Research Article

# Detection of Major Mastitis Pathogens by Bacterial Culture and Multiplex Polymerase Chain Reaction Assay

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**Abstract** | Bovine mastitis is a major disease of high economic importance in dairy farmers worldwide. A rapid and reliable diagnosis of the main pathogens causing bovine mastitis is essential for successful sanitation programs. This study aimed to standardize a multiplex PCR assay for simultaneous detection of eight bacteria associated with mastitis such as *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Escherichia coli* and *Klebsiella pneumoniae* directly from milk and to compare it with bacterial culture. A total of 200 milk samples collected from cows with clinical or subclinical mastitis were used for diagnosis of pathogens by conventional bacterial culture and multiplex PCR. Bacterial culture identified one or more pathogens in 84% (n=168) of total milk samples. For the target bacteria, this technique identified these bacteria in 44.5% (n = 89) of samples, whereas PCR identified them in 50% (n = 100) of samples. Out of these samples, 131 isolates of target bacteria were detected by PCR compared to 93 isolates by culture. The kappa value between the two methods varies from low to good depending on the species. The PCR assay detected pathogens in many samples that were negative for the corresponding species or showing no growth in culture. However, some positive-culture samples showed negative PCR results for the corresponding pathogens. Indeed, PCR assay did not detect *S. aureus* in 13 culture-positive samples. Despite that, PCR provided several advantages over bacterial culture. It enabled rapid identification of eight pathogens causing mastitis simultaneously. After some improvements, this test will be useful for monitoring and maintaining the bovine udder health and ensuring the bacteriological safety of milk.

**Keywords** | Bovine mastitis, Multiplex PCR assay, Bacterial culture, Pathogens

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## INTRODUCTION

Mastitis is a dominant pathology in dairy cattle. Besides the potential risk for food safety (Artursson *et al*., 2018), mastitis induces severe economic losses in all dairy systems. These losses are due to the decrease in milk production (Heikkilä *et al*., 2018; Aparicio-Roque *et al*., 2025), reduction in milk quality (Narváez-Semanate *et al*., 2022) and higher costs of treatment products (Nicholas *et al*., 2009). It is the primary cause of the use of antimicrobial in dairy cattle (Deng *et al*., 2020). Mastitis can be caused by a variety of bacteria, which differ depending on the pathogenicity and prevalence frequency. These bacteria can be broadly classified into two groups; contagious (such as *Staphylococcus aureus* and *Streptococcus agalactiae*) or environmental (*Streptococcus uberis*, *Streptococcus dysgalactiae* and coliforms), depending on their primary reservoir and mode of transmission (Ruegg, 2012). The contagious bacteria are transmitted among cows by contact with infected milk during the milking process. Environmental bacteria arise from the environment of cows (manure, mud and dirty bedding materials) entering into the udder between milking or during the milking process, they can multiply and cause mastitis. Several studies in different countries showed that the most frequently isolated bacteria in cases of clinical and subclinical mastitis are staphylococci (*Staphylococcus aureus* and Coagulase negative Staphylococci), *Streptococcus uberis* and *Escherichia coli* (Fadlelmula *et al*., 2009; Mekonnen *et al*., 2017; Vakkamaki *et al*., 2017; Heikkilä *et al*., 2018; Maalaoui *et al*., 2021). The frequency and origin of udder infection vary from country to country and even between herds in the same country (Olde Riekerink *et al*., 2008), this is due to the difference in the preventive measures and milking procedures on each farm (Bradley, 2002).

Mastitis diagnostic methods such as clinical inspection, somatic cell counting, the California mastitis test (CMT), electrical conductivity or the detection of body enzymes released due to tissue damage, are important for determining mastitis cases in a dairy farm (Stanek *et al*., 2024) but are unable to provide information on the identity of the causative agent and severity of the infection. Therefore, the identification and characterization of the various microorganisms responsible for this disease are important steps for mastitis control, better herd management and improving the treatment strategy, which decreases the use of inappropriate antimicrobials (Taponen *et al*., 2009) and the infection incidence. The widely used identification methods are based on bacterial culture and biochemical tests. Bacterial culture is the gold standard for routine analysis of milk samples (Koskinen *et al*., 2010). It allows the identification of a large number of bacteria and costs are low compared with more advanced molecular methods. However, this standard method is heavy, long and ambiguous (Hegde, 2011; Prabhu *et al*., 2013). In addition, it may not show any bacterial growth in milk samples from truly infected glands due to low concentration of pathogens (Phuektes *et al*., 2001). Furthermore, the lack of unique biochemical markers for identification of species makes identification based on phenotypic differences unreliable (Capurro *et al*., 2009). The DNA-based molecular method, such as multiplex PCR, has been developed for rapid detection of major pathogens causing mastitis (Hegde, 2011; Shome *et al*., 2011; Kajdanek *et al*., 2024). Indeed, in multiplex PCR two or more loci are simultaneously amplified in the same reaction tube that helps to save time and minimize reagents used. In addition, PCR may improve the level of detection due to its high sensitivity (Phuektes *et al*., 2003).

In this context, the aim of the current study was to evaluate the multiplex PCR method for the simultaneous detection of mastitis pathogens collected from cow's milk from smallholder farms and to compare it with the conventional culture method. Finding a less expensive, specific and fast method to facilitate and improve the detection of bacteria causing bovine mastitis in smallholder farms in Tunisia is an important issue.

