FAQ SHEET

What techniques can be used to identify bacteria other than traditional milk culture?

Reliable identification of causal bacteria is a vital part of a mastitis investigation. Similarly, accurate identification of an active intramammary infection is important in the routine management and treatment of dairy cows on-farm.

The culture of affected milk has long been the accepted "gold standard" for identification of mastitis-causing bacteria (as well as other microbes such as yeast and algae) (NMC 2017). Milk is spread onto agar plates and bacteria identified by their colony size, shape, colour, changes to the agar, staining, and various enzymatic tests.

Bacterial growth is classified into:

- Pure growth: presence of only one type of bacteria
- Mixed growth: presence of 2 types of bacteria
- Contaminated growth: 3 or more types of bacteria.

The degree of growth can also be assessed, from light to moderate to very heavy growth.

Milk samples can be submitted to certified laboratories or veterinary clinics for bacterial culture. Quality control is vital and trained and experienced operators will provide the most accurate results. The major disadvantage of laboratory milk culture is the time that elapses between sample collection, transport to the laboratory and subsequent bacterial growth and analysis. There may be loss of bacterial viability during sample handling and it important that efforts are made to minimise the growth of contaminants. The costs of laboratory culture vary but can be relatively high when small numbers of samples are submitted.

A increasing focus on antibiotic stewardship in dairy cattle has seen significant changes in bacterial identification methods. To enable targeted treatment of clinical mastitis during lactation, on-farm culture systems have been implemented that allow identification of an active infection in less than 24 hours (Lago *et al* 2011). In the laboratory, new technology involving DNA and protein analysis has led to faster and more detailed bacterial identification, with many laboratories adding these systems to their standard milk culture processes (McDougall *et al* 2018, Ferreira et al 2018).

These newer methods of bacterial identification – those with published data on their use and accuracy – are described and discussed below. It is important for the milk quality advisor to become familiar with new diagnostic systems as they enter the market and make a reasoned judgement of their benefits and pitfalls. They should look for peer-reviewed validation studies of new technology and seek help from an experienced mentor if having difficulty interpreting the results. Key statistics that determine a diagnostic test's performance are detailed in Box 1.

Box 1 Key statistics to consider when assessing diagnostic test performance

		Disease state (results from an acceptable reference "gold standard" test)	
		Positive	Negative
Test results	Positive	True positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)

- Sensitivity (Se), the ability of a test to identify infected animals correctly: TP/(TP+FN)
- Specificity (Sp), the ability of a test to identify non-infected animals correctly: TN/(TN+FP)
- Positive predictive value (PPV), the likelihood that a positive test is truly positive: TP/(TP + FP)
- Negative predictive value (NPV), the likelihood that a negative test is truly negative: TN/(TN + FN)
- Accuracy (Ac): (TP+TN)/(TP+TN+FP+FN)
- Kappa (κ) coefficient: the strength of agreement between the new diagnostic test and the reference test (controls for correct agreement that might be due to chance): 0 = none, 0.01–0.20 poor, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 good, and 0.81–1 very good agreement.

Rapid culture plates

Rapid culture plates have been designed to distinguish between common mastitis pathogens without the need for further enzymatic testing (such as coagulase, catalase etc). This enables incubation and identification of bacteria to be undertaken on-farm or within a veterinary clinic. Identification is usually possible within 24 hours. Rapid culture plates can be used to make selective treatment decisions, for example a cow with no growth may not receive antibiotic treatment. Rapid culture plates are bi-, tri- or quad plates. That is, they have 2, 3 or 4 sectors each containing a different culture media. Rapid culture plates are most reliable when used to classify infections into broad diagnostic categories, such as no growth, Gram-positive, or Gram-negative growth. However, some products are able to identify some pathogens at the genus level and even down to species level (e.g. *Staphylococcus aureus*, *E. coli*) (Lago and Godden, 2018).

There are many different rapid culture plates on the market with a different array of agars (see Figure 1 below). The various agars used in these products are described in Table 1.

Table 1 Agars used in rapid culture plates

Agar	Description
Blood agar	A "control" that grows most bacteria
MacConkey agar	For growth of Gram-negative bacteria and identification of coliforms
Factor™ media	Allows Gram-positive growth only
Modified Edwards agar and Focus™ media	Selective for *Streptococcus* and *streptococcus*-like organisms (SSLO)
Vogel Johnson or modified mannitol salt agar	Identify *Staph aureus* specifically
Chromogenic agars	These contain chromogens: colourless chromophores joined to a substrate for a target bacterial enzyme. When the chromogen enters the target bacteria, the enzyme splits it apart. The chromophore forms a coloured, insoluble precipitate.

