

Evaluation of an automated on-farm device to identify groups of bacteria associated with clinical mastitis on a United States dairy farm

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Abstract

Recent studies suggest that selectively treating non-severe clinical mastitis during lactation, guided by on-farm culture, can be prudent. However, on-farm culture presents logistical challenges. This study investigated Mastatest's® (MT; Mastaplex, New Zealand) ability to identify broad groups of pathogens in clinical mastitis milk samples compared to laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry (LC+M) on one U.S. dairy farm. Milk samples from quarters with clinical mastitis were collected on a dairy farm located in Colorado (n = 162). The test's ability to identify groups of pathogens was assessed against LC+M (reference method) using contingency tables. Test characteristics (sensitivity [Se], specificity [Sp], positive predictive value and negative predictive value) and their 95% confidence intervals were estimated. Agreement was assessed using the Kappa statistic. Prevalences (0.20-0.60) for Gram-positive and Gram-negative bacteria were used to estimate predictive values. Before analysis, contaminated or missing samples in either MT or LC+M were excluded (n = 36). Our findings indicated moderate to substantial agreement, with Kappa values ranging from 0.53 to 0.66. For Gram-positive pathogens, Se and Sp estimates, and their 95% confidence intervals were: Se: 0.83 (0.70, 0.92), Sp: 0.81 (0.70, 0.89), and for Gram-negative bacteria (Se: 0.73 [0.50, 0.89], Sp: 0.93 [0.87, 0.97]). Negative predictive value remained above 0.70 across different prevalence scenarios, particularly where values exceeded 0.90 for prevalences below 0.30. Our findings suggest MT is a suitable technology for on-farm detection of mastitis pathogens commonly identified on U.S. dairy farms in clinical mastitis milk samples.

Key words: udder health; antimicrobial stewardship; intramammary infections

Introduction

Mastitis is a highly prevalent and costly disease for dairy farms, and the most frequent reason for administering antibiotics to dairy cows in the United States.^{1,2} In recent decades, United States dairy farms have seen significant reductions in bulk tank somatic cell counts and a shift in clinical mastitis pathogens from udder-adapted to environmental microorganisms.³ At the same time, there has been growing pressure on

the dairy industry to improve antimicrobial stewardship on dairy farms.^{4,5} Studies have shown that selective treatment of non-severe clinical mastitis (CM) during lactation is a feasible alternative to blanket therapy without negatively impacting udder health or milk production.^{6,7} For this reason, the use of milk culture has become crucial for identifying broad categories of CM pathogens, and directing antimicrobial treatment to cows and quarters that could most benefit from antimicrobial therapy.⁸

Given the limitations of laboratory culture, including slow turnaround times dependent on proximity to the laboratory, using on-farm culture (OFC) has emerged as a viable alternative for identifying quarters that need treatment.^{7,8} On-farm culture can be successfully used to identify groups of bacteria, such as Gram-positive vs. Gram-negative and *Streptococcus* spp. vs. *Staphylococcus aureus* vs. non-*aureus* *Staphylococci*.⁹⁻¹¹ Previous studies have shown, however, that the diagnostic accuracy of OFC can vary depending on the observer's level of training, with more trained and experienced individuals achieving higher agreement with laboratory reference methods than less formally trained observers.¹² As a result, there has been interest in developing new technologies to simplify on-farm mastitis diagnostics, increasing their repeatability and reducing the need for specialized training.¹³

Mastatest® (MT, United States cartridge; Mastaplex) is a novel method that addresses some of the limitations of OFC through the implementation of an automated testing and reporting system of milk samples.¹⁴ Although it has been investigated in New Zealand,^{15,16} it has not been examined considering the distribution of mastitis pathogens typically encountered on a dairy farm in the United States. Therefore, the objective of this study was to investigate the ability of MT to identify broad groups of pathogens present in CM milk samples as compared to laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry (LC+M) on a dairy farm in the United States. We hypothesized that the use of MT would provide good agreement with LC+M for the identification of broad groups of mastitis pathogens.