## MATERIALS AND METHODS

### Sampling

Two hundred milk samples were collected aseptically from cows suffering from clinical and subclinical mastitis belonging to smallholder farmers, according to the guidelines of the National Mastitis Council (NMC, 1999) for the bacteriology of milk. The farmers were informed about the purpose and the methods of the study. Oral consent was obtained from the owner of the animals before commencement of the study. All the procedures used were non invasive and in addition all the results were communicated to animal owners. There is no specific law for milk sample collection and hence no approval was mandatory.

In subclinical mastitis cases, milk samples were taken from cows with a score of California Mastitis Test (CMT) greater than or equal to two. In clinical mastitis cases, samples were collected from cows with clinical signs such as altered milk secretion (clots, flakes and bloody or purulent appearance), swollen, painful udder and/or with visible injury. These milk samples were originated from one central-eastern region (Mahdia, 77 samples) and three northern regions of Tunisia (Bizerte, Beja and Jendouba, 123 samples). Milk samples were subjected to isolation and identification of mastitis pathogens using bacterial culture in the diagnostic bacteriology laboratory at Veterinary

Research Institute of Tunis (IRVT). After that, an aliquot of each sample was deep-frozen (-20°C) immediately after culturing for one week. Then, it was sent to the research laboratory Toxicology, Environmental Microbiology and Health at the Faculty of Sciences of Sfax to identify mastitis bacteria by multiplex PCR.

## Bacterial culture

To identify bacteria present in mastitis milk samples, 10µL of milk were plated onto blood agar (tryptone soy agar with 5% sheep blood) and BromoCresol Pourpre agar (BCP). The inoculated plates were incubated at 37°C for 18-24 hours. After incubation, the number and the morphology (form and hemolytic activity) of the colonies were evaluated. From each agar, one colony per morphology was Gram stained to define the gram (+) and gram (-) bacteria and to choose the type of rapid identification gallery (API20E, API20NE, API20Staph, API20Strep, bioMérieux, France) to be used next. In addition, other tests have been carried out such as catalase and oxidase tests, and also the mobility test to confirm the mobility of some gram (-) bacteria. Other characteristics have been sought such as free coagulase to confirm the presence of *S. aureus*. Finally, bacterial isolates were stored at -80°C in brain/heart infusion broth (BHI) supplemented with 15% glycerol.

## Bacterial strains

Bacterial strains derived from milk samples from clinical and subclinical mastitis cases were used in this study to standardize multiplex PCR and assess its accuracy in identifying species. These strains were previously well identified by phenotypic and molecular methods. These strains are *S. aureus*, *S. chromogenes*, *K. pneumoniae*, *E. coli*, *S. epidermidis*, *S. haemolyticus*, *Str. agalactiae* and *Str. uberis*. All bacterial strains were cultured on sheep blood agar and nutrient agar at 37°C for 24 hours to be used for further DNA extraction.

## Primers

The oligonucleotide primer sequences used to amplify and identify the main bacteria causing mastitis were derived from Shome *et al.* (2011). Detailed information on targeted organisms, primer sequences and size of PCR amplicons is presented in Table 1.

## Extraction of bacterial genomic DNA

Bacterial genomic DNA was extracted from bacterial cultures using Zymo «Quick-gDNA™ MiniPrep» Kit (Zymo Research, D 3024, USA) according to manufacturer's instructions. Afterwards, the quantity and quality of genomic DNA was determined using Nanodrop 2000 spectrophotometer (IMPLEN) and then DNA was stored at -20°C until further use.

## Multiplex PCR assay

A multiplex PCR assay was used for simultaneous detection of eight bacterial species causing bovine mastitis. A two-tube multiplex PCR assay was developed. Each tube includes four sets of primers targeting different bacteria. The final result of sample was a combination of the results found in the two tubes.

**Table 1:** Oligonucleotide primers sequences and predicted sizes of PCR products (Shome *et al.*, 2011).

| Organisms | Gene | Oligonucleotide primer 5´– 3´ | Location within gene | Amplicon size (bp) |
|---|---|---|---|---|
| *Staphylococcus aureus* | *23S rRNA* | AGCGAGTCTGAATAGGGCGTTT<br>CCCATCACAGCTCAGCCTTAAC | 678-699<br>1571-1550 | 894 |
| *Staphylococcus chromogenes* | *sodA* | GCGTACCAGAAGATAAACAAACTC<br>CATTATTTACAACGAGCCATGC | 134-157<br>355-334 | 222 |
| *Staphylococcus epidermidis* | *rdr* | AAGAGCGTGGAGAAAAGTATCAAG<br>TCGATACCATCAAAAAGTTGG | 400 016-400 039<br>400 145-400 125 | 130 |
| *Staphylococcus haemolyticus* | *sodA* | CAAATTAAATTCTGCAGTTGAGG<br>AGAGCCCCATTGTTCTTTGA | 63-85<br>276-257 | 214 |
| *Streptococcus uberis* | *cpn60* | TCGCGGTATTGAAAAAGCAACAT<br>TGCAATAATGAGAAGGGGACGAC | 57-79<br>456-434 | 400 |
| *Streptococcus agalactiae* | *16S rRNA* | GCTAATACCGCATAAGAGTAATTAAC<br>GGTAGATTTTCCACTCCTACCAA | 132-158<br>448-425 | 317 |
| *Klebsiella pneumoniae* | *Khe* | GGAAGTGTGGATAAACGGCT<br>CTCCTGCTCGGTGTTATTGA | 4800987-4801006<br>4801076-4801094 | 108* |
| *Escherichia coli* | *phoA* | GGTAACGTTTCTACCGCAGAGTTG<br>CAGGGTTGGTACACTGTCATTACG | 433-456<br>900-877 | 468 |

*(Cressier and Bissonnette, 2011).