If considering using one of these rapid culture plates, milk quality advisors should look for published information validating their performance. Studies will differ in terms of sample selection and transport, the people involved in collection and analysis of the samples, pathogen prevalence, the reference test, and the basis of determining agreement (Gram status vs genus vs species level).

Griffioen *et al* (2018) investigated how well four commercial rapid culture plates – read by technicians – agreed with routine bacterial culture of milk samples submitted to a laboratory. This study evaluated both clinical, subclinical and non-clinical milk samples and found that overall, the rapid plates had poor to fair agreement for Gram-positive bacteria (κ = 0.14 to 0.25) and good agreement for Gram-negative bacteria (κ approximately 0.70, for all four products). The agreement for no growth was fair (κ = 0.22 to 0.34).

In another study (Ferreira *et al* 2018), milk samples from clinically affected quarters were cultured on four different rapid culture plate systems and by two additional reference laboratories. Sample collection and plate interpretation was undertaken by trained research personnel. In this study, the reference test was the agreeing results from the two laboratories. Of 211 milk samples, for the broad categories of Gram-positive and Gram-negative, the accuracy of the different plates ranged from 73–90%. The strength of agreement for these categories ranged from moderate to very good (κ = 0.53 to 0.81). The ability to categorize bacterial growth to the genus and species varied amongst the commercial plates, from moderate to good agreement with the reference test (κ = 0.45–0.79).

McDougall *et al* (2018) compared the results of on-farm use of a rapid culture plate by farm staff against laboratory identification, when implemented in a selective antibiotic treatment protocol for cases of clinical mastitis. In the protocol, diagnostic categories were used to assign treatments on-farm (Gram-negative, no growth, *Staph aureus*, and "other" bacteria). The overall agreement between on-farm and laboratory results was 188/331 (57%; kappa=0.31, poor) but varied between farms from 45 to 88%. The authors noted that the level of agreement between on-farm and laboratory culture would have been even lower if the categorisation was undertaken to genus or species level.

While the use of rapid culture plates is promoted for selective antibiotic treatment of clinical mastitis, veterinarians and farmers may also consider using them as part of the diagnostic workup of a herd mastitis problem. In this situation, it is important to consider the quality of the information that these plates may provide as, as shown in the studies above, the ability of these plates to identify bacteria to the genus/species level varies considerably. Sipka *et al* (2019) looked at the strength of agreement between three different on-farm culture plates and laboratory culture when interpreted by a trained technician versus five veterinary students using the products' brochures as guides. The results varied with pathogen group and product. The trained reader consistently performed better, with moderate to very good agreement with the reference test, while the veterinary students had poor to good agreement. The authors noted that it is important to provide "*training beyond the instruction manual*" and ensure quality control of these rapid culture systems. McDougall *et al* (2018) also noted the variability in implementation of rapid culture plates on-farm and the need for specific training of staff and on-going monitoring of the results. For veterinarians considering sourcing and using these systems in-house, continuing education in microbiology is essential.

It is important to provide training beyond the instruction manual to ensure quality control of these rapid culture systems. ✓

Petrifilm system

Petrifilms are tools commonly used in food safety and have been adapted as a way of assessing bacterial infection in cows' milk. Two Petrifilms are used: Aerobic Count (AC)–Petrifilm™ and Coliform Count (CC)–Petrifilm™. Milk is diluted and spread between two thin films, incubated and then the number of bacteria counted after 24 hours.

