



Establishment and Application of a TaqMan-MGB Multiplex Real-time Fluorescent Quantitative PCR Assay for Detecting Common Pathogens Causing Bovine Mastitis

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ABSTRACT

Background: The aim of this study was to establish a TaqMan-MGB multiplex real-time fluorescent quantitative PCR assay for the simultaneous detection of four pathogens, enabling rapid and effective identification of the etiological agents of bovine mastitis.

Methods: Specific primers and TaqMan-MGB probes were designed targeting the conserved gene sequences of *Staphylococcus aureus*, *Escherichia coli*, *Salmonella spp* and *Pseudomonas aeruginosa*. After optimizing reaction conditions including primer/probe concentrations and annealing temperature, a multiplex real-time PCR assay was established. Specificity, sensitivity and repeatability tests were conducted and the established method was applied to detect 192 clinical samples.

Result: The amplification efficiencies of the 4-plex real-time PCR assay were all above 90%, with correlation coefficients R^2 exceeding 0.99, indicating high amplification efficiency. The assay specifically amplified *S. aureus*, *E. coli*, *Salmonella spp* and *P. aeruginosa*, with no cross-reactivity with *Bacillus cereus*, *Klebsiella spp*, or *Listeria spp*, demonstrating strong specificity. The assay exhibited high sensitivity, with the minimum detection limits of 1.85×10^1 copise/ μ L for *S. aureus*, 2.02×10^1 copise/ μ L for *E. coli*, 1.93×10^1 copise/ μ L for *Salmonella spp* and 1.89×10^1 copise/ μ L for *P. aeruginosa*. Repeatability tests revealed intra-assay coefficients of variation (CV) less than 1.86% and inter-assay CV less than 2.68%, confirming good reproducibility. When applied to 192 clinical milk samples, the coincidence rate of the 4-plex assay with conventional single-plex PCR for detecting mixed infections with the four bacteria was 83.30% and the overall coincidence rate between the two methods was 95.70%. The established 4-plex real-time PCR assay provides a valuable tool for the detection of bovine mastitis pathogens, guiding clinical medication and facilitating molecular epidemiological studies.

Key words: Bovine mastitis, Detection method, Multiplex real-time fluorescent quantitative PCR, TaqMan-MGB.

INTRODUCTION

Bovine mastitis (BM) is a major economic burden to the dairy industry, causing substantial production losses in the dairy sector (Zouharova *et al.*, 2023). Mastitis is typically a host response to intramammary infection, predominantly caused by a variety of bacterial pathogens (Hamed *et al.*, 2023). Currently, the main etiological agents of bovine mastitis include Gram-negative bacteria such as *E. coli* and *Klebsiella spp.* and Gram-positive bacteria such as *Streptococcus uberis* and *S. aureus*-many of which also act as human pathogens (Trung *et al.*, 2023; Anna *et al.*, 2023; Feng *et al.*, 2023; Abd *et al.*, 2023). Accurate, cost-effective detection of mastitis pathogens is crucial for the diagnosis, monitoring and control of bovine mastitis.

Various methods are available for detecting bovine mastitis, including analysis of milk physicochemical properties, somatic cell count, microbial isolation and culture, immunological diagnosis and molecular biological techniques. Microbial identification from milk is regarded as the "gold standard" for mastitis diagnosis; however, this method is time-consuming, cumbersome and low in sensitivity, making it difficult to meet clinical needs. Additionally, many pathogens share similar morphological characteristics and induce overlapping clinical symptoms

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in infected cows, posing challenges for the rapid diagnosis of multiple and secondary infections via culture-based methods. In contrast, multiplex real-time PCR-an approach independent of pathogen culture-has emerged as a mainstream technique for detecting mastitis pathogens

due to its ability to rapidly and specifically identify multiple bacterial species simultaneously (Tingrui *et al.*, 2022; Ke *et al.*, 2022).

In this study, four clinically common pathogens causing bovine mastitis, including *S. aureus*, *E. coli*, *Salmonella spp.* and *P. aeruginosa*, were selected as research objects. According to previous epidemiological investigations, *Salmonella spp.* and *P. aeruginosa* are frequently isolated from mastitis milk samples in dairy farms and have become important conditional pathogens for bovine mastitis. Infections caused by the four pathogens show similar clinical signs and pathological lesions and mixed infections are commonly observed in clinical cases, which aggravate disease severity and bring difficulties to differential diagnosis and prevention.