### Optimization of multiplex PCR assay

Individual PCR assays for *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *Str. agalactiae*, *Str. uberis*, *E. coli* and *K. pneumoniae* were initially optimized separately. Different concentrations of Taq polymerase, MgCl2, dNTP and primers were used in 25 µL reaction volume, to define the optimal PCR conditions for each individual PCR assay. To optimize the multiplex PCR assay, different combinations of individual PCR with varying concentrations of primers and template DNA were used (Figure 1). The final protocol included two separate tubes. Each tube was comprised of four sets of primers targeting different bacteria. The first tube reaction consisted of *S. aureus*, *E. coli*, *S. chromogenes* and *K. pneumoniae* and the second tube reaction included *Str. uberis*, *S. haemolyticus*, *Str. agalactiae* and *S. epidermidis*. Several assays were made until a final result was obtained which was a combination of the results found. In this study, we relied on the protocol of Shome *et al.* (2011) with slight modifications.

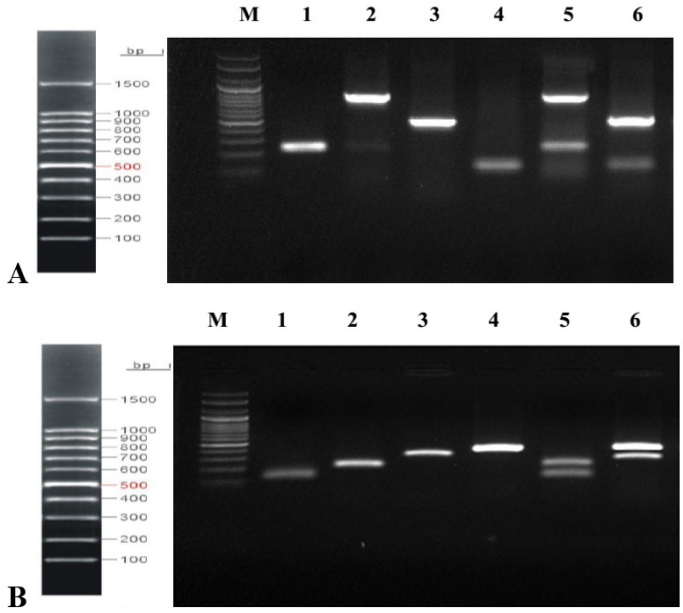


**Figure 1:** Optimization of multiplex PCR assay for bacterial strains. (A) Tube 1: Lane M: 100 bp DNA marker, Lane 1: *S. chromogenes* (222bp), Lane 2: *S. aureus* (894bp), Lane 3: *E. coli* (468bp), Lane 4: *K. pneumoniae* (108bp), Lane 5: Combination of *S. chromogenes* and *S. aureus*, Lane 6: Combination of *E. coli* and *K. pneumoniae*. (B) Tube 2: Lane M: 100 bp DNA marker, Lane 1: *S. epidermidis* (130bp), Lane 2: *S. haemolyticus* (214bp), Lane 3: *Str. agalactiae* (317bp), Lane 4: *Str. uberis* (400bp), Lane 5: Combination of *S. epidermidis* and *S. haemolyticus*, Lane 6: Combination of *Str. agalactiae* and *Str. uberis*.

Each optimized reaction mixture for PCR had a total volume of 25 µL composed of 1.25U of Taq polymerase (Promega, France), 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs,1× reaction buffer (Promega, France), combination of optimized primer concentrations (0.5µmol/l of *S. chromogenes, S. aureus and K. pneumoniae* and 0.2µmol/l of *E. coli* for tube 1 reaction and 0.5µmol/l of *S. epidermidis, S. haemolyticus, Str. agalactia*e and 0.2µmol/l of *Str. uberis* for tube 2 reaction) and 100ng of DNA extract. The amplification was performed in an automated thermocycler (UNO96,VWR) with initial denaturation at 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds; 60°C for 30 seconds, 72°C for 45 seconds and finally once at 72°C for 10 minutes. The amplified products were analysed by electrophoresis on 1.5% agarose gel containing 0.5% ethidium bromide. After an electrophoresis run-time of 40 minutes, the gels were photographed under UV light.

