Establishment of a Duplex Fluorescent Quantitative PCR Assay for Staphylococcus aureus Detection Based on Pangenome Analysis

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Article

Establishment of a Duplex Fluorescent Quantitative PCR Assay for *Staphylococcus aureus* Detection Based on Pangenome Analysis

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Abstract

(1) Background: *Staphylococcus aureus* is a common human respiratory pathogen. Its widespread antimicrobial resistance often leads to severe infections. However, the high genomic diversity among strains frequently causes false negatives in conventional PCR assays. (2) Methods: To address this limitation, we developed a highly sensitive and specific duplex fluorescent quantitative PCR (qPCR) method for *S. aureus* detection based on pangenome analysis. Conserved core genome sequences of *S. aureus* were identified using a pangenomic approach. Two highly conserved and species-specific genes—*isdA* and *recQ*—were selected as target loci for the duplex qPCR assay. A TaqMan probe-based duplex fluorescent qPCR assay was established, enabling simultaneous detection of both *isdA* and *recQ* genes in a single reaction. (3) Results: The assay demonstrated detection sensitivities of 1.335×10^3 copies/μL for *isdA* and 7.265×10^2 copies/μL for *recQ*. The intra-assay and inter-assay coefficients of variation (CV) for Ct values were below 1.86% and 2.87%, respectively, indicating excellent reproducibility. Specificity analysis revealed no cross-reactivity with genomic DNA from 14 common respiratory pathogens, including *Pseudomonas aeruginos*a and *Streptococcus pneumoniae*. (4) Conclusions: Due to its high specificity and sensitivity, this duplex qPCR assay effectively overcomes limitations of single-target PCR methods, such as missed detections and prolonged turnaround times, and holds significant potential for practical diagnostic applications.

Keywords: *Staphylococcus aureus*; pangenome analysis; *isdA* gene; *recQ* gene; duplex qPCR

1. Introduction

Staphylococcus aureus is a common, widely distributed human respiratory pathogen capable of causing gastrointestinal and pyogenic infections [1], including pneumonia, pericarditis, pseudomembranous colitis, septicemia [2,3], toxemia, and bacterial endocarditis [4,5]. Consequently, early detection of *S. aureus* infection is critical for effective treatment [6,7].

Current methods for detecting *S. aureus* include microbial culture, immunoassays [8], and molecular biology technique [9]. Microbial culture is time-consuming, potentially delaying clinical diagnosis. Immunoassays based on antibodies often incur high costs related to antibody production. Although molecular biology techniques offer high sensitivity, they require sophisticated instrumentation and skilled personnel [10]. With the advancement of molecular diagnostics, traditional PCR is increasingly being replaced by real-time quantitative PCR (qPCR) due to limitations such as contamination risks [11]. While qPCR has been applied for rapid *S. aureus* diagnosis, single-target assays are vulnerable to false-positive or false-negative results caused by residual nucleic acids or bacterial genome mutations [12].

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In this study, a pangenome-based approach identified conserved and specific core genomic targets of *S. aureus*. Two highly conserved genes, *isdA* and *recQ*, were selected as dual targets for a real-time duplex fluorescent qPCR assay. This dual-target strategy addresses the limitations of existing methods, such as false results and prolonged detection times, improving diagnostic accuracy compared to conventional single-target qPCR.

2. Materials and Methods

Bacterial Strains and DNA Extraction

Two *S. aureus* strains (ATCC 12600 and ATCC 25923) served as positive controls. *S. aureus* strains were cultured in broth medium at 37 °C for 18~24 hours. Bacterial concentrations (CFU/mL) were determined using standard plate counts. After dilution to working concentrations, genomic DNA was extracted using the Bacteria Genomic DNA Kit (CW0552S, Kangwei Century Biotechnology, Jiangsu, China) according to the manufacturer's instructions. Extracted DNA was stored at -20 °C.

For specificity evaluation, genomic DNA was also extracted from 14 common respiratory pathogens: *Pseudomonas aeruginosa* (ATCC 10145), *Klebsiella pneumoniae* (ATCC 13883), *Acinetobacter baumannii* (ATCC 19606), *Streptococcus pyogenes* (ATCC 12344), *Proteus mirabilis* (ATCC 29906), *Neisseria meningitidis* (ATCC 13077), *Stenotrophomonas maltophilia* (ATCC 13637), *Streptococcus salivarius* (ATCC 7073), *Klebsiella oxytoca* (ATCC 49131), *Moraxella catarrhalis* (ATCC 25238), *Haemophilus influenzae* (ATCC 49247), *Citrobacter freundii* (ATCC 13316), *Streptococcus pneumoniae* (ATCC 49619), and *Mycoplasma pneumoniae* (ATCC 15531). DNA extraction used the same kit (CW0552S). DNA concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, China), and all samples were stored at -20 °C.

