by traditional culture methods was also detected by PCR in 98% of cases indicating good agreement between the positive results of bacterial culture and PCR. A mastitis pathogen could not be recovered from approximately 30% of samples by bacterial culture, however, an aetiological agent was identified by PCR in 79% of these samples. Therefore a mastitis pathogen was detected in significantly more milk samples by PCR than by bacterial culture (92% and 70% respectively) although the clinical relevance of PCR positive culture negative results remains controversial. A mixed infection of 2 or more mastitis pathogens was also detected more commonly by PCR. Culture negative samples due to undetected *S. aureus* infections were rare. The use of PCR technology may assist in rapid mastitis diagnosis however accurate interpretation of PCR results in the absence of bacterial culture remains problematic.

## Introduction

Mastitis is an endemic disease of dairy cows and is estimated to be one of the most costly diseases to the dairy industry in Ireland and internationally (Barkema and others 2009; More and others 2010). Mastitis is commonly caused by an intramammary infection (IMI) and can be clinical, where the animal displays overt symptoms of disease, or more commonly sub-clinical where the animal displays no outward signs of disease but has an elevated somatic cell count (SCC) (Akerstedt and others 2007). Mastitis is generally caused by a single bacterial pathogen and in order to tailor mastitis control and treatment protocols, reliable identification of the infectious agent is important. Almost 150 different bacterial species have been implicated in bovine mastitis (Radostits 2007) although the majority of cases in Ireland and internationally are caused by a smaller number of bacterial species, including *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae* and coagulase negative Staphylococci (CNS) (Barrett and others 2005; Bradley and others 2007; DAFM 2006-2009, 2010; Makovec and Ruegg 2003). Isolation of a bacterial pathogen from the milk of an infected cow is considered the gold-standard in mastitis diagnosis (NMC 1999, 2004).

While bacterial culture (BC) remains the definitive diagnosis of intramammary infection, it has a number of disadvantages. Culturing of mastitis-causing microorganisms is relatively slow, taking a minimum of 24-48 hours before a pathogen can be reliably identified. The absence of rapid results may result in the use of broad-spectrum antibiotic therapy in many cases, where a narrow-spectrum antibiotic therapy or no antibiotic at all may in fact be more appropriate. Additionally, a sizeable number of mastitic milk samples fail to yield any culturable organisms. For published clinical and sub-clinical mastitis studies the proportion of milk samples yielding no bacterial growth varies between 27% and 50% (Barrett and others 2005; Bradley and others 2007; Hogan and others 1989; Keane and others 2013; Makovec and Ruegg 2003; Olde Riekerink and others 2008). Bacterial culture is primarily used in Ireland to identify the on-farm pathogen challenges rather than for individual animal treatment selection and so pathogen negative samples can be frustrating for the clinician and farmer as they do not indicate a causative organism, and thus the most suitable type of prevention or control measures.

Molecular methods of mastitis pathogen identification have been proposed as an alternative to bacteriological culturing (Gillespie and Oliver 2005). Such methods, based on the detection of bacterial DNA in milk by Polymerase Chain Reaction (PCR), allow rapid (<6 hours) and accurate identification of mastitis pathogens including pathogens that do not grow using conventional culturing techniques (Koskinen and others 2009). Additionally, PCR technology has the potential to detect bacterial DNA in milk from which a viable pathogen cannot be cultured, although the clinical relevance of this remains controversial. Molecular methods target specific pathogens and so must be appropriate for the local major mastitis pathogens. Molecular methods must also be robust and work routinely with clinical samples, many of which contain PCR inhibitors (Chakravorty and Tyagi 2001).

A commercial real-time PCR test for the identification of mastitis pathogens in milk is now available (Pathoproof™ Mastitis PCR Assay, Finnzymes). This test targets the most common mastitis pathogens and was shown to have 100% specificity and sensitivity when detecting bovine mastitis pathogens from pure culture (Koskinen and others 2009). This PCR test has previously been used to determine the prevalence of the common mastitis pathogens, identify cows requiring dry cow treatment and diagnose intramammary infection in BC negative samples (Bexiga and others 2011; Cederlof and others 2012; Katholm and others 2012; Taponen and others 2009). The objective of this study was i) to evaluate the PathoProof test for use in Ireland with clinical mastitic milk samples including those from which no pathogen could be cultured and ii) to evaluate whether the pathogen profile differed between bacterial culture positive/PCR positive samples and bacterial culture negative/PCR positive samples.