### Detection of pathogens in milk samples by multiplex PCR assay

The aliquot of each 200 milk samples frozen at -20°C was thawed out and processed for DNA extraction directly from milk using the Zymo "Quick-gDNA ™ MiniPrep" extraction Kit (Zymo Research, D 3024, USA) with a few modifications to eliminate fats. Before starting the DNA extraction protocol, two essential steps were carried out due to the milk fat content:

- Of each milk sample, centrifuge 1 mL of milk for 20 minutes at 13000 rpm. After centrifugation, discard the surface lipid layer and the supernatant and conserve the pellet.
- Add 200 µL of sterile water to the pellet then shake with an intense vortex to homogenize the mixture.
- After DNA extraction from the milk samples, a multiplex PCR assay was applied to all samples to identify the mastitis causative agents. The detection of PCR products was carried out by electrophoresis on agarose gel (1.5%). The obtained results were compared with those of culture method.

### Statistical analysis

Data were transferred to an Excel spreadsheet (Microsoft Corp., Redmond, WA) for analysis. The diagnostic sensitivities of culture and PCR on milk samples were compared using an adjusted McNemar's X2 test. The test result agreement between the two methods was evaluated using Kappa statistics (Kappa value below 0.40 "weak" agreement, 0.4-0.6 "moderate", 0.6-0.8 "substantial", and greater than 0.8 "almost perfect"; McGINN *et al.*, 2004). Analysis was performed using SPSS version 20.0. A P-value < 0.05 was considered statistically significant.

## RESULTS

Bacterial culture enabled the isolation of several bacteria (Gram positive and Gram negative) directly from the milk, including the eight target organisms detected by multiplex PCR. These organisms are *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*,

*Staphylococcus haemolyticus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Escherichia coli*, and *Klebsiella pneumoniae*.

### Bacteriological culture of milk samples

Out of 200 milk samples detected positives for mastitis by CMT and clinical examinations of the udder and milk, 37 were from clinical cases of bovine mastitis and 163 were from subclinical cases. Conventional bacterial culture identified one or more udder pathogens in 84% (168/200) of total milk samples.

Out of the 37 clinical samples, 89.2% (n = 33) were found positive for the presence of bacteria. Hence, 10.8% (n = 4) provided culture-negative results (Table 2). Forty bacterial isolates could be cultured and identified by biochemical tests that consist of 48.6% (n= 18) isolates of *S. aureus*, 27% (n= 10) isolates of CNS and 5.4% (n= 2) isolates for each of *Str. uberis*, *Aerococcus viridians*, *Enterococcus* spp., *Micrococcus* spp., *Pseudomonas luteola* and *Pasteurella* spp. CNS species identified from all samples (Table 2) were as follows: *S. chromogenes* (10.8%), *S. epidermidis* (2.7%), *S. haemolyticus* (2.7%), *S. saprophyticus* (2.7%), *S. lentus* (2.7%), *S. warneri* (2.7%) and *S.* spp. (2.7%).

From the 163 subclinical samples, 82.8% (n = 135) had a detectable bacterial infection. However, no growth was observed in the remaining samples. One hundred eighty six strains were isolated. *S. aureus* was isolated from 20.9% ( n = 34) of samples and coagulase negative staphylococci from 40.5% (n = 66) of samples including *S.* spp (10.4%), *S. xylosus* (8.6%), *S. chromogenes* (11.7%), *S. lentus* (2.5%), *S. epidermidis* (1.8%), *S. haemolyticus* (1.2%), *S. cohnii ssp* (1.2%), *S. capitis* (1.2%), *S. sciuri* (<1%), *S. saprophyticus* (<1%) and *S. auricularis* (<1%). *Str. uberis*, *E. coli* and

**Table 2:** Bacterial culture results of bacteria isolated from subclinical and clinical mastitis samples.

| Organism | Mastitis | | | | Total | |
|---|---|---|---|---|---|---|
| | Clinical mastitis (N=37) | | Subclinical mastitis (N=163) | | N= 200 | |
| | N | % | N | % | N | % |
| Positive samples | 33 | 89.2 | 135 | 82.8 | 168 | 84 |
| *S. aureus* | 18 | 48.6 | 34 | 20.9 | 52 | 26 |
| Coagulase-negative staphylococci | 10 | 27 | 66 | 40.5 | 76 | 38 |
| *S. chromogenes* | 4 | 10.8 | 19 | 11.7 | 23 | 11.5 |
| *S. epidermidis* | 1 | 2.7 | 3 | 1.8 | 4 | 2 |
| *S. haemolyticus* | 1 | 2.7 | 2 | 1.2 | 3 | 1.5 |
| *S. sciuri* | 0 | 0 | 1 | 0.6 | 1 | 0.5 |
| *S. xylosus* | 0 | 0 | 14 | 8.6 | 14 | 7 |
| *S. saprophyticus* | 1 | 2.7 | 1 | 0.6 | 2 | 1 |
| *S. auricularis* | 0 | 0 | 1 | 0.6 | 1 | 0.5 |
| *S. cohnii ssp* | 0 | 0 | 2 | 1.2 | 2 | 1 |
| *S. lentus* | 1 | 2.7 | 4 | 2.5 | 5 | 2.5 |
| *S. capitis* | 0 | 0 | 2 | 1.2 | 2 | 1 |
| *S. warneri* | 1 | 2.7 | 0 | 0 | 1 | 0.5 |
| *S.* spp | 1 | 2.7 | 17 | 10.4 | 18 | 9 |
| *Str. uberis* | 2 | 5.4 | 3 | 1.8 | 5 | 2.5 |
| *E. coli* | 0 | 0 | 3 | 1.8 | 3 | 1.5 |
| *K. pneumoniae* | 0 | 0 | 3 | 1.8 | 3 | 1.5 |
| *Aerococcus viridians* | 2 | 5.4 | 24 | 14.7 | 26 | 13 |
| *Enterococcus* spp | 2 | 5.4 | 4 | 2.5 | 6 | 3 |
| *Pseudomonas luteola* | 2 | 5.4 | 9 | 5.5 | 11 | 5.5 |
| *Aeromonas hydrophila* | 0 | 0 | 4 | 2.5 | 4 | 2 |
| *Micrococcus* spp | 2 | 5.4 | 4 | 2.5 | 6 | 3 |
| *Pasteurella* spp | 2 | 5.4 | 8 | 4.9 | 10 | 5 |
| *Kocuria varians/rosea* | 0 | 0 | 2 | 1.2 | 2 | 1 |
| *Burkholderia cepacia* | 0 | 0 | 4 | 2.5 | 4 | 2 |
| No growth | 4 | 10.8 | 28 | 17.2 | 32 | 16 |