Materials and methods

This study was conducted between November 2021 and March 2022 on a commercial free-stall dairy farm located in Colorado, housing approximately 5,400 Holstein lactating cows bedded with sand. Cows were milked 3 times a day and the average bulk tank milk SCC was 166,000 cells/mL during the study period. This work was designed and written considering the guidelines for reporting of diagnostic accuracy studies (STARD).¹⁷ Members of the research team trained farm personnel on CM detection and aseptic milk sample collection prior to the start of the study. Clinical mastitis was defined as the presence of abnormal milk, with or without the presence of inflamed quarters and/or systemic signs.¹⁸ All lactating cows during the study period were monitored daily by farm staff for CM through visual examination of forestripped milk.

Upon detection of CM, farm records were evaluated. Quarters with non-severe cases (i.e., no systemic signs) that did not receive intramammary treatment within 21 days of diagnosis were sampled by trained farm personnel following National Mastitis Council guidelines.¹⁹ In brief, after pre-dipping teats, 3-4 streams of milk were discarded, teat end was disinfected with a gauze soaked with 70% ethanol, and approximately 10 mL of milk were collected into sterile vials. Milk samples were immediately placed in a cooler with ice, and within 30 minutes of sample collection divided into two aliquots. The first aliquot containing 5 mL was used for on-farm testing using MT. The second aliquot was stored at 4 °C and submitted within 24 hours to a reference laboratory for milk culture and taxonomic assignment using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

According to the manufacturer's instructions,¹⁴ farm personnel filled MT cartridges with approximately 1.4 mL of milk. Subsequently, cartridges were placed inside the device. This device uses an electronic eye technology to automatically detect color changes associated with presence of different bacteria in milk samples.¹⁴ Results were sent to the researchers within 24 hours via an automated online platform. Test results were reported as: coagulase negative *Staphylococcus*, *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Streptococcus* species (including *Streptococcus*-like organisms), other Gram-positive bacteria, *Escherichia coli*, *Klebsiella* spp., or no growth (i.e., no bacteria detected). Samples were classified as contaminated if the device identified more than 2 different microorganisms.

Laboratory milk culture was performed at The Dairy Authority LLC laboratory (Greeley, CO, USA). Approximately, 0.01 mL were inoculated onto the plates using a calibrated loop. Samples were processed using 4% washed bovine blood and 0.1% esculin plates, and MacConkey agar plates. Plates were incubated at 37 °C and read twice at both 18-24 hours and 36-48 hours to evaluate the presence of bacterial growth. If more than 2 different isolates were identified on the plates, samples were classified as contaminated and excluded from further analyses. Identity of isolates from non-contaminated samples was investigated using MALDI-TOF MS (MALDI Microflex LT Biotyper, Bruker Daltonics Inc.). A confidence score of 1.95 was used for genus and species level identification. Isolates with a confidence score below 1.95 were re-analyzed. An IMI was defined as the presence of visible growth of any colony in the used plates (i.e., 100 colony forming units/mL).

All statistical analyses were performed using R version 4.3.2. Code, data and output are available online: https://fepenamosca.github.io/mastatest_validation/. The unit of analysis was quarter-level milk samples, and all samples collected from clinical mastitis cases during the observation period were included. No priori sample size calculation was performed. Before data analysis, contaminated or missing samples in either MT or LC+M were excluded (n = 36). The overall prevalence of IMI, as well as the prevalence of each bacterium in these samples was estimated and reported for each test. Analyses were performed to investigate the ability to identify broad groups of bacteria, including Gram-positive and Gram-negative, and sub-groups or genera present in at least 10 samples, in comparison to LC+M (reference test). Diagnostic performance was evaluated using contingency tables using functions implemented in the “epiR” package.²⁰ The true prevalence (i.e., estimated using the reference test), apparent prevalence (i.e., estimated using MT), and test characteristics (sensitivity [Se], specificity [Sp], negative predictive value [NPV], positive predictive value [PPV]) along with their exact binomial 95% confidence intervals (CI) were estimated. Agreement in the identification of bacteria was estimated using the Cohen Kappa Statistic through functions implemented in the “fmsb” package.²¹

For sensitivity analyses, scenarios with varying prevalence, Se and Sp for Gram-positive and Gram-negative bacteria were considered to estimate NPV and PPV that could be observed when using MT, in different hypothetical scenarios. Investigated prevalences for Gram-positive and Gram-negative bacteria varied between 0.20 and 0.60.