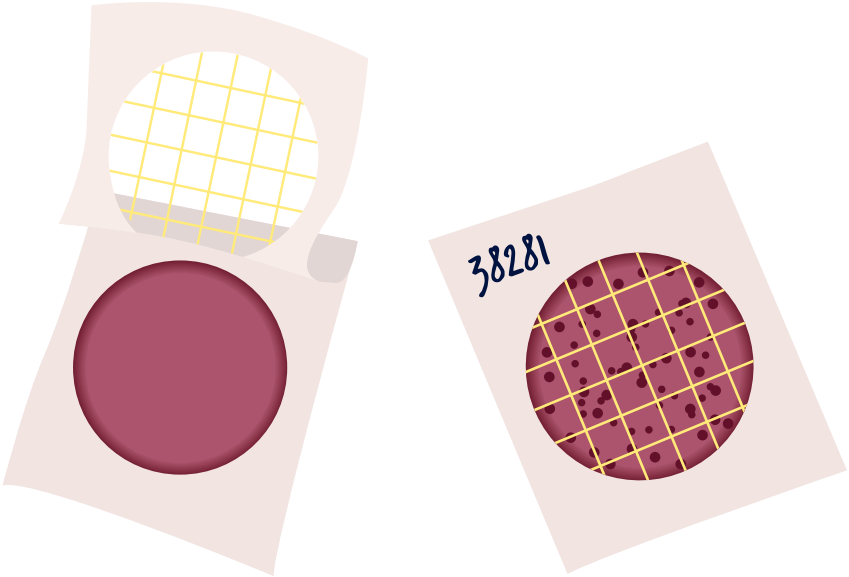
McCarron *et al* (2009) investigated the performance of the Petrifilm system to class clinical milk samples into Gram-positive and Gram-negative categories. They found that test sensitivity was highest when a case was classed as Gram-negative if there were ≥20 colonies present on the CC and classed as Gram-positive if there were <20 colonies present on the CC and ≥5 colonies present on the AC.

Mansion-de Vries *et al* (2014) compared assessment by Petrifilm™ on-farm to laboratory bacterial culture for 616 cases of clinical mastitis. When divided into three categories – Gram-positive, Gram-negative and no growth – the Petrifilm™ had an accuracy of 71%. The biggest discrepancy was for no growths. Bacteria were detected on-farm in 128 (59%) of the 217 samples that grew nothing in the laboratory. The authors suggest that the greater volume of milk inoculated onto the petrifilms compared to bacterial culture as well as the time taken to transport samples to the laboratory may have contributed to the discrepancy.

Kock *et al* (2018) examined 129 clinical mastitis samples with a Rapid Petrifilm™ product, where bacterial growth can be assessed after 12 hours of incubation. While the authors found the sensitivity for Gram-negative and Gram-positive bacteria to be high (93% and 89% respectively), the specificity for Gram-positive bacteria was low, at 39%. In this situation, if a farm was doing selective antibiotic treatment, a high proportion (61%) of animals would be false positives and would be treated with antibiotics where a Gram-positive infection was not present.

Silva *et al* (2005) evaluated the Petrifilm Staph Express plates in diagnosing *Staph aureus*. They found the count of colony number and interpretation of colour intensity around bacterial colonies varied considerably between readers, resulting in variable specificity. The authors recommended having standardised instructions in order to achieve consistent interpretation and encouraged having people with "excellent visual abilities and ability to discern colours" be responsible for reading the Petrifilms.

Both Mansion-de Vries *et al* (2014) and McCarron *et al* (2009) highlight the weakness of the Petrifilm™ system in determining if a milk sample was collected hygienically or whether it was contaminated. They recommend regularly laboratory culture of duplicate samples to assess milk sample collection and plating technique.



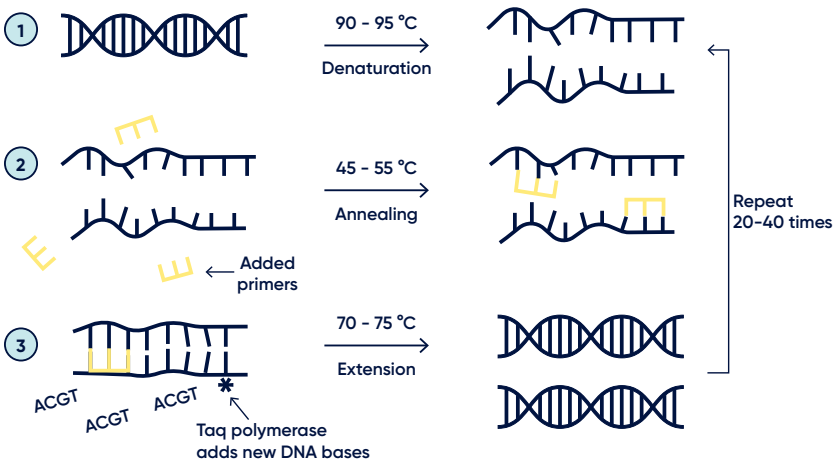
Real Time PCR

Polymerase Chain Reaction (PCR) is a diagnostic test that looks for the DNA of mastitis bacteria in a milk sample. It can be used on individual cows or as a herd screening tool when used on bulk milk or waste milk. Carried out in referral laboratories, the benefits of PCR over traditional culture are touted to be speed, automated interpretation of results, and increased sensitivity. However, the cost of the test is currently up to four times that of rapid diagnostic systems.