**Table 3:** Multiplex PCR results of target bacteria isolated from subclinical and clinical mastitis samples.

| Organism | Mastitis | | | | Total | |
|---|---|---|---|---|---|---|
| | Clinical mastitis (N=37) | | Subclinical mastitis (N=163) | | N= 200 | |
| | N | % | N | % | N | % |
| Positive samples | 26 | 70.3 | 74 | 45.4 | 100 | 50 |
| *S. aureus* | 9 | 24.3 | 31 | 19 | 40 | 20 |
| Coagulase-negative staphylococci | 11 | 29.7 | 38 | 23.3 | 49 | 24.5 |
| *S. chromogenes* | 10 | 27 | 31 | 19 | 41 | 20.5 |
| *S. epidermidis* | 0 | 0 | 2 | 1.2 | 2 | 1 |
| *S. haemolyticus* | 1 | 2.7 | 5 | 3.1 | 6 | 3 |
| *Str. uberis* | 10 | 27 | 8 | 4.9 | 18 | 9 |
| *Str. agalactiae* | 0 | 0 | 2 | 1.2 | 2 | 1 |
| *E. coli* | 0 | 0 | 7 | 4.3 | 7 | 3.5 |
| *K. pneumoniae* | 3 | 8.1 | 12 | 7.4 | 15 | 7.5 |
| No growth | 11 | 29.7 | 89 | 54.6 | 100 | 50 |

*K. pneumoniae* were found in 1.8% (n= 3) of samples. *Aerococcus viridians*, *Pseudomonas luteola* and *Pasteurella* spp. were isolated from 14.7%, 5.5% and 4.9% of samples, respectively. Other samples contained *Micrococcus* spp. (2.5%), *Enterococcus* spp. (2.5%) and *Aeromonas hydrophila* (2.5%), *Kocuria varians/ rosea* (1.2%) and *Burkholderia cepacia* (2.5%).

## Optimization of multiplex PCR assay

Individual PCR assays specifically amplified the eight bacterial strains tested in this study in the same conditions. Species of each reaction mixture were tested individually and then in pairs until the detection of four target pathogens in a single reaction. The evaluation of eight bacterial strains showed that all the pairs of primers designed were specific to the species. PCR product sizes obtained for *S. aureus*, *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *Str. uberis*, *Str. agalactiae*, *K. pneumoniae* and *E. coli* were 894, 222, 130, 214, 400, 317, 108, 468 bp, respectively, as shown in Figure 1. Finally, all the eight target pathogens were detected simultaneously in two reactions (Figure 2). In each reaction mixture, when the primers were added at equal concentrations, some target species were not detected. So, the concentrations of the primers were modified to detect all target strains in each reaction.

## Pathogen detection in milk samples by multiplex PCR assay

The multiplex PCR assay was tested to detect target pathogens using DNA directly extracted from milk. The test effectively identified target bacteria. In clinical mastitis cases, *S. aureus*, *Str. uberis*, *S. haemolyticus*, *S. chromogenes* and *K. pneumoniae* were detected in 24.3%, 27%, 2.7%, 27% and 8.1% of samples, respectively. None of these milk samples was positive for *S. epidermidis*, *E. coli*, *Str. agalactiae*. From 163 subclinical samples, all target bacteria are isolated such as 19% *S. aureus*, 19% *S. chromogenes*, 3.1% *S. haemolyticus*, 1.2% *S. epidermidis*, 4.9% *Str.uberis*, 1.2% *Str. agalactiae*, 4.3% *E. coli* and 7.4% *K. pneumoniae* (Table 3).

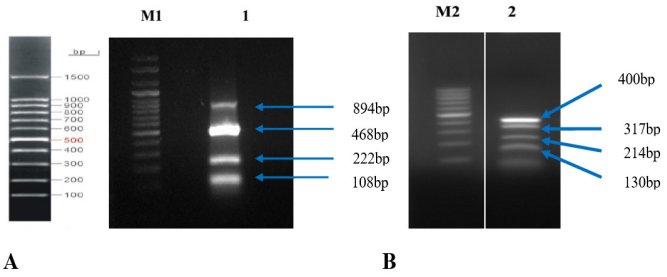


**Figure 2:** Multiplex PCR assay for simultaneous detection of 8 mastitis pathogens.
(A) Tube 1: Lane M1: 100 bp DNA marker, Lane 1: Combination of *S. aureus* (894 bp) + *E. coli* (468 bp) + *S. chromogenes* (222bp) + *K. pneumoniae* (108bp). (B) Tube 2: Lane M2: 100 bp DNA marker, Lane 2: Combination of *Str. uberis* (400bp) + *Str. agalactiae* (317bp) + *S. haemolyticus* (214bp) + *S. epidermidis* (130bp)

According to the multiplex PCR assay, 35% of the total milk samples were positive for a single target species, 15% were positive for more than one target species, while 50% were negative for all target bacterial species.