Results

One hundred sixty-two quarter-level samples from 142 distinct cows with CM were collected across different stages of lactation: 42.0% (68/162) were obtained from cows with less than 100 DIM, 30.2% (49/162) between 100 and 200 DIM, and 27.8% (45/162) from cows with more than 200 DIM. Approximately 15% (24/162) of the samples were obtained from primiparous cows and 85% (138/162) from multiparous cows. A total of 17.9% (29/162) of the milk samples were contaminated, and 4.3% (7/162) of the results were missing in either LC+M or MT. These samples were excluded from the statistical analysis.

Out of the 126 quarter level milk samples used for statistical analysis, 41.3% (52/126) showed no growth, 41.3% (52/126) growth of Gram-positive, and 17.5% (22/126) Gram-negative bacteria when submitted for LC+M. From samples processed using MT, 36.5% (46/126) did not detect any bacteria, 45.2% (57/126) identified Gram-positives, and 18.3% (23/126) identified Gram-negatives. The most prevalent bacteria within Gram-positives were *Streptococcus* spp. and *Streptococcus*-like organisms (SSLO) (LC+M: 25.4% [32/126]; MT: 36.5% [46/126]) and within Gram-negatives *Escherichia coli* (LC+M: 15.9% [20/126]; MT: 17.5% [22/126]) (Table 1).

Moderate to substantial agreement (Kappa ranging from 0.53 to 0.66) on the identification of Gram-positive and Gram-negative mastitis bacteria in milk samples was observed between MT and LC+M. When looking at specific tests' characteristics, MT exhibited a Se of 0.83 for Gram-positive (95%CI: 0.70, 0.92) and a Sp of 0.73 (95%CI: 0.50, 0.89) for Gram-negative when compared to LC+M. The Sp for detection of Gram-positive and Gram-negative bacteria were 0.81 (95%CI: 0.70, 0.89) and 0.93 (95%CI: 0.87, 0.97), respectively. Positive predictive values

Table 1: Descriptive results from 126 non-contaminated quarter milk samples from cows with non-severe clinical mastitis, submitted for identification using laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry (LC+M; reference method) and Mastatest® (MT).

Item	LC+M ^{*1}	MT ²
Any IMI	58.7% (74)	63.5% (80)
GP	41.3% (52)	45.2% (57)
<i>Staph aureus</i>	0% (0)	0% (0)
NAS	5.6% (7)	1.6% (2)
<i>Staph chromogenes</i>	4.8% (6)	–
SSLO	25.4% (32)	36.5% (46)
<i>Strep uberis</i>	2.4% (3)	8.7% (11)
<i>Strep dysgalactiae</i>	11.1% (14)	2.4% (3)
Other Strep and SLO	11.9% (15)	25.4% (32)
<i>Enterococcus spp.</i>	5.6% (7)	–
<i>Lactococcus spp.</i>	3.2% (4)	–
Other Gram-positive	11.1% (14)	7.1% (9)
<i>Corynebacterium spp.</i>	10.3% (13)	–
GN	17.4% (22)	18.2% (23)
<i>Escherichia coli</i>	15.9% (20)	17.5% (22)
<i>Klebsiella spp.</i>	0.8% (1)	0.8% (1)
No bacteria	41.3% (52)	36.5% (46)

¹ LC+M: identification of bacteria in milk samples using laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry. *One milk sample submitted for LC+M showed the growth of two distinct bacteria.

² MT: identification of bacteria in milk samples using Mastatest (Mastaplex).

GP: Gram-positive. *Staph*: *Staphylococcus*. *Strep*: *Streptococcus*. NAS: *non-aureus Staphylococci*. SSLO: *Streptococcus spp.* and *Streptococcus*-like organisms. SLO: *Streptococcus*-like organisms. GN: Gram-negative. *E. coli*: *Escherichia coli*.

ranged between 0.59 and 0.75 for different bacteria. Negative predictive values were high for all analyzed mastitis associated bacteria (> 0.87). Test characteristics results are presented in Table 2, including results for prevalent CM pathogens in our dataset (n > 10).

When estimating PPV and NPV at varying prevalence of Gram-positives or Gram-negatives (i.e., 0.2 to 0.6), we estimated that NPV were in most circumstances relatively high. For instance, estimated NPV point-estimates were above 0.9 for a prevalence under 0.3 and above 0.7 for a prevalence between 0.3 and 0.6. The estimated PPV and NPV at varying prevalences are presented in Figure 1.