## Materials and Methods

### *Sample collection*

As part of a study looking at the aetiology of clinical mastitis in Irish milk-recording dairy herds, a total of 630 quarter milk samples were taken aseptically from affected quarters before antibiotic treatment over a one year period from cows displaying symptoms of clinical mastitis (clots, flecks or blood in the milk or heat or swelling in the udder) from 30 farms as described previously (Keane and others 2013). Samples were frozen and submitted monthly for bacterial culture.

### *Bacteriological analysis*

In order to identify the pathogen causing mastitis, standard microbiological methods recommended by the National Mastitis Council were used (NMC 1999). Briefly, 10 µl of thawed milk were plated directly on aesculin blood agar. The plates were incubated at 37 °C in aerobic conditions and examined after 24 and 48 hours. Plates with no visible colonies were deemed negative for mastitis-associated pathogens. Plates containing three or more colony types were deemed contaminated and discarded, in accordance with NMC guidelines. Remaining colonies were putatively identified based on colony morphology, haemolysis, Gram stain, catalase test and growth on MacConkey lactose indicator, Baird-Parker and Mannitol Salt Agar plates. Putative *Staphylococcus aureus* was distinguished from coagulase negative staphylococci (CNS) by the above tests in addition to the coagulase test. Putative *Streptococcus uberis* was identified by its ability to hydrolyse aesculin while remaining streptococci were identified using the API 20 Strep strips (BioMerieux). *Escherichia coli* were distinguished from other coliforms using the API 20E strips (BioMerieux). At the end of the study period contaminated milk samples were discarded and the remaining 615 frozen milk samples shipped to a separate lab for real-time PCR analysis. From the 615 available milk samples 141 were randomly selected for real-time PCR analysis.

### *Real-time PCR Assay*

A commercial real-time PCR kit was used to identify mastitis pathogens in milk samples from cows displaying signs of clinical mastitis (PathoProof™ Mastitis PCR assay, Finnzymes). This kit can identify 11 mastitis-causing species or species groups, namely *S. aureus*, coagulase negative *Staphylococcus* species, *S. uberis*, *S. dysgalactiae*, *S. agalactiae*, *Enterococcus* spp, *E. coli*, *Serratia marcescens*,

*Klebsiella* spp, *Corynebacterium bovis*, *Trueperella pyogenes* and *Peptoniphilus indolicus*. In addition the test detects the Staphylococcal  $\beta$ -lactamase gene (*blaZ*), responsible for penicillin resistance. Bacterial genomic DNA was extracted from 350  $\mu$ l of thawed milk by first lysing the cells, followed by DNA purification through a Qiagen spin-column according to the manufacturer's instructions with 5% of the final volume used as template genomic DNA. Four separate multiplex real-time PCR reactions were set up for each genomic DNA sample according to manufacturer's instructions. Each multiplex reaction consisted of an Internal Amplification Control (IAC) and three target reactions. Samples were set up in 96 well plates with each plate containing an extraction negative sample and a no template control sample. PCR plates were run on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems). The PCR involved an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 5 sec followed by annealing/extension at 60 °C for 1 minute. Samples were finally cooled to 25 °C for 5 sec. Results were analysed with Norden Lab Mastitis Studio software. Milk samples were considered as positive or negative based on their threshold cycle (Ct) value and visual checking of the amplification plots. The Ct is defined as the minimum number of PCR cycles required to reach a pre-determined fluorescence threshold value, with a lower Ct indicating a greater quantity of DNA in the sample. Where >1 species was detected by RT-PCR the species with the lowest Ct (highest abundance) was considered the predominant species.

#### *Penicillin resistance*

Penicillin and oxacillin susceptibility tests were performed for all *Staphylococcus aureus* and coagulase negative Staphylococcus (CNS) isolates by the disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2008). Penicillin (6  $\mu$ g/10 IU) and oxacillin (1  $\mu$ g) were tested. The *S. aureus* American Type Tissue Collection (ATCC) 25923 (penicillin susceptible) and *E. coli* ATCC 25922 (penicillin resistant) strains were used as negative and positive controls respectively and isolates were classified as resistant or susceptible based on comparison of the measured zone diameter with the zone diameter interpretive standards for Staphylococcal veterinary pathogens ((CLSI) 2008).