## Comparison of bacteriological culture and PCR results

Our results showed that of 200 milk samples cultured, 89 samples (44.5%) yielded one or two of the eight target pathogens, 79 samples presented microorganisms that were not targeted by the PCR test and 32 samples with negative culture. Comparing PCR with bacterial culture, 100 samples (50%) were positive and 131 strains of target bacteria were detected by PCR. Furthermore, comparing

**Table 4:** Comparative detection of target pathogens in 200 milk samples by bacterial culture and multiplex PCR.

| | Bacterial culture | | Multiplex PCR | | McNemar (P) | Kappa value (95 % CI) |
|---|---|---|---|---|---|---|
| **Organism** | **Results** | **N** | **Positive** | **Negative** | | |
| *Staphylococcus aureus* | Positive | 52 | 39 | 13 | | |
| | Negative | 116 | 1 | 115 | 0.002 | 0.803 |
| | Total | 168 | 40 | 128 | | [0.705-0.897] |
| *Staphylococcus chromogenes* | Positive | 23 | 20 | 3 | | |
| | Negative | 145 | 16 | 129 | 0.004 | 0.625 |
| | Total | 168 | 36 | 132 | | [0.447-0.769] |
| *Staphylococcus epidermidis* | Positive | 4 | 1 | 3 | | |
| | Negative | 164 | 1 | 163 | 0.625 | 0.324 |
| | Total | 168 | 2 | 166 | | [-0.017-0.798] |
| *Staphylococcus haemolyticus* | Positive | 3 | 3 | 0 | | |
| | Negative | 165 | 3 | 162 | 0.250 | 0.660 |
| | Total | 168 | 6 | 162 | | [0-1] |
| *Streptococcus uberis* | Positive | 5 | 4 | 1 | | |
| | Negative | 163 | 10 | 153 | 0.012 | 0.399 |
| | Total | 168 | 14 | 154 | | [-0.009-0.673] |
| *Streptococcus agalactiae* | Positive | 0 | 0 | 0 | | |
| | Negative | 168 | 2 | 166 | - | - |
| | Total | 168 | 2 | 166 | | |
| *Escherichia coli* | Positive | 3 | 1 | 2 | | |
| | Negative | 165 | 5 | 160 | 0.453 | 0.206 |
| | Total | 168 | 6 | 162 | | [-0.027-0.660] |
| *Klebsiella pneumoniae* | Positive | 3 | 2 | 1 | | |
| | Negative | 165 | 9 | 156 | 0.021 | 0.268 |
| | Total | 168 | 11 | 157 | | [ -0.015-0.558] |
| No growth | | 32 | 9* | 23 | | |

*4 samples: *Str. uberis*, 1 sample: *S. chromogenes*, 3 samples: *S. chromogenes* + *K. pneumoniae* and 1 sample: *K. pneumoniae* + *S. chromogenes* + *E. coli*

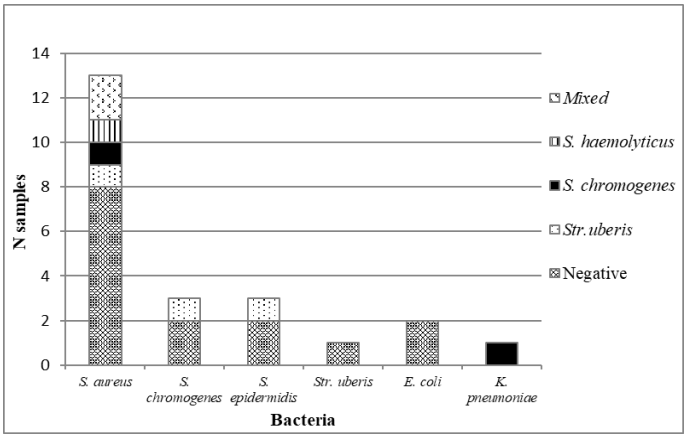


**Figure 3:** Culture-positive samples found negative by PCR for each target bacterial species.
Mixed: Association between two germs (one association between *S. chromogenes* + *Str. uberis* and one association between *Str. uberis* + *S. epidermidis*).