The present study aimed to design specific primers and TaqMan-MGB probes targeting conserved gene sequences of the four pathogens, optimize reaction conditions to establish and verify a 4-plex real-time fluorescent quantitative PCR assay. This work intends to provide a novel and reliable technical tool for rapid differential diagnosis, molecular epidemiological investigation, mixed infection analysis and clinical medication guidance of bovine mastitis.

MATERIALS AND METHODS

Bacterial strains

Four standard strains were used in this study: *S. aureus*, *E. coli*, *Salmonella spp.* and *P. aeruginosa*, all purchased

from the China General Microbiological Culture Collection Center (CGMCC).

Main reagents and instruments

Nuclease-free water (P1193, Promega); Premix Ex Taq™ (Probe qPCR) (RR390L, Takara Biomedical Technology (Beijing) Co., Ltd.); Nucleic Acid Extraction Kit (Xi'an Tianlong Technology Co., Ltd.).

ABI 7500 real-time PCR system (Thermo Fisher Applied Biosystems, USA); NP968-S Automatic Nucleic Acid Extractor (Xi'an Tianlong Technology Co., Ltd.); 3k15 High-Speed Refrigerated Centrifuge (SIGMA, Germany); BSC-150 Constant Temperature and Humidity Incubator (Shanghai Boxun Industrial Co., Ltd.); QB-9002 Micro Oscillator (Kylin-Bell Lab Instruments Co., Ltd.); DZKW-4 Water Bath (Zhongxing Instrument Co., Ltd.).

Sample collection and processing

All sample collection procedures complied with relevant animal welfare regulations. In June 2023, a total of 192 milk samples were collected from mastitis cows in 12 dairy farms located in Shijiazhuang, Baoding, Tangshan and Qinghuangdao (Table 1). Fresh milk samples were transferred into sterile centrifuge tubes pre-filled with 5 mL pH 7.2 PBS solution, vortexed vigorously for 1 min and centrifuged at 5,000 r/min for 10 min. The obtained supernatants were stored at -20°C for subsequent experiments.

Design and synthesis of primers and probes

Conserved genes of *S. aureus*, *E. coli*, *Salmonella spp.* and *P. aeruginosa* were selected and their gene sequences were downloaded from the NCBI database. Specific and conserved regions were chosen to design primers and probes using BeaconDesigner 7.9 software in accordance with primer design principles. To avoid false positive or false negative results in subsequent amplification, BLAST sequence alignment was performed to verify 100% specific binding of primers and probes to target fragments. All primers and probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Table 2).

Table 1: Results of milk sample collection for bovine mastitis.

Region	Number of cattle farms	Number of milk samples tested
Tangshan	5	80
Baoding	3	48
Shijiazhuang	2	32
Qinghuangdao	2	32
Total	10	192

Table 2: Primer and TaqMan probe sequences.

Strain	Primer and probe sequences (5'-3')	Amplified fragment length/bp
<i>Staphylococcus aureus</i>	F: GTGGTTCTGAAGATCCAACAGTAT R: CTTGCTTCAGGACCATATTTCTCT P: VIC-TGGTGATACGGTTAAATT-MGB	205
<i>Escherichia coli</i>	F: AGGTATAGATGCCTTACCGCTT R: TTGACCAGGCGGTTGCTGAT P: ROX-ATGCGCTGAATAAA-MGB	113
<i>Salmonella</i>	F: AACTGCGGGAGCTTGGCTAT R: CGCTCCAGTTGGTCCAGCAT P: FAM-TTGCGGAACGCGC-MGB	137
<i>Pseudomonas aeruginosa</i>	F: ACGATACCTGGGAAGGCAAGAT R: TAGCCGCACTGCTCCAGTTGTT P: QUASAR 670-TCAAGCCACGGTCAT-MGB	217

Preparation of recombinant plasmid standards

Genomic DNA of the four standard strains was extracted using the NP968-S automatic nucleic acid extractor. The genomic DNA was used as amplification templates with corresponding specific primers and sterile deionized water was set as negative control. The reaction system and procedure are shown in Table 3.