2.1. Pangenome Analysis

The pan-genome, representing the complete genomic repertoire of a species, comprises the core genome (genes shared by all strains) and the variable genome (strain-specific genes or sequence variations). The core genome is associated with essential biological functions and conserved phenotypic traits, reflecting genetic stability. The variable genome contributes to environmental adaptation and diversification.

The Bacterial Pan Genome Analysis tool (BPGA) is a high-efficiency platform for microbial pangenomic studies, suitable for large-scale analyses to assess gene content variation, delineate core and variable genomes, and trace horizontal gene transfer.

Complete *S. aureus* genome sequences were obtained from NCBI. A local genomic database was constructed using 200 downloaded *S. aureus* genomes and analyzed using BPGA. Candidate core gene specificity was evaluated using NCBI Protein BLAST [13]. Conserved core genes with high specificity were further analyzed for sequence conservation using local BLAST against the database.

2.1. Primer and Probe Design

S. aureus isdA and *recQ* gene sequences were retrieved from NCBI Gene. Multiple sequence alignments assessed conservation, and primers were designed within conserved regions. Criteria included[14]: GC content 45~55%; amplicon length 80~300 bp; primer length 17~25 nucleotides; ≤3 bases self-complementarity; ≤3 consecutive G or C nucleotides; melting temperature (Tm) 50~65 °C (forward/reverse Tm difference ≤1 °C). Specificity was evaluated using Primer BLAST to eliminate non-specific amplification.

TaqMan probes were designed between primer pairs using Primer Express 3.0. The isdA-qP probe was labeled with VIC (5') and BHQ1 (3'). The recQ-qP probe was labeled with 6-FAM (5') and BHQ1 (3').

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2.1. Validation of Primer Specificity Using Conventional PCR and SYBR Green qPCR

Genomic DNA from the 16 respiratory pathogens (Section 2.1) served as template to evaluate *isdA* and *recQ* primer specificity by conventional PCR, followed by SYBR Green qPCR [15].

The conventional PCR reaction mixture (50 μL) contained: 45 μL Gold Mix enzyme (Qingke Biotechnology, Wuhan, China), 2 μL forward primer, 2 μL reverse primer, 1 μL template DNA, nuclease-free water to 50 μL. Cycling conditions: 98 °C for 2 min; 40 cycles of 98 °C for 10 s, 60 °C for 10 s, 72 °C for 10 s; 72 °C for 5 min; hold at 4 °C.

The SYBR Green qPCR reaction mixture (25 μL) contained: 12.5 μL TB Green Premix Ex Taq II FAST qPCR (2×) (TaKaRa Biotechnology, Dalian, China), 1 μL forward primer, 1 μL reverse primer, 1 μL template DNA, nuclease-free water to 25 μL. Cycling conditions: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 10 s; melt curve: 60 °C to 95 °C at 0.5 °C/s.

2.1. Preparation of Standard Plasmids

isdA and *recQ* gene fragments were amplified from *S. aureus* genomic DNA by conventional PCR and cloned into the pMD-18T vector. Recombinant plasmids (pMD-18T-isdA and pMD-18T-recQ) were purified and quantified.

Plasmid copy numbers were calculated using the formula:

$$\text{Copy number (copies/μL)} = \frac{\text{Plasmid concentration(ng/μL)} \times 10^{-9} \times 6.02 \times 10^{23}}{660 \times \text{Length of the plasmid}}$$

Equal volumes of pMD-18T-isdA and pMD-18T-recQ were mixed and serially diluted 10-fold using EASY Dilution II buffer. These dilutions served as templates for generating standard curves in the duplex qPCR assay [16].

2.1. Optimization of Duplex Fluorescent Quantitative PCR Assay

To enhance stability, sensitivity, and efficiency, annealing temperature, primer concentration, and probe concentration were optimized using *S. aureus* genomic DNA as template and sterile water as negative control.

A matrix approach optimized annealing temperature (56~63 °C), primer concentrations (0.2~1.0 μmol/L), and probe concentrations (0.2~1.0 μmol/L). Optimal conditions were defined by the strongest fluorescence signal and lowest Cq value.

Ten-fold serial dilutions of the standard plasmid mixture (10^{-1} to 10^{-10}) were amplified under optimized conditions, with sterile water as negative control.

2.1. Evaluation of Specificity, Repeatability, and Sensitivity

Specificity: Genomic DNA from 15 respiratory pathogens (including *S. aureus*) was tested under optimized duplex qPCR conditions [17].