the results obtained by the two methods for the detection of the eight target bacteria using the McNemar test (Table 4), we found no significant difference between conventional methods and multiplex PCR for the detection of *S. epidermidis* (P= 0.625), S. *haemolyticus* (P=0.250), and *E. coli* (P=0.453). However, a significant difference was reported between the two methods for the detection of *S. aureus* (P=0.002), S. *chromogenes* (P= 0.004), *Str. uberis* (P=0.012) and *K. pneumoniae* (P=0.021). Also, the kappa value between the two methods varies from weak to almost perfect agreement depending on the species (Table 4). Despite the relatively good concordance observed between culture and PCR results, PCR identified species that were not detected in culture in many samples. The PCR test detected *S. chromogenes* in 16 samples, *Str. uberis* in 10 samples, *K. pneumoniae* in nine samples, *E. coli* in five samples, *S. haemolyticus* in three samples and *S. aureus* and *S. epidermidis* in one sample (Table 4). In addition, amongst the target species, *Str. agalactiae* was not isolated in any sample by bacterial culture but it was detected by PCR in two samples. In total, PCR detected 14 microorganisms in nine samples showing no growth in culture. These included *Str. uberis* (n=4), *S. chromogenes* (n=5), *K. pneumoniae* (n=4) and *E. coli* (n=1). Twenty-three samples were negative for both cultural and PCR detection. Despite the higher detection of target bacteria observed in PCR when compared to culture, some positive-culture samples showed negative PCR results for

the corresponding pathogens. For most species, except *S. aureus*, these samples ranged from one to three. For *S. aureus*, the PCR assay did not detect this pathogen in 13 culture-positive samples, eight of which showed no species and five presented species other than *S. aureus* including *S. chromogenes* (n=1), *S. haemolyticus* (n=1), *Str. uberis* (n=1), association between *S. chromogenes* and *Str. uberis* (n=1) and association between *Str. uberis* and *S. epidermidis* (n=1, Figure 3).

## DISCUSSION

Since several bacterial species can be involved in mastitis, inadequate techniques for detecting or identifying pathogens often delay timely interventions in disease treatment and control. The current study evaluated a multiplex PCR assay, which uses many primers in the same reaction, for rapid and accurate detection of eight bacterial pathogens frequently associated with bovine mastitis. The multiplex PCR method was compared with bacterial culture and the difference between these tests was noted. Results reported a corresponding level of agreement between Bacteriological Culture and PCR diagnosis for some samples. These results are in agreement with those reported by Shome *et al.* (2011). However, PCR detected species in samples found culture-negative for the corresponding pathogens and others showing no growth. *Str. agalactiae* was diagnosed only by multiplex PCR in two milk samples but not detected by bacterial culture. In the same way, *S. chromogenes*, *Str. uberis*, *K. pneumoniae* detection presented higher sensitivity by multiplex-PCR (41, 18 and 15 positive milk samples detected, respectively), when compared to the detection by microbiological culture (23, 5 and 3 positive samples, respectively). Furthermore, the PCR assay improved the detection of mastitis bacteria in nine milk samples that may not show any bacterial growth under bacterial culture conditions. These results are in agreement with those found by Kahya Demirbilek *et al.* (2024), who showed that a bacterial and/or yeast gene was found by rPCR in 187 of 246 (76.01%) samples with no bacterial growth. In addition, several studies have reported the presence of a large proportion of culture-negative milk samples from cows with clinical or subclinical mastitis ranging from 27 % to 50% (Makovec and Ruegg, 2003; Barrett *et al.*, 2005; Bradley *et al.*, 2007; Taponen *et al.*, 2009). These negative results might be explained by the use of non-specific culture media for these bacteria, the low bacterial concentration, and the unknown use of antibiotics prior to collection or to the presence of several substances that could have inhibited the growth of bacteria on agar plates (Phuektes *et al.*, 2001; Taponen *et al.*, 2009). In addition, the host immune system may have effectively affected the viability of a microorganism, but this microorganism could maintain the status of inflammation by releasing toxins (Taponen *et al.*, 2009; Krômker *et al.*, 2010). The case of two species samples detected only by PCR might be due to a competition between germs for nutrients and space and a low concentration of some pathogens compared to others or as in culture. These results might be also explained by bacterial colonies that are selected based on morphological characteristics, species with similar phenotype can be found and, in this case, the risk of missing a species is probable (Shome *et al.*, 2011). Similarly, possible reasons for no growth in milk samples may be due to the absence of viable bacterial cells. PCR can detect viable or non-viable bacteria present in milk. In this case, although the non-viable bacteria have no role in the initiation of infection, they are identified as positive by PCR. Thus, these samples may be considered as false positives. Therefore, multiplex PCR can be used as an alternative method to bacteriological culture in the routine diagnosis of all mastitis pathogens regardless of fastidious, slow growing and non-culturable microorganisms or easy-to-culture pathogens.

Despite the higher detection of target bacteria observed in PCR when compared to culture, 23 positive-culture samples showed negative PCR results. Discordance was observed mainly for *S. aureus* which was not detected in 13 samples. These results agree with studies of Troncarelli *et al.* (2015) and Singh *et al.* (2019) where *S. aureus* was isolated by culture but not by multiplex PCR. These culture-positive/PCR-negative cases could be explained by the presence of PCR inhibitors that have been found in milk samples such us antibiotics, detergents, enzymes, polysaccharides, fats, proteins, plasmin and bacterial debris (Wilson, 1997; Murphy *et al.*, 2002). Several protocols of DNA-extraction in milk were developed to eliminate these PCR inhibitors and to improve its sensitivity (Kim *et al.*, 2001; Cremonesi *et al.*, 2006; Kubota *et al.*, 2007). Phuektes *et al.* (2001) reported that applying different DNA extraction methods showed different PCR sensitivities. In addition, Cressier and Bissonnette (2011) showed a lower sensitivity of PCR due to the use of spin-columns method for DNA-extraction from milk. In our study, before extraction protocol we tried to remove fats from milk that make it difficult to extract DNA in high quantity and quality (Amills *et al.*, 1997; Cremonesi *et al.*, 2006) and therefore, the inhibition of PCR.