PCR involves several steps that are repeated 20 to 40 times:

- The sample is heated to separate double stranded DNA into two single strands
- The temperature is lowered to enable DNA primers (a short nucleotide sequences from target bacteria) to attach to the DNA
- Nucleotides and the enzyme **Taq polymerase** are added: these then complete the strands of DNA

Bacterial identification



Keane *et al* (2013) compared the results of bacterial culture and PCR for the identification of the bacterial causes of clinical mastitis. The pathogen identified by culture was also detected by PCR in 98% of cases. A mastitis pathogen was not recovered on bacterial culture from 30% of samples but in 79% of these "no growths" a pathogen was identified by PCR. Koskinen *et al* (2010) investigated the performance of a PCR kit capable of detecting 11 mastitis-causing bacteria. One thousand quarter milk samples were taken from cows with clinical or subclinical mastitis. Of the 780 samples from clinical mastitis, bacterial culture identified pathogens in 77% of cases, whereas PCR identified bacteria in 89% of the samples.

PCR identifies the presence of bacterial DNA, whether that bacteria is alive or dead. For bacterial culture, bacteria must be alive.

Unlike culture, PCR can identify the presence of dead pathogens in milk samples. Therefore, death of bacteria (e.g. due to recent treatment) prior to sample collection or between sampling and testing may lead to discrepancies between these testing approaches. Unnecessary treatments may be given if using PCR results to dictate treatment protocols (i.e. an active intramammary infection is not present: a false positive).

PCR will only identify bacteria whose primer is added. For example, the Pathoproof™ kit that is routinely used in Australia, contains primers that can identify the bacterial DNA of 15 organisms. If used on an animal infected with different bacteria it will not detect the infection (a false negative). In the study by Koskinen *et al* (2010), bacterial culture identified a species not targeted by the PCR test in 44/780 samples from clinical mastitis and in 9/220 samples from subclinical mastitis.

Intramammary infection is not the only source of bacterial DNA found in milk samples. Teat skin, the teat canal and sampling processes can contaminate milk with bacterial DNA. Hence, milk samples must be collected aseptically if being used at the cow level, and any result with the DNA of 3 or more bacteria should be treated as contaminated. When used as a herd screening tool it is important to decide whether the pathogens identified are likely to be responsible for a mastitis problem

Mastitis is the inflammation of one or more quarters of the udder. It is diagnosed by visible changes to the milk and/or udder or increases in the somatic cell count of the milk. While the inflammation is most commonly the result of an intramammary infection with bacteria (and on occasion a yeast or algae) the two terms – inflammation and infection – are not interchangeable.

Technote 4.3 describes collecting and submitting milk samples for culture.

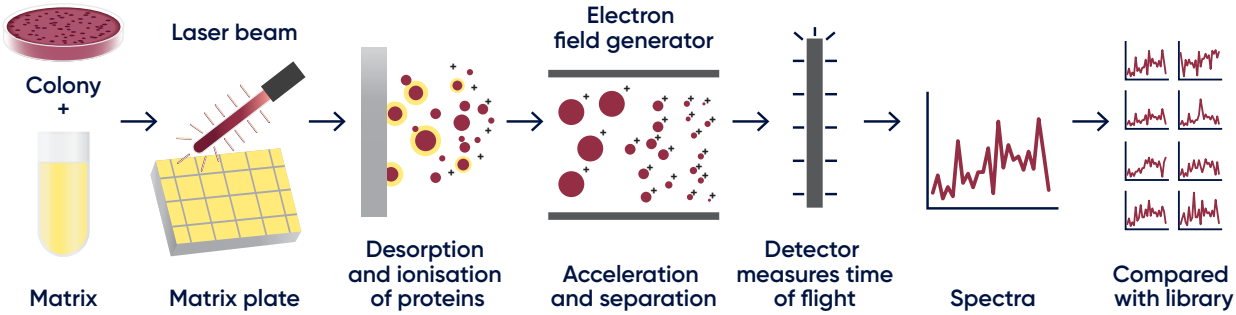
in the herd and whether further action needs to be taken. For example, *Strep uberis*, *Staph aureus* or *E. coli* isolated from a bulk milk sample may have originated from the skin surface, in the mammary gland or from post-milking contamination. Whereas for *Mycoplasma spp.* and *Strep agalactiae* it can be assumed that infected cows are in the herd as an infected mammary gland is the primary reservoir of these bacteria. With the increased ability to detect the presence of bacteria (alive or dead), PCR may be more likely to yield results that could be classified as contaminated: Koskinen *et al* (2010) found that PCR detected 3 or more bacterial species in 137/780 samples from clinical mastitis, whereas bacterial culture identified 3 or more species in only 60/780 samples from clinical mastitis.