#### *16S rDNA sequencing*

DNA was extracted from three Streptococcal isolates that were identified as putative *S. uberis* based on aesculin hydrolysis but identified by the PathoProof assay as *S.*

*dysgalactiae* using the PurElute™ Bacterial Genomic Kit (Edge Biosystems) as described in the manufacturer's protocol. The hypervariable region of the 5' end of the bacterial 16S rDNA gene was amplified from genomic DNA using the primers 16SFa: 5' GCTCAGATTGAACGCTGG 3' and 16SR: 5' TACTGCTGCCTCCCGTA 3' as described previously (Harris and Hartley 2003). PCR products were purified with the QIAamp PCR purification kit (Qiagen) and sent to Source BioScience (Dublin) for Sanger sequencing. Sequence chromatograms were checked for quality using BioEdit V7.0.0 and bacterial DNA species identified by comparison of the 16S rDNA sequence with the GenBank database (<http://www.ncbi.nlm.nih.gov>) using BLAST (Altschul and others 1990). If species-specific sequences in the database matched the query sequence with 100% identity then the query sequence was determined to come from that species (Harris and Hartley 2003).



## Results

A total of 141 milk samples from cows with clinical mastitis were randomly selected for PCR testing. The 141 randomly selected samples were representative of the larger dataset of 615 samples as no pathogen was significantly under or over-represented on bacterial culture. However, there was a higher proportion of culture negative samples in the larger dataset. Of the 141 samples, conventional bacterial culture identified a single udder pathogen in 94 samples (67%), a mixture of 2 udder pathogens in 4 samples (3%) while no viable pathogens were cultured from the remaining 43 samples (30%). PCR testing detected a single udder pathogen in 89 samples (63%), 2 udder pathogens in 38 samples (27%), three udder pathogens in 3 samples (2%) while no pathogens were detected in 11 (8%) of samples. Therefore, significantly more samples were positive for at least one mastitis pathogen when tested by PCR than by bacterial culture ( $P < 0.0001$ ; Chi-square test).

### Single isolate samples

A single udder pathogen was isolated by bacterial culture and identified using standard biochemical tests from 94 milk samples. The occurrence of the different bacterial pathogen species (Table 1) reflects their frequency in the population from which the samples were derived (Keane and others 2013). The pathogen identified by traditional culture methods was also detected by PCR in 92/94 (98%) of cases (Table 1). However, in 3 of these cases the pathogen was not detected as the predominant pathogen in the sample but was detected as an additional pathogen. For the remaining 2 samples no udder pathogens were detected by PCR despite being culture positive. PCR testing also indicated that a substantial proportion (23/94; 24%) of the milk samples contained DNA from an additional bacterial species that was not detected by conventional culture. Three udder pathogens were detected by PCR in 2/94 (2%) of the samples. Milk samples positive for *E. coli* by BC were significantly more likely to indicate the presence of an additional pathogen by PCR compared to samples positive for the other pathogens ( $P < 0.001$ ; Chi-square test). The results of the bacterial culture and PCR testing are shown in Table 1 and the additional pathogens detected by PCR but not by culture are summarised in Table 2.

Three samples, originally identified as putative *S. uberis* based on aesculin hydrolysis, were identified as *S. dysgalactiae* by PCR testing. In order to definitively identify the isolates, genomic DNA was extracted from the 3 isolates and the

hypervariable 5' end of the 16S rDNA gene amplified and sequenced (Harris and Hartley 2003). The amplified 16S rDNA sequence matched *S. dysgalactiae* 16S rDNA sequences deposited in GenBank with 100% sequence identity, unambiguously identifying these isolates as *S. dysgalactiae*.

### **Double isolate samples**

Two udder pathogens were isolated by bacterial culture from four milk samples (2 positive for *S. dysgalactiae*/CNS and 2 positive for *S. uberis*/CNS). For 2/4 of these samples there was complete agreement between the results of bacterial culture and PCR testing (1 *S. dysgalactiae*/CNS and 1 *S. uberis*/CNS). For the remaining two samples, only the Streptococcus species were detected by PCR.

### **Antimicrobial susceptibility**

In total 42 isolates (36 *S. aureus* and 6 CNS) were tested for penicillin resistance or susceptibility by disk diffusion testing. A total of 17 isolates (40%) were found to express penicillin resistance *in vitro* with the remaining 25 isolates (60%) penicillin sensitive. For 39/42 isolates (93%) the phenotypic characteristic of the isolate was in agreement with the presence or absence of the Staphylococcal  $\beta$ -lactamase gene. However, for 3 isolates the genotypic and phenotypic tests did not agree. Two samples displayed positive PCR reactions for the *blaZ* gene, however, these isolates did not display penicillin resistance. The *blaZ* gene was not amplified from a further sample despite the isolate being penicillin resistant by disk diffusion testing. This isolate was further tested by the disk diffusion method and found to be resistant to ampicillin but susceptible to oxacillin.