Besides, incorrectly identified species by bacterial culture can be a cause of PCR-negative results (Pitkälä *et al.*, 2005; Koskinen *et al.*, 2010) where in several negative cases for the corresponding pathogen, we find the identification of other bacteria than those found by culture. In case of *S. aureus*, five samples positive for this pathogen in culture showed other pathogens by PCR. Indeed, we found that

these five strains identified as *S. aureus* by conventional culture, using the API STAPH identification gallery, were identified as *S. chromogenes* in the first case, *S. haemolyticus* in the second case, *Str. uberis* in the third case, an association between *S. chromogenes* and *Str. uberis* in the fourth case and an association between *S. epidermidis* and *Str. uberis* in the fifth case. In addition, the PCR-negative results for some gram-positive bacteria can be related to the complexity of the cell wall, which may hamperlysis during the extraction of DNA (Cremonesi *et al*., 2006). The cell wall of gram-positive bacteria contain several molecules which provide a rigid exoskeleton for protection against both mechanical and osmotic lysis (Singh *et al*., 2019).

Our study showed that twenty-three mastitis-samples were negative for both cultural and PCR detection. These results can be mainly explained by the fact that mastitis in these cases could be of traumatic origin due to the milking machine, or less probably caused by yeasts, algae or fungi (Krukowski *et al*., 2006; Fadlelmula *et al*., 2009; Lassa and Smulski, 2013).

Apart from the target species, we found several cases where bacterial culture method identified species not targeted by the PCR assay as for example *Aerococcus viridians*, *Enterococcus* spp., *Pseudomonas luteola*, *Aeromonas hydrophila*, *Micrococcus* spp. and *Pasteurella* spp. For this reason, it would be important to increase the number of target species to be detected by the multiplex PCR test. In this case many researchers developed multiplex PCR assay for simultaneous detection of several pathogens causing bovine mastitis in milk such us ten bacterial species in a study of Shome *et al*. (2011) and nine in a study of Ashraf *et al*. (2017).

The present study is in agreement with several researches, which confirmed that the diagnosis by PCR could be carried out in a few hours (Phuektes *et al*., 2001; Ashraf *et al*., 2017; Kajdanek *et al*., 2024). It takes less than 24 hours (while conventional culture requires more than 72 hours) thus it enables us to do the test monthly or even weekly, which allows the detection of the first cases of mastitis in herds. Rapid results can improve mastitis control and reduce costs through improved treatment strategy and oportum-time intervention (Pyörälä, 2002; Barkema *et al*., 2006) which improves the cure rates and decreases the use of inappropriate antimicrobials (Taponen *et al*., 2009) and discarded milk. However, despite these advantages and the concordance between multiplex PCR and bacterial culture shown in our study, the discordance found for the identification of *S. aureus* by PCR multiplex, should not be overlooked. In this case, it is important to revise this multiplex PCR protocol and attempt to improve its efficiency, by finding a solution to the problems of PCR inhibition and by developing efficient DNA extraction protocols so that it can be used for routine diagnosis of bovine mastitis and in herd decision pathways for smallholders

## CONCLUSIONS

The current study has shown that, as compared to the conventional culture method, the multiplex PCR assay can be applied for rapid and sensitive detection of *S. aureus*, *S. chromogenes*, *S. haemolyticus*, *S. epidermidis*, *Str. uberis*, *Str. agalactiae*, *E. coli* and *K. pneumoniae* simultaneously in milk samples collected from mastitis cows from smallholder farms. The widespread and systematic use of PCR tests to help in the management and control of mastitis on farm and particularly on small dairy farms, would allow rapid diagnosis of the mastitis origins, making treatment earlier and more effective and with lower losses. However, despite the benefits, this method has shown unacceptably low sensitivity for the identification of *S. aureus*. Furthermore, PCR methods are limited to detecting only the targeted bacterial species. In this case, it would be advantageous to improve the multiplex PCR protocol by finding a solution to the problems of PCR inhibition and by increasing the number of targeted species for detection of mastitis by this method.

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## NOVELTY STATEMENT

The development and evaluation of a multiplex PCR assay for simultaneous detection of eight mastitis-causing bacteria directly in milk. The assay will be useful for the detection of mastitis, testing bacteriological safety of milk and for species level differentiation. Finding a less expensive, specific and fast method to facilitate and improve the detection of bacteria causing bovine mastitis in smallholder farms is an important issue.

## AUTHOR'S CONTRIBUTION

All authors contributed to this study. AM designed the study, conducted all experiments, analysed data and wrote the manuscript. HS designed, conducted the experiments and revised the manuscript. AT supervised and coordinated the study and revised the manuscript. RG examined and evaluated the results and revised the manuscript. NS contributed to the experiments. PGM and AM revised

the manuscript. All authors read and approved the final manuscript.

## Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

## Conflict of interest

The authors have declared no conflict of interest.

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