Matrix-Assisted Laser Desorption/ Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

One aspect of laboratory bacterial identification is biochemical testing i.e. identifying bacteria based on the presence of certain enzymes. Analysing bacteria protein profiles is a recent but fast-growing alternative method of identification. The MALDI-TOF MS method compares ribosomal proteins from bacterial isolates to a searchable database. It can also analyse the protein profiles of other microbes, such as yeast. MALDI-TOF MS is becoming more commonplace for the genus- and/or species-level identification of bacteria and, in some laboratories, has replaced or is used in conjunction with traditional biochemical methods.

The MALDI-TOF MS procedure (Wilson *et al* 2019) is as follows:

1. An isolated colony (after 24–48 hours growth on conventional media) is mixed with a matrix solution. This crystallizes with the isolate and dries onto a target plate
2. The plate is placed in a high-vacuum chamber, where samples are exposed to short laser pulses that ionize the isolate's molecules
3. An electromagnetic field accelerates these ionized molecules within a flight tube. The times of flight (TOF) taken for the various molecules to reach an ion detector are recorded as a mass spectra profile. The TOF is unique for most bacterial species
4. The observed profile is then compared to those of a reference database with spectra from over 4000 known bacteria
5. Software calculates a log score between zero (no similarity with any known bacterium) and a maximum of 3 (100% similarity). Scores between 1.7 and 1.99 are considered reliable to the genus level (e.g. *Staphylococcus spp.*) and scores of >2.0 are considered reliable to the genus and species level (e.g. *Staphylococcus chromogenes*)



Braga *et al* (2018) analysed 305 isolates of bacteria from cows with subclinical mastitis by both conventional microbiological culture and MALDI-TOF MS. Approximately 89% of the identifications performed by MALDI-TOF MS were consistent with results obtained by culture. From the remaining isolates the misclassification was at genus and/or species level. The disagreements were mostly associated with identification of *Streptococcus* and *Enterococcus* species, thought to be due to the close similarity between these two genera and the difficulty in differentiating them with conventional bacterial culture. The authors suggest that MALDI-TOF MS therefore may be an alternative to overcome incorrect species-specific identification that occurs with traditional biochemical testing.

Wilson *et al* (2019) carried out a blinded comparison of three methods of identification of bacterial isolates. Milk samples were submitted from a commercial dairy farm from recently calved cows or clinical mastitis cases. Samples were cultured, and 181 isolates were identified by biochemical testing, MALDI-TOF MS, and 16S rRNA sequence analysis. The agreement in bacterial identification between MALDI-TOF MS and 16S rRNA testing was high, 98%. The agreement of biochemical testing with each of the other 2 methods was 95%. In most cases of disagreement, biochemical testing had a different result while the MALDI-TOF MS and 16S rRNA agreed.

Barreiro *et al* (2017) investigated the use of MALDI-TOF MS to identify bacteria directly from milk, rather than from bacterial colonies. Milk samples were experimentally contaminated with *Staph aureus*, *Strep uberis*, *Strep agalactiae*, *Strep dysgalactiae*, and *E. coli* to have bacterial counts ranging from 103 to 109 CFU/mL. For MALDI-TOF MS to identify bacterial infection adequately, bacterial counts had to be high. The counts also varied with the species of bacteria: ≥106 CFU/mL of *Staph aureus*, ≥107 CFU/mL of *E. coli*, and ≥108 CFU/mL of *Streptococcus species*. This is an area that will undoubtedly undergo further work.

What is 16S rRNA sequence analysis?

Bacteria contain genes called 16S rRNA genes. The gene sequence data from an unknown isolate are compared with databases from known bacterial isolates. Even small genetic differences can be used to differentiate bacterial species, and the test is considered a reference test.