Recombinant plasmids were constructed to prepare quantitative standards. The PCR products were purified and recovered by gel extraction, then ligated into pUC57 vector and transformed into competent *E. coli* DH5± cells. Positive clones were screened on LB solid medium and cultured. Recombinant plasmids were extracted and identified by PCR, restriction enzyme digestion and sequencing. After determining the concentration of positive recombinant plasmids, the copy number was calculated according to the following formula:

$$\text{Copy number} = \frac{\text{Plasmid concentration} \times 6.02 \times 10^{23}}{660 \times \text{total plasmid length}}$$

Single-plex real-time PCR and standard curve construction

Recombinant plasmid standards of the four bacteria were serially diluted 10-fold (from 10^7 to 10^0 copies/ μL) and subjected to single-plex real-time PCR, with three replicates per dilution. Sterile water was used as a negative control. Standard curves were generated by plotting copy number (x-axis) against Ct value (y-axis) using analytical software. The reaction system and procedure are shown in Table 4.

Optimization of the 4-plex real-time PCR assay

Based on the reaction conditions of single-plex real-time PCR, the reaction temperature and system of the 4-plex assay were optimized. The plasmid standards of four target bacteria were used as templates. A matrix experiment was carried out to optimize the final concentration of probes (0.2-0.8 $\mu\text{mol/L}$), primers (0.2-0.8 $\mu\text{mol/L}$) and annealing temperature (55-62°C). The final 25 μL reaction system of the 4-plex assay contained 12.5 μL Premix Ex Taq™ (Probe qPCR), 0.5 μL of each primer, 1 μL template and nuclease-free water to make up the total volume. After mixing and brief centrifugation, amplification was performed on ABI 7500 real-time PCR system. Combined with the low-concentration template amplification characteristics in clinical samples, the cycle threshold was set as $C_t \leq 45$ cycles for result judgment and the optimal reaction system and procedure were finally determined.

Specificity test of the 4-plex real-time PCR assay

Genomic DNA of *Bacillus cereus*, *Klebsiella spp.* and *Listeria spp.* was extracted and used as templates for the 4-plex assay. A mixed plasmid of the four target bacteria served as a positive control and sterile water was used as a negative control. The specificity test was repeated three times.

Sensitivity test of the 4-plex real-time PCR assay

Serially diluted mixed plasmid standards (from 10^7 to 10^1 copies/ μL) were used as templates, with sterile water as a

negative control. The assay was performed in triplicate to determine the minimum detection limit for each pathogen.

Repeatability test of the 4-plex real-time PCR assay

Mixed plasmid standards at three concentrations (10^7 , 10^5 , 10^3 copies/ μL) were prepared by 10-fold serial dilution, with sterile water as a negative control. Intra-assay repeatability was evaluated by testing each concentration three times in the same run; inter-assay repeatability was assessed by testing the same concentrations in three independent runs. CV values were calculated to analyze reproducibility.

Clinical sample detection

Genomic DNA were extracted from 192 clinical milk samples. All samples were detected by the established 4-plex real-time PCR assay and conventional multiplex PCR simultaneously. The conventional multiplex PCR was performed in a 25 μL system: 12.5 μL 2× PCR Mix, 0.5 μL of each upstream and downstream primer of four pathogens, 2 μL nucleic acid template and nuclease-free water to supplement volume. Reaction procedure: pre-denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 40 s; final extension at 72°C for 10 min. Amplification products were detected by agarose gel electrophoresis. The positive rates and coincidence rates of the two detection methods for four pathogens were statistically analyzed.

RESULTS AND DISCUSSION

Preparation of recombinant plasmid standards

Specific amplification of cDNA from *S. aureus*, *E. coli*, *Salmonella spp.* and *P. aeruginosa* yielded amplicons of 205 bp, 113 bp, 137 bp and 217 bp, respectively, consistent

Table 3: Reaction system.

Component	Dosage (μL)
2×EsTaqMasterMix reaction solution	10
dd H ₂ O	6
Upstream primer	1
Downstream primer	1
DNA template	2

Note: Reaction procedure: 94°C, pre-denaturation for 5 min; 35 cycles {95°C, 30 s; 65°C, 30 s; 73°C, 1 min}, extension at 72°C for 10 min.

Table 4: Reaction system.

Component	Dosage (μL)
Premix Ex Taq™ (Probe qPCR)	7.5
Nuclease-free water	4.5
Upstream primer	0.5
Downstream primer	0.5
DNA template	1

Note: Reaction procedure: 95°C, pre-denaturation for 30s; 40 cycles {95°C, 5 s; 60°C, 30 s}.

with expected sizes. Sequencing of recombinant plasmids confirmed 100% homology with corresponding GenBank sequences. The copy numbers of recombinant plasmids were calculated as 1.85×10^7 copies/ μL for *S. aureus*, 2.02×10^7 copies/ μL for *E. coli*, 1.93×10^7 copies/ μL for *Salmonella spp.* and 1.89×10^7 copies/ μL for *P. aeruginosa*, which were used as standard templates.