Repeatability: Recombinant plasmid mixtures at 10^{-1}, 10^{-2}, and 10^{-3} dilutions served as templates. Intra-assay repeatability was assessed with three technical replicates per run. Inter-assay repeatability was assessed across three independent runs [18].

Sensitivity: Serial dilutions of the plasmid mixture (10^{0} to 10^{-10}) were amplified. Standard curves were generated; amplification efficiency (E) and correlation coefficient (R^2) were calculated [19]. Genomic DNA was extracted from 1 mL *S. aureus* suspension (3.59×10^{9} CFU/mL), serially diluted 10-fold, and tested by single-gene qPCR to determine the limit of detection (LOD) for cultured cells.

3. Results

3.1. Pan-Genome Analysis

Pan-genome analysis of 200 *S. aureus* strains using BPGA (Figure 1) showed the number of core genes decreased gradually as more genomes were added, though the rate of decrease slowed. This

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indicates an open pan-genome with a tendency towards convergence, supporting the adequacy of our genome sample size. 11409 core gene sequences were identified.

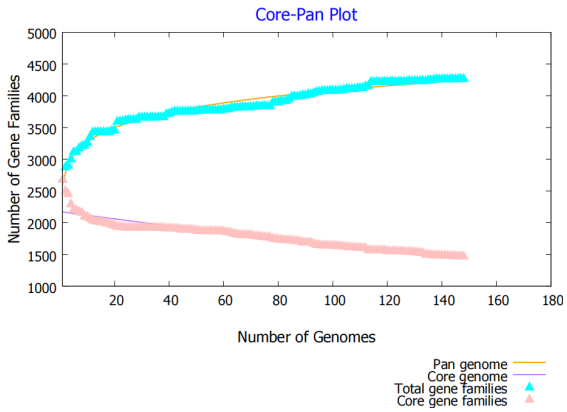

Figure 1. Pan-genome analysis results of *S. aureus.*

Table 1. Analysis results of specificity and conservation of target genes.

Fungus name	*isdA* gene		*recQ* gene	
	highest score	similarity%	highest score	similarity%
S. aureus	1945	93~100	948	95~100
P. aeruginosa	0	0	0	0
K. pneumoniae	0	0	0	0
A. baumannii	0	0	0	0
P. mirabilis	0	0	0	0
K. oxytoca	0	0	0	0
M. catarrhalis	0	0	0	0
L. pneumophila	0	0	0	0
H. influenzae	0	0	0	0
N. meningitidis	0	0	0	0
C. freundii	0	0	0	0
S. pneumoniae	0	0	0	0
M. pneumonia	0	0	0	0
S. pyogenes	0	0	0	0
S. salivarius	0	0	0	0

Specificity analysis (NCBI-BLAST) identified two species-specific proteins: IsdA (WP_180821735.1) and RecQ (WP_194085592.1) (Table 1). Both genes were exclusive to *S. aureus* and absent in other common pathogens.

Iron-regulated surface determinant A (IsdA) facilitates bacterial iron acquisition by binding host iron-containing molecules (e.g., hemoglobin, heme), playing a crucial role in *S. aureus* survival and pathogenicity [20].

The *S. aureus recQ* gene encodes a helicase essential for genome stability, involved in DNA replication, recombination, and repair [21]. RecQ helicase recognizes single-stranded DNA during homologous recombination repair, helping maintain genomic stability under stress [22].

Local BLAST conservation analysis (Table 1) showed both *isdA* and *recQ* genes exhibited >90% sequence similarity across the database, confirming wide distribution and high conservation in *S. aureus*.

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3.2. Primer and Probe Design Results

The specific primers and probes were designed using NCBI-BLAST and Primer Express 3.0 software (Table 2). Multiple sequence alignments confirmed primers and probes were located within conserved regions of the target genes (Figures 2.1, 2.2).

Table 2. Primer and probe sequences.

Target gene	Primers/probes	sequences (5′→3′)	Fragment size(bp)
isdA	isdA-qF	ACAGCACAAACTGCTCAAGA	229bp
	isdA-qR	GTGTTGCGAAGGCAACTGTG	
	isdA-qP	VIC-CAAAATAAAGTTCAAACACC-BHQ1	
recQ	recQ-qF	ACGTTTCGACCAGGACAAGAA	80bp
	recQ-qR	CCACCAGTTGGTAAGACACCA	
	recQ-qP	6-FAM-ATTAGCAAAGTATTAGACCATCG-BHQ1	