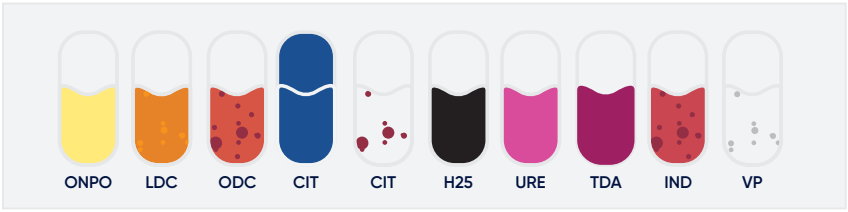
Nonnemann *et al* (2019) analysed 500 isolates from clinical and subclinical mastitis cases using MALDI-TOF MS: 94% could be identified to the species level, and 7% were identified only to the genus level. Isolates identified to the genus level required further identification to the species level by conventional methods or 16S rDNA sequencing. A total of 24 genera and 61 species were identified in this study. This included both genera and species not routinely identified as mastitis pathogens. As this diagnostic technology continues to improve and become more readily available, milk quality advisors will need to learn about the treatment and prognosis prospects of these 'newly' identified bacteria.

MALDI-TOF MS requires microbial colonies for testing, and so it may not always offer the speed required for targeted treatment decisions for individual cows. The speed of results will depend on the distance from a capable laboratory. One major advantage of using MALDI-TOF MS for identification is that limited information is needed prior to submitting the isolate for analysis. Disregarding the initial investment (of hundreds of thousands of dollars), MALDI-TOF MS can be cheaper and faster than some traditional methods of bacterial identification. It is semi-automated and so more likely to provide consistent results, within and between labs. However, it is important that technology is not relied upon implicitly as incorrect identifications may occur without the careful review and comparison of MALDI-TOF MS results to the original bacterial colony characteristics. Accuracy may be poor if the MALDTI-TOF MS library does not include mastitis pathogens. Commercial laboratories conducting milk cultures should expand their library by isolating mastitis pathogens from field samples, identifying them using a reference test (such as 16s rRNA) and then adding the mass spectra profile to the existing library (Wanecka *et al* 2019). As technology moves forward from traditional microbiology, it is important that these basic microbiological skills are not lost from within our industry.

Colour changes of media (colorimetry)

Over the years, microbiologists have developed technology to enable easier and quicker completion of biochemical tests that allow identification of bacteria, such as Analytical Profile Index (API) strips. An example is the API 20E strip which differentiates Enterobacteriaceae and other Gram-negative rods. Twenty microtubules containing dehydrated substrates are inoculated with a bacterial suspension. During incubation and bacterial growth and metabolism, colour changes occur that are either spontaneous or revealed by the addition of reagent.

The Vitek 2 system continues this method but is automated and on a larger scale. As with MALDI-TOF MS, it is being used as part of bacterial identification in milk quality laboratories. With this system, reagent cards have 64 tiny wells that each contain an individual test substrate. These substrates measure various metabolic activities such as acidification, enzyme hydrolysis and growth in inhibitory substances. Different cards are available for different organism classes (such as Gram-negative, Gram-positive, yeasts). After inoculation with a suspension of the organism, each card is incubated and read by the machine's internal optics which detect changes in colour or turbidity. Results are compared to a data base of known species-specific reactions. Results are available in 4–6 hours.



API strips - manual interpretation

Change No change

Vitek2 - automated interpretation

At a farm level, several products have been created that are based on colour change.

Leimbach and Kromker (2018) looked at a test tube system for on-farm treatment decisions (mastDecide®). The system consists of 2 test tubes containing pink test medium that can discriminate between Gram-positive cocci, coliform bacteria, and no growth. A visible discolouration of the test medium after inoculation with 100µL of sample and incubation at 37°C for a defined time is considered as a positive reaction. A discolouration of both test tubes indicates the growth of Gram-positive cocci, a discolouration of only the first tube indicates the growth of coliform bacteria, and no change in colour indicates no microbiological growth. However, as the authors note, some microorganisms (e.g. yeasts, *Prototheca spp.*, *Pseudomonas spp.*, *Bacillus spp.*, *Corynebacterium spp.*, or *Trueperella pyogenes*) are not able to grow in the system and the test will result in a false negative. The evaluation of the tube test result after 14 hours of incubation at 37°C resulted in sensitivities for the different categories of 71–84%, specificity of 83–94% (with an overall tube test sensitivity = 81%; specificity = 71%).