Standard curve construction

Fluorescent signals were detected for all four recombinant plasmid standards at concentrations ranging from 10^7 to 10^1 copies/ μL . Standard curves exhibited good linearity, with amplification efficiencies $> 90\%$ and $R^2 > 0.99$ for all pathogens (Fig 1). The linear equations were:

S. aureus: $y = -3.519 + 41.766x$, $R^2 = 0.996$, $E = 92.389\%$.

E. coli: $y = -3.536 + 39.232x$, $R^2 = 0.999$, $E = 91.795\%$.

Salmonella spp.: $y = -3.03 + 38.33x$, $R^2 = 0.999$, $E = 113.829\%$.

P. aeruginosa: $y = -3.547 + 40.238x$, $R^2 = 0.99$, $E = 91.401\%$.

Optimal reaction system and procedure for the 4-plex assay

The optimal conditions were determined as follows: primer and probe concentrations of $0.5 \mu\text{mol/L}$ and a $25 \mu\text{L}$ reaction system (Table 5).

Specificity analysis of the 4-plex assay

Positive amplification curves were observed for the four target pathogens, while no amplification was detected for *Bacillus cereus*, *Klebsiella spp.*, *Listeria spp.*, or the negative control (Fig 2), confirming high specificity with no cross-reactivity.

Sensitivity analysis of the 4-plex assay

The minimum detection limits were 1.85×10^1 copies/ μL for *S. aureus*, 2.02×10^1 copies/ μL for *E. coli*, 1.93×10^1 copies/ μL

for *Salmonella spp.* and 1.89×10^1 copies/ μL for *P. aeruginosa* (Fig 3).

Repeatability analysis of the 4-plex assay

Intra-assay CV values were 0.31% - 1.47% for *S. aureus*, 0.42% - 1.76% for *E. coli*, 0.22% - 2.31% for *Salmonella spp.* and 0.27% - 1.86% for *P. aeruginosa*. Inter-assay CV values were 0.81% - 2.68% for *S. aureus*, 0.91% - 1.78% for *E. coli*, 0.89% - 1.99% for *Salmonella spp.* and 1.00% - 2.65% for *P. aeruginosa* (Table 6). All CV values were within the acceptable range (intra-assay CV $< 10\%$, inter-assay CV $< 20\%$), indicating that this 4-plex assay possessed excellent stability and reproducibility.

Clinical sample detection

Among 192 clinical milk samples, 18 samples (9.38%) were detected as quadruple mixed infection by the 4-plex assay, while 15 samples (7.81%) were positive by conventional multiplex PCR. The coincidence rate for mixed infection detection was 83.30% and the overall coincidence rate of the two methods was 95.70% . The Kappa value was calculated as 0.82 , suggesting a substantial statistical consistency between the two methods (Table 7). The 4-plex real-time PCR assay detected 8 more mixed infection positive samples, which proved its higher sensitivity and good application prospect in clinical detection.

Molecular biological techniques represented by real-time PCR present prominent advantages in sensitivity, specificity and stability compared with conventional pathogen detection methods. It features simple operation, low contamination risk and acceptable cost and supports both qualitative and quantitative detection of pathogens. This technology has been widely applied in pathogen identification and disease diagnosis and serves as an