### **Culture negative samples**

Of the 141 samples cultured, no viable pathogens were identified in 43 samples (30%). Of these culture-negative samples, 34 were positive by PCR for mastitis pathogens with no mastitis pathogens detected in the remaining 9 samples. Of the 34 PCR positive samples, a single mastitis pathogen was detected in 20 samples, two pathogens were detected in 13 samples while three pathogens were detected in a single sample. The most common pathogen detected in the culture negative samples was *S. uberis* which was detected in 16 samples (47%) and was the predominant pathogen in 14 samples (41%). *S. aureus*, which was the most common pathogen in culture positive samples (40%), was found in only 5 (15%) BC-negative PCR-positive samples and was the predominant mastitis pathogen in just three samples (9%). Culture-positive milk samples were therefore significantly more likely

to indicate the presence of *S. aureus* than culture-negative PCR-positive samples ( $P < 0.001$ ; Chi-square test). The pathogens detected by PCR in culture negative samples are shown in Table 3 and the pathogen profile in BC positive and BC negative/PCR positive samples is displayed in Figure 1. With the exception of *S. aureus*, no pathogen was detected significantly more or less often in culture positive compared to culture negative PCR positive samples.

## Discussion

An udder pathogen was detected in 70% of quarter milk samples from cows with clinical mastitis by conventional bacterial culture. In contrast, 92% of samples were positive for udder pathogen DNA by PCR. Hence 30% and 8% of the milk samples provided culture and PCR negative results respectively. In total 79% of culture-negative milk samples gave positive results by real-time PCR analysis. The frequency of mastitis pathogen detection was significantly higher with PCR compared with bacterial culture. It must be noted, however, that the sample volume for the PCR test is equivalent to 1.75 x that of bacterial culture and this may account for increased pathogen detection in some of these samples. Many studies report a large proportion of culture-negative milk samples from cows with clinical or sub-clinical mastitis with values typically ranging from 27-50% (Barrett and others 2005; Bradley and others 2007; Makovec and Ruegg 2003; Petrovski and others 2011). In our study 30% of samples were negative by bacterial culture. The use of PCR technology may assist in detection of intramammary bacteria, although its usefulness in improving mastitis diagnosis and informing treatment decisions in such cases remains to be further evaluated.

A number of studies from other countries have evaluated the PathoProof assay using a variety of sample types. A study from Finland which tested 79 culture negative mastitic milk samples found 43% yielded positive results for bacterial DNA by PCR (Taponen and others 2009). This is in contrast to our study in which 79% of culture negative mastitic milk samples gave positive PCR results. In Finland the most commonly detected species in culture negative PCR positive samples were *S. uberis*, CNS and *C. bovis*. A second study consisting of samples from Finland and the Netherlands from 780 cows with clinical mastitis, found 76% of culture negative clinical mastitis milk samples were positive by PCR (Koskinen and others 2010). This figure is in close agreement with our study. Koskinen *et al* also found CNS, *C. bovis*, *S. uberis* and *S. dysgalactiae* were the species most commonly identified in culture negative PCR positive samples. We also commonly found *S. uberis* and *S. dysgalactiae* in culture negative PCR positive milk. Two other studies from Portugal and Germany found 47% and 57% of culture negative samples gave PCR positive results respectively. The predominant species detected were CNS and *C. bovis* in both studies with *E. coli* in Portugal and *S. aureus* in Germany also commonly

detected (Bexiga and others 2011; Spittel and Hoedemaker 2012). Neither study, however, included clinical mastitis samples and consisted of samples with varying SCC. Such variation between studies in detection and identification of mastitis pathogens demonstrates the complexity in interpreting PCR positive results.

The samples used in this study were frozen prior to bacterial culture and PCR analysis. It has previously been demonstrated that short-term freezing of milk (~24 h) increases the proportion of culture negative samples (Bexiga and others 2011) although freezing for up to 6 weeks was reported to have no effect on the viability of the major mastitis pathogens (Murdough and others 1996). It has also been reported that freezing for 4 weeks had no effect on *S. aureus*, streptococci and *T. pyogenes* isolation rates although it did increase the number of CNS positive samples and decrease the number of *E. coli* positive samples (Schukken and others 1989). Our milk samples were collected and cultured monthly and the possibility that freezing had an effect on the relative frequency of some of the isolated mastitis pathogen species cannot be excluded.