Jones *et al* (2019) assessed the performance of an automated colorimetric system on 292 milk samples from cows with clinical mastitis. The testing system (Mastatest) is comprised of six wells containing colorimetric media to which the milk sample is added. After 24 hours of incubation, computer analysis of the combination of colour changes classes growth into Coliform, *Strep uberis*, *Staph aureus*, Coagulase negative *Staph*, "other" Gram-positive bacteria, or no growth. The system was compared with laboratory culture, which used conventional biochemical tests and MALDI-TOF MS for any unclear results. Assessing the study's initial data, there was fair agreement between the system and bacterial culture (κ = 0.30–0.37) for the Coliform, *Strep uberis* and *Staph aureus* and no growth categories. This system was also compared to laboratory based antibiotic sensitivity testing that followed the Clinical and Laboratory Standards Institute (CLSI) guidelines. The methodology used by this system to select the most appropriate antibiotics requires further investigation. Given current understanding, there is evidence that the use of antimicrobial susceptibility testing for mastitis samples should not be recommended to guide treatment decisions (Constable and Morin, 2003).

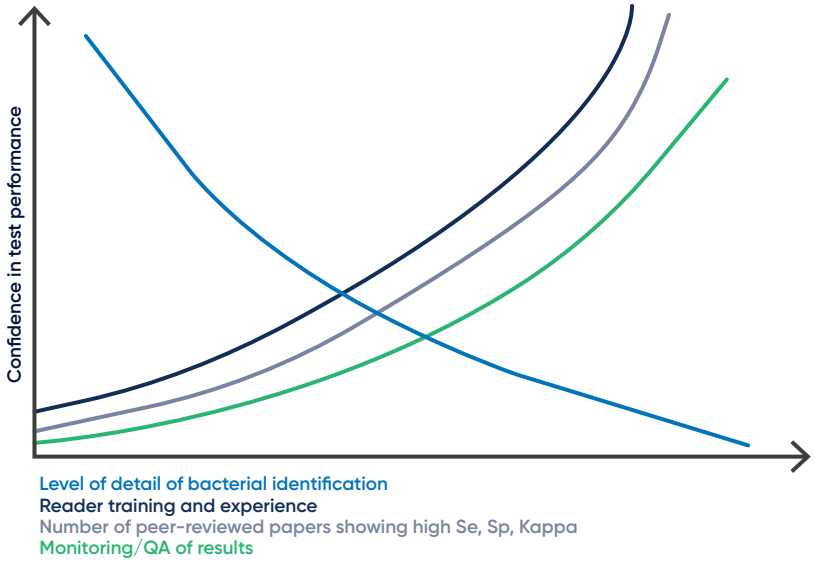
In summary

Milk quality advisors (and farmers) should clearly define the reason for carrying out a diagnostic test: such as identifying pathogens at a herd level for targeted preventative actions or identifying the most appropriate treatment and management for individual cows with clinical mastitis.

When deciding which diagnostic test to use for identification of bacterial infection, there are various points to consider:

- Validity: choose a product with peer-reviewed published research on its use, with comparison to a recognised reference test. The results should include sensitivity, specificity and level of agreement with the reference test.
- The advisor should consider the implications of a false negative or false positive result – will a cow be culled when she could be cured? Or treated when cure is unlikely? It is important to be aware of what pathogens these systems are unable to detect, such as Mycoplasma sp.
- Ease of use: a product that has clear instructions and is easy to use will better enable standardised, repeatable results.
- Detail required: is categorisation into Gram-positive and Gram-negative suitable or is further differentiation required? A bi-plate or Petrifilm may identify a Gram-positive infection but won't distinguish between *Staph aureus* and *Strep uberis*. They may be useful for treatment decisions but not to identify key steps for prevention of infection.
- Speed: time until diagnosis is important for selective treatments.
- Cost: per individual test and the cost and practicalities of the set up required (fridge, incubator, shelf space etc).

- Capacity: some farms or veterinary clinics may not have the capacity to use these systems well. Ongoing training and quality control are a necessity. Rapid diagnostic culture plates may be prone to misinterpretation by untrained staff, and so for a mastitis investigation the cost and time involved in using a laboratory culturing service may be of best value. Veterinarians interested in milk quality microbiology should consider further training



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An Index to the contents of the *Countdown Downunder Technotes* and associated FAQs can be found between the Introduction section and Technote 1.