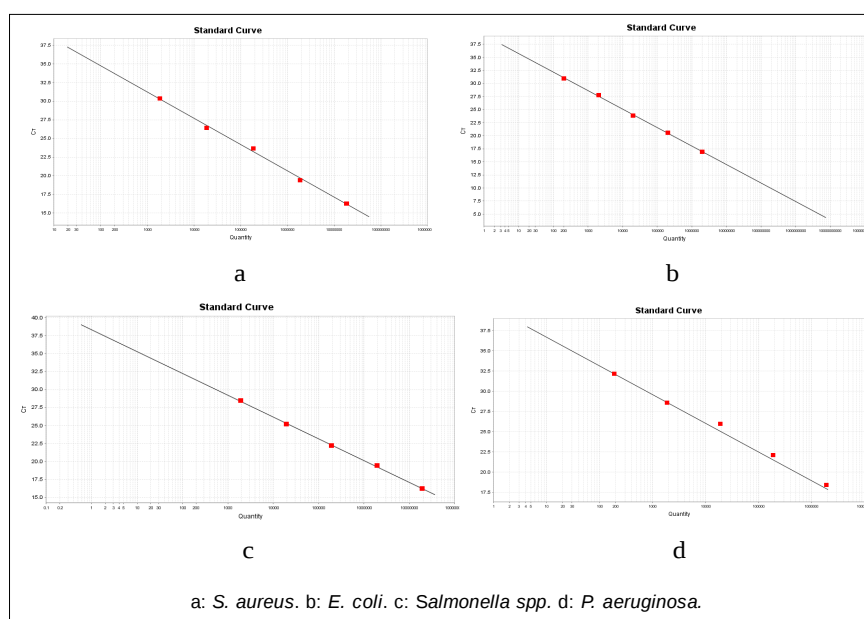


Fig 1: Standard curves of the 4-plex real-time PCR assay.

effective tool for the simultaneous detection of multiple pathogens.

Multiplex real-time PCR can achieve accurate quantification of target nucleic acids and bypass agarose gel electrophoresis, which simplifies the experimental procedure and reduces laboratory pollution (Zhou *et al.*, 2017). Specifically, TaqMan real-time PCR improves detection specificity through specific hybridization between probes and target templates and realizes quantitative detection of nucleic acid copy numbers. Previous comparative studies demonstrated that the limit of detection of TaqMan real-time PCR is between 10^1 to 10^2 copies/ μ L, which is 100-fold and 1000-fold lower than that of SYBR green I-based real-time PCR and conventional PCR, respectively (Noemí *et al.*, 2022). Nevertheless, TaqMan probes and matched consumables are relatively expensive, leading to a higher cost for single detection. Combining multiple

primer-probe sets in one reaction system for multiple detection can greatly shorten operation duration, reduce reagent consumption, simplify sample handling and lower contamination risks (Letian *et al.*, 2022), simplifying sample loading procedures, minimizing contamination and saving time.

In this study, we successfully established a 4-plex TaqMan real-time PCR assay targeting four prevalent pathogens of bovine mastitis, which cause similar clinical symptoms in infected cows. The optimized assay produced a single specific amplification peak with uniform T_m values, no primer dimers and no non-specific amplicons, making it suitable for the rapid detection, differential diagnosis and epidemiological investigation of *S. aureus*, *E. coli*, *Salmonella spp.* and *P. aeruginosa* in large batches of milk samples. The assay showed excellent specificity with no cross-reactivity to *Bacillus cereus*, *Klebsiella spp.*, or *Listeria spp.* It also had high sensitivity (limit of detection: 10^1 copies/ μ L for all four pathogens) and good reproducibility, with intra-assay CV below 1.86% and inter-assay CV below 2.68%.

A total of 192 clinical milk samples were tested in this study. The 4-plex assay achieved an 83.30% coincidence rate with conventional multiplex PCR in detecting mixed infections and the overall coincidence rate reached 95.70%. Epidemiologically, mixed infections caused by the four pathogens are widespread in local dairy farms, which aggravates disease severity, increases treatment difficulty and raises economic losses for the dairy industry. Rapid multiplex detection is therefore critical for the prevention and control of bovine mastitis. Compared with traditional PCR, the multiplex real-time PCR assay enables both qualitative and quantitative detection of the four bacteria, with higher sensitivity, no need for electrophoresis, shorter operation time and simpler procedures. This 4-plex assay improves detection efficiency and provides a reliable technical support for early diagnosis and epidemiological research on mastitis caused by the four pathogens (Wen *et al.*, 2022).

Table 5: Reaction system.

Component	Dosage (μ L)
Premix Ex Taq™ (Probe qPCR)	12.5
<i>Staphylococcus aureus</i> -F	0.5
<i>Staphylococcus aureus</i> -R	0.5
<i>Staphylococcus aureus</i> -P	1
<i>Escherichia coli</i> -F	0.5
<i>Escherichia coli</i> -R	0.5
<i>Escherichia coli</i> -P	1
<i>Salmonella</i> -F	0.5
<i>Salmonella</i> -R	0.5
<i>Salmonella</i> -P	1
<i>Pseudomonas aeruginosa</i> -F	0.5
<i>Pseudomonas aeruginosa</i> -R	0.5
<i>Pseudomonas aeruginosa</i> -P	1
Nuclease-free Water	3.5
DNA template	1

Note: Reaction procedure: 95°C, pre-denaturation for 30 s; 40 cycles {95°C, 5 s; 60°C, 30 s}.