Multiple pathogens in milk samples from cows with clinical mastitis were detected more frequently with PCR than bacterial culture. PCR has previously been reported to detect multiple pathogens more commonly than conventional culture (Bexiga and others 2011; Spittel and Hoedemaker 2012). In our study, a mixture of 2 bacterial species was detected in 3% of milk samples by bacterial culture. However, a mixed infection was detected in 29% of samples with PCR. A sample from which 3 or more bacterial species are cultured is considered contaminated (NMC 1999). Three milk samples indicated the presence of three mastitis pathogens by PCR and so could be considered contaminated on the basis of PCR analysis alone, although the validity of applying culture-based standards to molecular based methods of mastitis pathogen detection remains questionable. The most common additional pathogens found were *S. dysgalactiae* and *S. uberis*. Although both species are environmental pathogens they may not be contaminants in this instance as other environmental species such as *E. coli*, CNS and Enterococcus spp were not commonly identified as additional pathogens.

Milk samples positive for *E. coli* by BC were significantly more likely to indicate the presence of an additional pathogen by PCR. *E. coli* can be a common contaminant of milk samples (DAFM 2010), therefore the detection of an additional major mastitis pathogen by PCR in these samples may be clinically significant.

*Staphylococcus aureus* was recovered from two samples which were PCR negative and species identification was confirmed genotypically. These samples showed a low level of PCR amplification of many targets but did not reach the threshold value for any target species. The internal amplification control was positive for both samples indicating that the PCR reaction did not fail. There are a number of possible reasons for BC positive PCR negative results, for example these samples may have had bacterial numbers below the limit of detection of PCR but cultured an organism by chance, there may have been polymorphisms in the PCR target region resulting in amplification failure or the culture plate may have been contaminated. Further work would be required to elucidate the reason for these BC positive PCR negative samples.

In this study 40% of mastitis-associated Staphylococcal isolates displayed penicillin resistance. The percentage of penicillin resistant isolates is in good agreement with the percentage of resistant isolates reported in the Irish All-island Animal Disease Surveillance Report (DAFM 2010). The PCR test utilised in this study detects the presence of the Staphylococcal *blaZ* gene and expression of *BlaZ* has been demonstrated to confer penicillin resistance (Rowland and Dyke 1990). The results of the antimicrobial susceptibility and PCR tests agreed for the vast majority of isolates (93%). However, a number of discrepancies were observed. The *blaZ* gene was amplified from two isolates that did not display penicillin resistance. It has previously been reported that 10% of penicillin susceptible *S. aureus* isolates possess the *blaZ* gene (El Feghaly and others 2012). The *blaZ* gene could not be amplified from a further isolate despite the fact that this isolate displayed penicillin resistance. The *mecA* gene, which confers meticillin/oxacillin resistance, also confers penicillin resistance. In order to determine if this isolate was potentially penicillin resistant due to carriage of *mecA* it was tested for phenotypic oxacillin resistance. Phenotypic testing was used due to the presence of *mecA* homologues which can also encode meticillin resistance (Garcia-Alvarez and others 2011). The isolate was oxacillin sensitive indicating that the observed penicillin resistance is unlikely to be due to the presence of *mecA* or *mecA* homologues.

For culture-positive samples this study shows a very high concordance between results of bacterial culture and PCR indicating the potential of PCR testing to provide rapid, clinically relevant information, at least for this subset of samples. No pathogen was detected in 30% of samples by BC, however, PCR detected a mastitis

pathogen in 79% of these samples. An evaluation of the clinical relevance of PCR testing in the case of culture negative samples is problematic as the current gold-standard is bacterial culture. However, this gold-standard has limitations, which will penalise the PCR test should it correctly identify an infecting pathogen when bacterial culture does not. A minimum of 24-48 hours are required for pathogen detection and identification with culture-based methods. With PCR, results may be available within 4-6 hours. Therefore identification of bacterial species by PCR could provide rapid information to the farmer on the bacterial challenges encountered on that farm. However, the detection of pathogen nucleic acid in milk samples does not indicate the presence of viable pathogens, required to cause disease and so the full clinical significance of PCR based testing in the absence of confirmatory bacterial culture requires further study.