Discussion

This investigation aimed to explore the prognostic potential of an automated on-farm device for the identification of groups of bacteria on quarter milk samples collected from quarters with CM. Under our study conditions, MT exhibited moderate to high Se and Sp, and high NPV for the detection of Gram-positive and Gram-negative bacterial organisms. These test characteristics remained relatively high under varying prevalences. Our findings suggest that MT could serve as an effective tool for detection of groups of bacteria in CM milk samples.

As our understanding of mastitis epidemiology deepens, we increasingly recognize the unique epidemiological profiles of different mastitis pathogens.^{22,23} In consequence, identifying bacteria types within milk samples is vital for determining the prognosis of clinical cases and targeting cows that could benefit from antimicrobial treatment.⁸ Automated on-farm devices have the potential to simplify the logistics of on-farm mastitis pathogen identification.¹³ The results from our study suggest that MT effectively identifies broad groups of mastitis pathogens such as Gram-positive or Gram-negative. Its performance is comparable to that of other OFC systems,^{9,10,12} with outcomes in the latter dependent on the training of the technician reading the plates.^{9,12} For instance, Se ranged from 0.58 to 0.86 and Sp from 0.79 to 0.93 in studies evaluating bi- and tri-plates for identifying Gram-positive bacteria.^{9,12} Meanwhile, Se for studies using chromogenic media to identify *Streptococcus spp.* ranged from 0.34 to 0.90, with Sp ranging from 0.90 to 0.93.^{10,12} Given that different studies may vary in their methodologies and prevalence of pathogens in study populations, comparisons should be interpreted with caution. In the context of guiding treatment decisions, it is also useful to consider the proportion of clinical mastitis cases that would have been misclassified based on MT results, particularly for pathogen groups typically requiring treatment. For instance, in the study farm, the Se for detection of Gram-positive IMI was 0.83, suggesting that 17.3% (9/52) of the clinical mastitis

Table 2: Test characteristics for identification of bacteria present in 126 non-contaminated quarter milk samples from cows with clinical mastitis using Mastatest® (MT) compared to laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry (LC+M;reference test). Only bacteria identified in at least 10 samples using both LC+M and MT were evaluated.