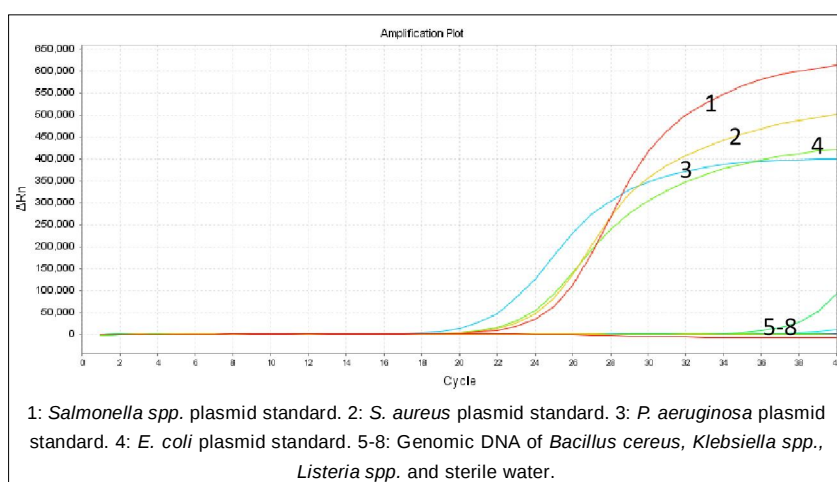


Fig 2: Specificity test of the 4-plex real-time PCR assay.