A number of studies have examined the milk microbiome using metagenomic methods (Bhatt and others 2012; Kuehn and others 2013; Oikonomou and others 2012). DNA from a large array of bacterial species was detected in samples from both mastitic and healthy quarters, although differences were observed between the microbiome of healthy and mastitic milk (Kuehn and others 2013; Oikonomou and others 2012). In one study, Staphylococci and Streptococci DNA was found in almost all culture negative samples, including those from healthy quarters (SCC < 10,000) (Oikonomou and others 2012), indicating the potential of culture-independent methods to generate false positive results. The quantity of starting material used for metagenomic methods exceeds that required for conventional culture, which may lead to a higher proportion of pathogen positive samples. In our study, bacteria were detected in higher abundance (lower Ct) in BC positive samples than in BC negative samples warranting further studies on the role of bacterial quantification in determining clinical significance of culture-independent methods. Molecular pathogen identification could also be combined with disease indicators such as clinical symptoms or somatic cell count to inform PCR result interpretation.

In our study, the distribution of mastitis pathogens detected by PCR in BC negative samples was not significantly different from that of mastitis pathogens in BC positive samples with the exception of *S. aureus* which was identified significantly less often in BC negative samples. This was an interesting finding, as traditionally practitioners have often interpreted BC negative samples from cows with clinical mastitis symptoms, or an elevated SCC as a probable indicator of *S. aureus* infection,

not detected with culture due to intermittent bacterial shedding. Our results do not support this theory, however, further work is required to confirm this finding due to the low number of culture negative samples in this study.

Molecular methods of mastitis pathogen diagnosis offer a number of advantages: i) they allow more rapid detection of mastitis-causing pathogens than traditional bacterial culture methods ii) they are more amenable to high-throughput analysis and automation than bacterial culture methods and iii) they have the potential to diagnose the cause of intramammary infection when no viable pathogens can be cultured. However, the gold standard for mastitis diagnosis remains bacterial culture as the clinical relevance of detecting bacterial nucleic acid in the absence of viable pathogens remains controversial. The PathoProof™ Mastitis PCR assay targets all the major pathogens associated with clinical mastitis in Irish milk-recording herds in a recent study (Keane and others 2013) although the cost of the test exceeds that of bacterial culture. In this study we found good general agreement between the positive results of BC and PCR. The use of PCR resulted in an increased number of samples positive for one or multiple pathogens and therefore under present criteria has the potential to result in more samples being discarded due to contamination. The overall number of positive pathogen detections was increased with the use of PCR, however, there is currently no method to reliably distinguish which of these PCR positive results are clinically relevant.



## **Acknowledgements**

Funding for this study was provided by Teagasc core funding. We would like to thank the farmers for their participation in the study and Noel Byrne (Teagasc) for sample collection.

**Figure 1.** Distribution of mastitis pathogens in BC-positive and BC-negative/PCR positive samples.

**Table 1.** Identification of udder pathogens by bacterial culture (BC) and PCR in samples from which a single isolate was recovered by BC.

Pathogen detected by BC	No. of BC positive samples (%)	No. of PCR positive samples (%)†	No. of PCR positive samples‡	No. of PCR negative samples	No. of samples positive for additional species
<i>S. aureus</i>	36 (38)	33 (37)	1	2	4
<i>S. uberis</i>	22 (23)	22 (25)	0	-	5
<i>E. coli</i>	16 (17)	16 (18)	0	-	11
<i>S. dysgalactiae</i>	10 (11)	9 (10)	1	-	1
CNS	6 (6)	5 (6)	1	-	3
<i>T. pyogenes</i>	4 (4)	4 (4)	0	-	1
Total	94	89	3	2	25

† Species was the predominant pathogen

‡ Species was detected but was not the predominant pathogen

**Table 2.** Additional species detected in samples with >1 mastitis pathogen by PCR

Pathogen detected by BC	Additional species detected by PCR						
	<i>S. aureus</i>	<i>S. uberis</i>	<i>E. coli</i>	<i>S. dysgalactiae</i>	CNS	<i>T. pyogenes</i>	Other
<i>S. aureus</i>	-	0	0	3	0	0	1
<i>S. uberis</i>	0	-	0	1	1	2	1
<i>E. coli</i>	1	7	-	3	2	0	0
<i>S. dysgalactiae</i>	0	0	0	-	0	1	0
CNS	0	2	0	1	-	0	0
<i>T. pyogenes</i>	1	0	0	0	0	-	0