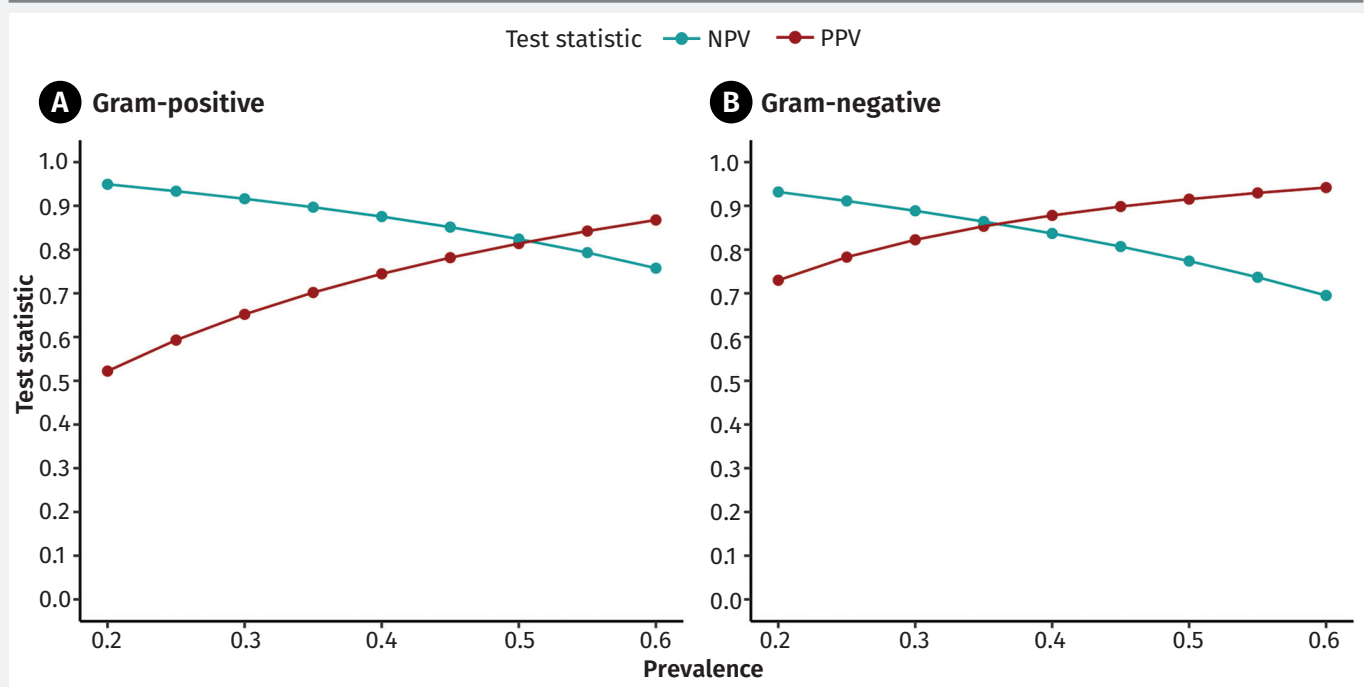
Outcome	LC+M ¹ Pr (95%CI)	MT ² Pr (95%CI)	Se (95%CI)	Sp (95%CI)	PPV (95%CI)	NPV (95%CI)	Kappa (95%CI)
Any IMI	0.59 (0.50, 0.67)	0.63 (0.54, 0.72)	0.85 (0.75, 0.92)	0.67 (0.53, 0.80)	0.79 (0.68, 0.87)	0.76 (0.61, 0.87)	0.53 (0.38, 0.69)
GP	0.41 (0.33, 0.50)	0.45 (0.36, 0.54)	0.83 (0.70, 0.92)	0.81 (0.70, 0.89)	0.75 (0.62, 0.86)	0.87 (0.77, 0.94)	0.63 (0.49, 0.77)
SSLO	0.25 (0.18, 0.34)	0.37 (0.28, 0.46)	0.84 (0.67, 0.95)	0.80 (0.70, 0.87)	0.59 (0.43, 0.73)	0.94 (0.86, 0.98)	0.56 (0.40, 0.72)
GN	0.17 (0.11, 0.25)	0.18 (0.12, 0.26)	0.73 (0.50, 0.89)	0.93 (0.87, 0.97)	0.70 (0.47, 0.87)	0.94 (0.88, 0.98)	0.65 (0.47, 0.83)
<i>E. coli</i>	0.16 (0.10, 0.23)	0.17 (0.11, 0.25)	0.75 (0.51, 0.91)	0.93 (0.87, 0.97)	0.68 (0.45, 0.86)	0.95 (0.89, 0.98)	0.66 (0.47, 0.84)
No bacteria	0.41 (0.22, 0.50)	0.37 (0.28, 0.46)	0.67 (0.53, 0.80)	0.85 (0.75, 0.92)	0.76 (0.61, 0.87)	0.79 (0.68, 0.87)	0.53 (0.38, 0.69)

¹ LC+M: identification of bacteria in milk samples using laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry.

² MT: identification of bacteria in milk samples using Mastatest (Mastaplex).

Pr: Prevalence. Se: Sensitivity. Sp: Specificity. PPV: Positive predictive. NPV: Negative predictive value. Predictive values were computed using contingency tables. Kappa: Cohen Kappa statistic. 95%CI: 95% confidence interval. GP: Gram-positive. SSLO: *Streptococcus spp.* and *Streptococcus*-like organisms. GN: Gram-negative. *E. coli*: *Escherichia coli*.

Figure 1: Sensitivity analyses used to estimate negative predictive values (NPV) and positive predictive values (PPV) in scenarios with varying prevalence for the detection of Gram-positive (Panel A) and Gram-negative bacteria (Panel B) using Mastatest®. Laboratory culture and matrix-assisted laser desorption ionization-time of flight mass spectrometry was used as the reference test.



cases requiring treatment would have been missed. Similarly, the Sp was 0.81, indicating that 18.9% (14/74) of the cases without Gram-positive IMI would have received unnecessary treatment. Evaluating the proportion of misclassified cases helps link diagnostic accuracy with practical treatment decisions. In addition, it provides important information about the likelihood that a positive or negative test result reflects the true infection status, which is critical for informed clinical decision-making.