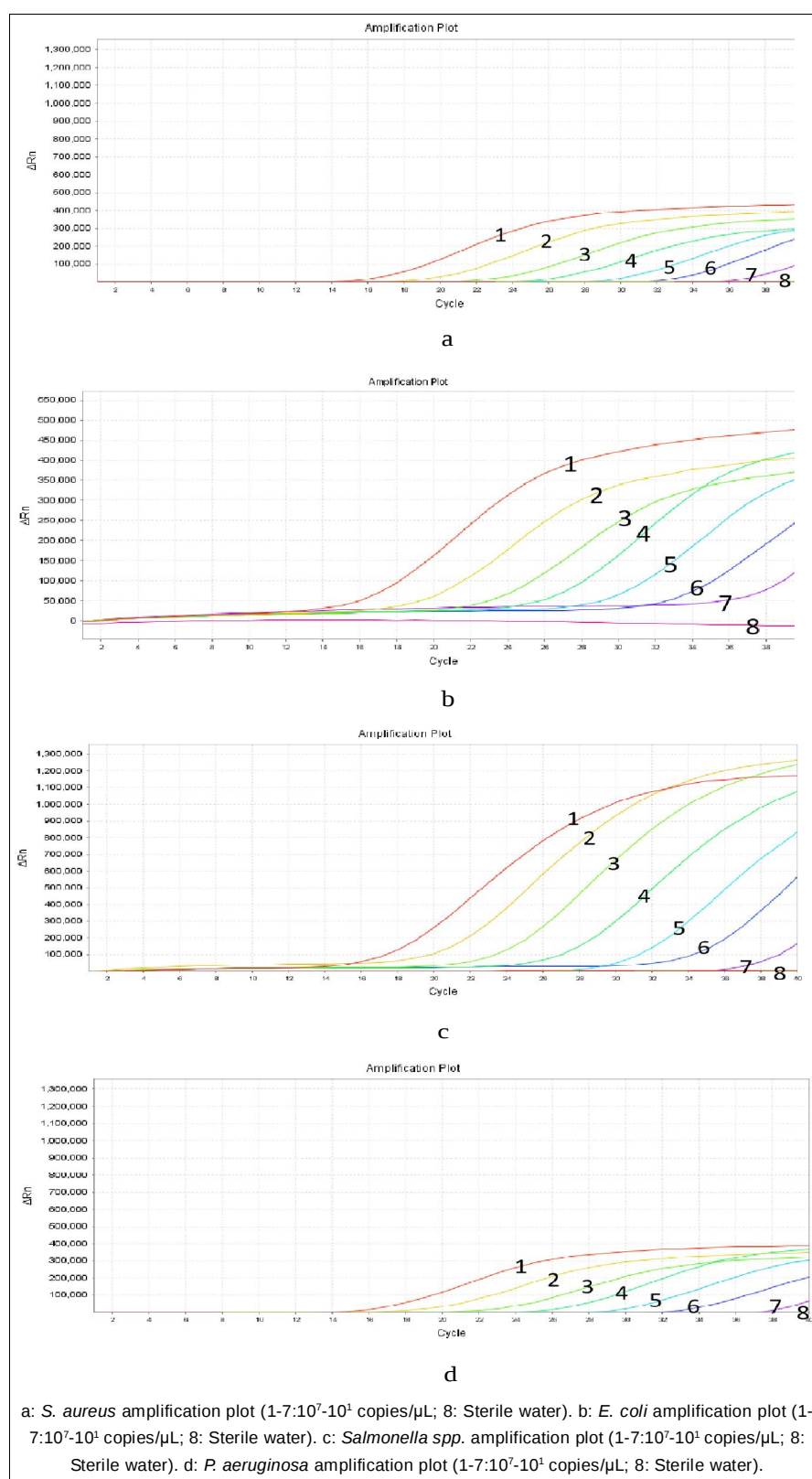


Fig 3: Sensitivity test of the 4-plex real-time PCR assay.

Table 6: Reproducibility results of 4-Plex fluorescent quantitative PCR.

Plasmid standard	Standard concentration (copies/ μ L)	n	Intra-group variation test		Intergroup variation test	
			Ct value (mean $\pm$ SD)	Coefficient of variation CV(%)	Ct value (mean $\pm$ SD)	Coefficient of variation CV(%)
<i>Staphylococcus aureus</i>	1.85×10^7	3	18.35 $\pm$ 0.27	1.47	19.61 $\pm$ 0.53	2.68
	1.85×10^5	3	24.79 $\pm$ 0.08	0.31	27.47 $\pm$ 0.22	0.81
	1.85×10^3	3	32.67 $\pm$ 0.47	1.44	34.27 $\pm$ 0.77	2.24
<i>Escherichia coli</i>	2.02×10^7	3	12.97 $\pm$ 0.22	1.71	14.99 $\pm$ 0.07	1.78
	2.02×10^5	3	23.30 $\pm$ 0.41	1.76	23.05 $\pm$ 0.04	0.91
	2.02×10^3	3	30.01 $\pm$ 0.13	0.42	34.82 $\pm$ 0.13	1.03
<i>Salmonella</i>	1.93×10^7	3	15.04 $\pm$ 0.18	1.22	15.27 $\pm$ 0.09	1.99
	1.93×10^5	3	24.58 $\pm$ 0.05	0.22	24.29 $\pm$ 0.05	0.89
	1.93×10^3	3	32.59 $\pm$ 0.75	2.31	33.03 $\pm$ 0.10	0.95
<i>Pseudomonas aeruginosa</i>	1.89×10^7	3	18.58 $\pm$ 0.05	0.27	19.05 $\pm$ 0.50	2.65
	1.89×10^5	3	26.29 $\pm$ 0.49	1.86	27.56 $\pm$ 0.62	2.25
	1.89×10^3	3	33.10 $\pm$ 0.12	0.37	35.68 $\pm$ 0.13	1.00

Table 7: Detection results of clinical milk samples.

Pathogen	Number of samples (pieces)	Multiplex fluorescent quantitative PCR detection		Conventional multiplex PCR assay	
		Number of positive samples (units)	Positivity rate (%)	Number of positive samples (units)	Positivity rate (%)
<i>Staphylococcus aureus</i> + <i>Escherichia coli</i> + <i>Salmonella</i> + <i>Pseudomonas aeruginosa</i>	192	18	9.38	15	7.81
<i>Staphylococcus aureus</i> + <i>Escherichia coli</i> + <i>Salmonella</i>	192	25	13.02	22	11.46
<i>Staphylococcus aureus</i> + <i>Escherichia coli</i> + <i>Pseudomonas aeruginosa</i>	192	12	6.25	12	6.25
<i>Staphylococcus aureus</i> + <i>Salmonella</i> + <i>Pseudomonas aeruginosa</i>	192	9	4.69	9	4.69
<i>Staphylococcus aureus</i> + <i>Escherichia coli</i>	192	22	11.46	20	10.42
<i>Staphylococcus aureus</i> + <i>Salmonella</i>	192	10	5.21	10	5.21
<i>Staphylococcus aureus</i> + <i>Pseudomonas aeruginosa</i>	192	12	6.25	12	6.25
<i>Escherichia coli</i> + <i>Salmonella</i>	192	9	4.69	9	4.69
<i>Escherichia coli</i> + <i>Pseudomonas aeruginosa</i>	192	11	5.73	11	5.73
<i>Salmonella</i> + <i>Pseudomonas aeruginosa</i>	192	6	3.13	6	3.13
<i>Staphylococcus aureus</i>	192	16	8.33	16	8.33
<i>Escherichia coli</i>	192	16	8.33	16	8.33
<i>Salmonella</i>	192	11	5.73	11	5.73
<i>Pseudomonas aeruginosa</i>	192	9	4.69	9	4.69
Total	192	186	96.88	178	92.71