**Table 3.** Predominant and additional pathogens detected by PCR in bacterial culture negative samples

Pathogen	Predominant pathogen	Additional pathogen
<i>S. aureus</i>	3	2
<i>S. uberis</i>	14	2
<i>E. coli</i>	7	3
<i>S. dysgalactiae</i>	6	3
CNS	2	3
<i>T. pyogenes</i>	1	0
Other	1	2
Total	34	15

## References

- (CLSI), C. A. L. S. I. (2008) Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals; Approved Standard - Third Edition. pp 65-72
- AKERSTEDT, M., PERSSON WALLER, K. & STERNESJO, A. (2007) Haptoglobin and serum amyloid A in relation to the somatic cell count in quarter, cow composite and bulk tank milk samples. *J Dairy Res* 74, 198-203
- ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. (1990) Basic local alignment search tool. *J Mol Biol* 215, 403-410
- BARKEMA, H. W., GREEN, M. J., BRADLEY, A. J. & ZADOKS, R. N. (2009) Invited review: The role of contagious disease in udder health. *J. Dairy Sci* 92, 4717-4729
- BARRETT, D. J., HEALY, A. M., LEONARD, F. C. & DOHERTY, M. L. (2005) Prevalence of pathogens causing subclinical mastitis in 15 dairy herds in the Republic of Ireland. *Ir Vet J* 58, 333-337
- BEXIGA, R., KOSKINEN, M. T., HOLOPAINEN, J., CARNEIRO, C., PEREIRA, H., ELLIS, K. A. & VILELA, C. L. (2011) Diagnosis of intramammary infection in samples yielding negative results or minor pathogens in conventional bacterial culturing. *J Dairy Res* 78, 49-55
- BHATT, V. D., AHIR, V. B., KORINGA, P. G., JAKHESARA, S. J., RANK, D. N., NAURIYAL, D. S., KUNJADIA, A. P. & JOSHI, C. G. (2012) Milk microbiome signatures of subclinical mastitis-affected cattle analysed by shotgun sequencing. *J Appl Microbiol* 112, 639-650
- BRADLEY, A. J., LEACH, K. A., BREEN, J. E., GREEN, L. E. & GREEN, M. J. (2007) Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet Rec* 160, 253-257
- CEDERLOF, S. E., TOFT, N., AALBAEK, B. & KLASS, I. C. (2012) Latent class analysis of the diagnostic characteristics of PCR and conventional bacteriological culture in diagnosing intramammary infection caused by *Staphylococcus aureus* in dairy cows at dry off. *Acta Vet Scand* 54, 65
- CHAKRAVORTY, S. & TYAGI, J. S. (2001) Novel use of guanidinium isothiocyanate in the isolation of Mycobacterium tuberculosis DNA from clinical material. *FEMS Microbiol Lett* 205, 113-117
- CLSI (2008) Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals
- DAFM (2006-2009) Regional Veterinary Laboratories Surveillance Report. <http://www.agriculture.gov.ie/rvlreport/>
- DAFM (2010) 2010 AFBI/DAFF All-island Surveillance Report. <http://www.agriculture.gov.ie/rvlreport/>
- EL FEGHALY, R. E., STAMM, J. E., FRITZ, S. A. & BURNHAM, C. A. (2012) Presence of the bla(Z) beta-lactamase gene in isolates of *Staphylococcus aureus* that appear penicillin susceptible by conventional phenotypic methods. *Diagn Microbiol Infect Dis* 74, 388-393
- GARCIA-ALVAREZ, L., HOLDEN, M. T., LINDSAY, H., WEBB, C. R., BROWN, D. F., CURRAN, M. D., WALPOLE, E., BROOKS, K., PICKARD, D. J., TEALE, C., PARKHILL, J., BENTLEY, S. D., EDWARDS, G. F., GIRVAN, E. K., KEARNS, A. M., PICHON, B., HILL, R. L., LARSEN, A. R., SKOV, R. L., PEACOCK, S. J., MASKELL, D. J. & HOLMES, M. A. (2011) Meticillin-resistant *Staphylococcus aureus* with a novel mecA homologue in human and bovine

populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis* 11, 595-603

GILLESPIE, B. E. & OLIVER, S. P. (2005) Simultaneous detection of mastitis pathogens, *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. *J Dairy Sci* 88, 3510-3518

HARRIS, K. A. & HARTLEY, J. C. (2003) Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service. *J Med Microbiol* 52, 685-691

HOGAN, J. S., SMITH, K. L., HOBLET, K. H., SCHOENBERGER, P. S., TODHUNTER, D. A., HUESTON, W. D., PRITCHARD, D. E., BOWMAN, G. L., HEIDER, L. E., BROCKETT, B. L. & ET AL. (1989) Field survey of clinical mastitis in low somatic cell count herds. *J Dairy Sci* 72, 1547-1556