For programs aiming for antimicrobial stewardship that aim to maintain udder health and performance sustainably, an important indicator is the NPV, especially for major pathogens such as SSLO and coliforms.²⁴ The NPV estimates the proportion of test-negative samples that are truly negative (in this case, using MT). A high NPV explains why imperfect tests (i.e., with moderate Se and Sp) such as those relying on OFC for selective treatment of CM⁸ or algorithm-based selective dry cow therapy,²⁵ can still be effective in guiding selective treatment decisions without adversely affecting udder health. It is important to note that NPV tends to be higher in populations with a lower prevalence of target pathogens. This scenario is common in well-managed farms, that should aim for selective treatment of mastitis during lactation,^{6,26} as the cost-effectiveness of OFC decreases with higher prevalences of Gram-positive bacteria.²⁷

Recognizing that not all farms have the same prevalence of Gram-positive and Gram-negative bacteria, we calculated how NPV and PPV would change under different prevalence scenarios. For instance, farms with a low prevalence of Gram-positive pathogens, often targeted for selective CM treatment,⁶ typically exhibit higher NPV for detection of these bacteria. However, in farms with higher prevalence of Gram-positive mastitis pathogens, NPV may be lower, potentially compromising udder health, as a substantial proportion of the quarters that tested negative could still harbor a Gram-positive IMI. Our results showed that NPV remained high at varying prevalences (NPV > 0.70), especially below 0.30 in which NPV was above 0.90. This finding indicates that MT could be used successfully to identify these bacterial groups under different prevalence scenarios. This could explain why a previous investigation that used MT for on-farm selective treatment of mastitis (n = 648 clinical cases) found no impact on mastitis cure rate or other udder health indicators.²⁸ However, it is important to recognize that the PPV was low, especially in scenarios with low prevalence of target bacteria (i.e., 0.20). Therefore, it is crucial to exercise caution in the interpretation of results, as in these circumstances, a significant proportion of positive results could represent non-infected quarters.

This study was conducted on one commercial dairy farm in the United States and agrees with investigations conducted in New Zealand.^{15,16} However, we recognize that the prevalence and distribution of mastitis pathogens might differ across farms, and this could affect the NPV and PPV of the test. To address this limitation and improve the external validity of results, we estimated predictive values across a range of plausible prevalences for Gram-positive or Gram-negative bacteria. However, this could still represent an issue, as some specific major mastitis pathogens, such as *Staphylococcus aureus* or *Klebsiella* spp., were not identified in our dataset or showed a very low prevalence. Despite this, the ability of MT to identify *Staphylococcus aureus* has been investigated previously in another country.¹⁶ Furthermore, factors such as farm management practices, seasonality and geographical location

may also influence pathogen prevalence, and further studies across different herd settings would help clarify the broader applicability of these findings on more U.S. dairy farms.

Another limitation of this diagnostic accuracy study is the small sample size, which was affected by the high proportion of contaminated samples. This led to imprecise estimates for certain subgroups of bacteria, such as Gram-negative bacteria. Despite efforts to prevent contamination through training farm personnel on sample collection, results were missing for samples classified as contaminated and had to be excluded from the analysis. Nevertheless, this is an issue related to inadequate collection of milk samples,²⁹ not test performance, as it could occur in any diagnostic system. In addition, it is important to note that the confidence score cut-off used for assigning taxonomy with MALDI-TOF MS differs from the commonly used cut-offs of 1.8 and 2.0 for genus and species level identification,³⁰ which represents a potential limitation. However, since MT and LC+M were evaluated at the genus level or for groups of bacteria (except for *Escherichia coli*), we expect minimal impact on the results.

Conclusions

Our findings indicate that the use of MT is a valuable tool for on-farm detection of mastitis pathogens in milk samples from CM cases, particularly those commonly identified on dairy farms in the United States. This technology does not require trained technicians or laboratory maintenance, minimizing the opportunity for bias from untrained personnel. The implementation of MT could offer a simple yet effective approach for the identification of broad categories of mastitis pathogens on-farm, facilitating informed decision-making, including the selective treatment of CM. Additional studies are needed to confirm these findings across different herd settings.

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Conflicts of interest

Susan Saila and Olaf Bork are employed by Mastaplex. The company and affiliated co-authors did not participate in study design, study implementation or analysis. The authors have not stated any other conflicts of interest.

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