CONCLUSION

In conclusion, we successfully established a 4-plex TaqMan-MGB real-time fluorescent quantitative PCR assay for the simultaneous detection of *S. aureus*, *E. coli*, *Salmonella spp.* and *P. aeruginosa* in milk samples. With high efficiency, specificity, sensitivity and reproducibility, this method is well suited for clinical detection of mixed infections of bovine mastitis and also

lays a foundation for further epidemiological research on these pathogens.

Despite its good performance, this assay still has obvious limitations. It relies on professional real-time PCR instruments and special fluorescent probes, so it cannot be applied to rapid field detection at grassroots dairy farms lacking supporting equipment. In addition, the high price of probes also restricts its large-scale promotion in primary veterinary laboratories.

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Conflict of interest

The authors declare no conflict of interest.

REFERENCES

- Abd, E.K.A., Amany, A.A., Ehab, A.F., Ashraf, H.S., Abeer, M.A. and Magdy, E. (2023). Phenotypic and genotypic characterization of erythromycin-resistant *Staphylococcus aureus* isolated from bovine subclinical mastitis in Egypt. *Vet. World*. **16(7)**: 1562-1571.
- Anna, D., Dagmara, G.W., Agata, M. et al. (2023). Detection of immunoreactive proteins of *Escherichia coli*, *Streptococcus uberis* and *Streptococcus agalactiae* isolated from cows with diagnosed mastitis. *Front. Cell. Infect. Microbiol.* **13**: 987842.
- Feng, Y., Wenli, S., Na, M. et al. (2023). Antimicrobial resistance and virulence profiles of staphylococci isolated from clinical bovine mastitis. *Front Microbiol.* **14**: 1190790.
- Hamed, R.S., Twfik, R.S. and Abdelall, S.E. (2023). Development of a lateral flow device for rapid simultaneous multiple detections of some common bacterial causes of bovine mastitis. *J. Adv. Vet. Anim. Res.* **10(2)**: 292-300.
- Ke, L., Mingyuan, H., Lin, Z. et al. (2022). Analysis of antimicrobial resistance and genetic correlations of *Escherichia coli* in dairy cow mastitis. *J. Vet. Res.* **66(4)**: 571-579.
- Letian, Z., Zhiwen, J., Zitong, Z. et al. (2022). A TaqMan probe-based multiplex real-time PCR for simultaneous detection of porcine epidemic diarrhea virus subtypes G1 and G2 and porcine rotavirus groups A and C. *Viruses*. **14(8)**: 1819.
- Noemí, M.C., Araceli, S., Lucila, M. et al. (2022). Diagnosis of bacterial meningitis caused by *Streptococcus pneumoniae*, *Neisseria meningitidis* and haemophilus influenzae using a multiplex real-time PCR technique. *Braz. J. Microbiol.* **53(4)**: 1951-1958.
- Tingrui, Z., Linli, T., Sukolrat, B. et al. (2022). Antimicrobial resistance of *Streptococcus uberis* isolated from bovine mastitis: A review. *Indian Journal of Animal Research*. **56(12)**: 1435-1441. doi: 10.18805/IJAR.B-1377.
- Trung, T.M., Van, L.T., Duy, V.M. et al. (2023). Antimicrobial resistance and molecular characterization of *Escherichia coli* isolated from bovine mastitis samples in Nghe An province, Vietnam. *Vet. World*. **16(4)**: 743-751.
- Wen, X.J., Sheng, T.H., Long, X. et al. (2022). Development of a diagnostic assay by three-tube multiplex real-time PCR for simultaneous detection of nine microorganisms causing acute respiratory infections. *Sci. Rep.* **12(1)**: 13306.
- Zouharova, M., Nedbalcova, K., Matiaskova, K. et al. (2023). Antimicrobial susceptibility and resistance genes in *Streptococcus uberis* isolated from bovine mastitis in the Czech Republic. *Antibiotics*. **12(10)**: 1-12.
- Zhou, X., Zhang, T., Song, D. et al. (2017). Comparison and evaluation of conventional RT-PCR, SYBR green I and TaqMan real-time RT-PCR assays for the detection of porcine epidemic diarrhea virus. *Mol. Cell. Probes*. **33**: 36-41.