KATHOLM, J., BENNEDSGAARD, T. W., KOSKINEN, M. T. & RATTENBORG, E. (2012) Quality of bulk tank milk samples from Danish dairy herds based on real-time polymerase chain reaction identification of mastitis pathogens. *J Dairy Sci* 95, 5702-5708

KEANE, O. M., BUDD, K. E., FLYNN, J. & MCCOY, F. (2013) Pathogen profile of clinical mastitis in Irish milk-recording herds reveals a complex aetiology. *Vet Record* In Press

KOSKINEN, M. T., HOLOPAINEN, J., PYORALA, S., BREDBACKA, P., PITKALA, A., BARKEMA, H. W., BEXIGA, R., ROBERSON, J., SOLVEROD, L., PICCININI, R., KELTON, D., LEHMUSTO, H., NISKALA, S. & SALMIKIVI, L. (2009) Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *J Dairy Sci* 92, 952-959

KOSKINEN, M. T., WELLENBERG, G. J., SAMPIMON, O. C., HOLOPAINEN, J., ROTHKAMP, A., SALMIKIVI, L., VAN HAERINGEN, W. A., LAM, T. J. & PYORALA, S. (2010) Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. *J Dairy Sci* 93, 5707-5715

KUEHN, J. S., GORDEN, P. J., MUNRO, D., RONG, R., DONG, Q., PLUMMER, P. J., WANG, C. & PHILLIPS, G. J. (2013) Bacterial community profiling of milk samples as a means to understand culture-negative bovine clinical mastitis. *PLoS One* 8, e61959

MAKOVEC, J. A. & RUEGG, P. L. (2003) Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J Dairy Sci* 86, 3466-3472

MORE, S. J., MCKENZIE, K., O'FLAHERTY, J., DOHERTY, M. L., CROMIE, A. R. & MAGAN, M. J. (2010) Setting priorities for non-regulatory animal health in Ireland: results from an expert Policy Delphi study and a farmer priority identification survey. *Prev Vet Med* 95, 198-207

MURDOUGH, P. A., DEITZ, K. E. & PANKEY, J. W. (1996) Effects of freezing on the viability of nine pathogens from quarters with subclinical mastitis. *J Dairy Sci* 79, 334-336

NMC (1999) *Laboratory Handbook on Bovine Mastitis*. Madison WI, NMC Inc

NMC (2004) *Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality*. Madison WI

OIKONOMOU, G., MACHADO, V. S., SANTISTEBAN, C., SCHUKKEN, Y. H. & BICALHO, R. C. (2012) Microbial diversity of bovine mastitic milk as described by pyrosequencing of metagenomic 16s rDNA. *PLoS One* 7, e47671

- OLDE RIEKERINK, R. G., BARKEMA, H. W., KELTON, D. F. & SCHOLL, D. T. (2008) Incidence rate of clinical mastitis on Canadian dairy farms. *J Dairy Sci* 91, 1366-1377
- PETROVSKI, K. R., WILLIAMSON, N. B., LOPEZ-VILLALOBOS, N., PARKINSON, T. J. & TUCKER, I. G. (2011) Culture results from milk samples submitted to veterinary diagnostic laboratories from August 2003 to December 2006 in New Zealand. *N Z Vet J* 59, 317-322
- RADOSTITS, O. (2007) *Diseases of the Mammary Gland*. London, Elsevier Ltd
- ROWLAND, S. J. & DYKE, K. G. (1990) Tn552, a novel transposable element from *Staphylococcus aureus*. *Mol Microbiol* 4, 961-975
- SCHUKKEN, Y. H., GROMMERS, F. J., SMIT, J. A., VANDEGEER, D. & BRAND, A. (1989) Effect of freezing on bacteriologic culturing of mastitis milk samples. *J Dairy Sci* 72, 1900-1906
- SPITTEL, S. & HOEDEMAKER, M. (2012) Mastitis diagnosis in dairy cows using PathoProof real-time polymerase chain reaction assay in comparison with conventional bacterial culture in a Northern German field study. *Berl Munch Tierarztl Wochenschr* 125, 494-502
- TAPONEN, S., SALMIKIVI, L., SIMOJOKI, H., KOSKINEN, M. T. & PYORALA, S. (2009) Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J Dairy Sci* 92, 2610-2617