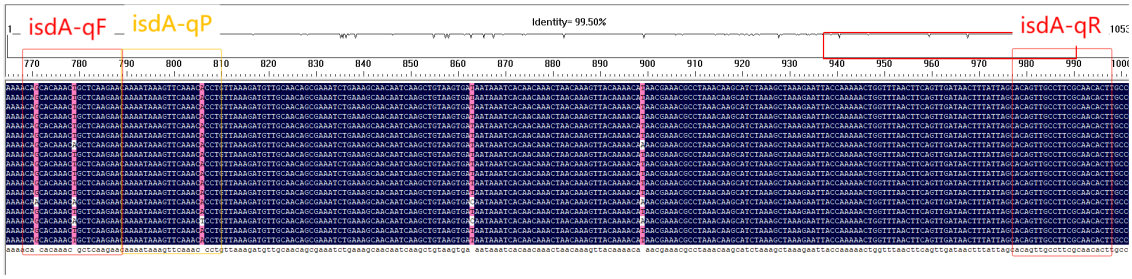

Figure 2.1. Analysis of the conservation of *isdA* gene and primers.

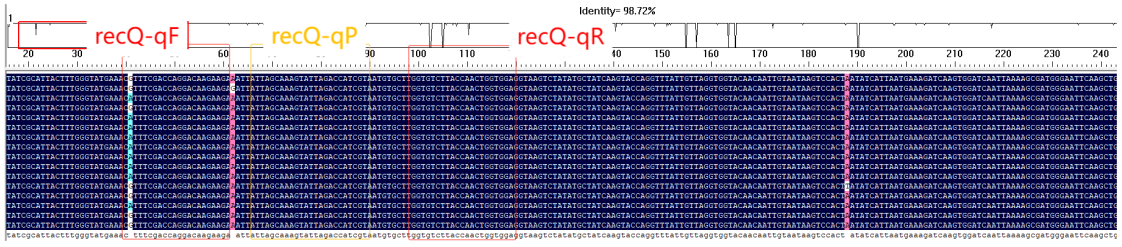

Figure 2.2. Analysis of the conservation of *recQ* gene and primers.

3.3. Specificity Validation of Primers by Conventional PCR and SYBR Green Assay

Genomic DNA from 16 respiratory pathogens was tested.

isdA Gene: Conventional PCR (Figure 3.1) showed a band around 250bp only for *S. aureus-1* and *S. aureus-2*. SYBR Green qPCR (Figure 3.2) showed amplification curves only for these strains.

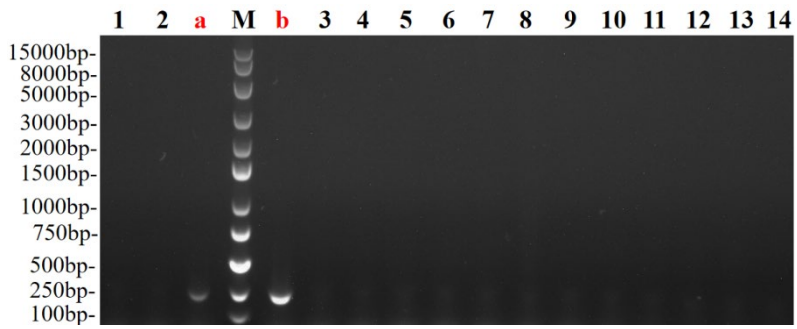

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Figure 3.1. Conventional PCR results of *isdA* gene. "M" lane represents DNA marker, "a" lane represents *S. aureu-1*, "b" lane represents *S. aureu-2*, and "1~14" lanes represent respectively *P. aeruginosa, K. Pneumoniae, A. baumannii, S. pyogenes, P. mirabilis, N. meningitidis, S. salivarius, K. oxytoca, M. catarrhalis, L. pneumophila, H. influenzae, C. freundii, S. pneumoniae, M. pneumoniae.*

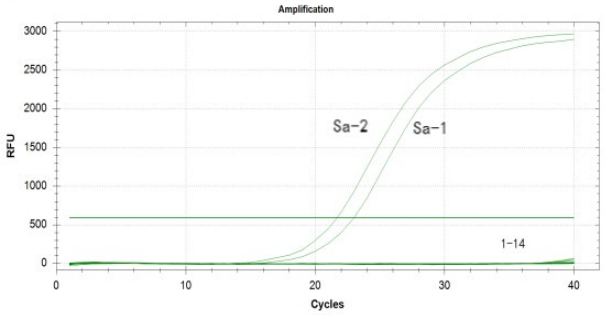

Figure 3. 2. Results of isdA gene qPCR (SYBR Green). The curve "Sa-1" represents S. aureu-1, "Sa-2" represents S. aureu-2, and "1~14" respectively represent P. aeruginosa, K. Pneumoniae, A. baumannii, S. pyogenes, P. mirabilis, N. meningitidis, S. salivarius, K. oxytoca, M. catarrhalis, L. pneumophila, H. influenzae, C. freundii, S. pneumoniae, M. pneumoniae.

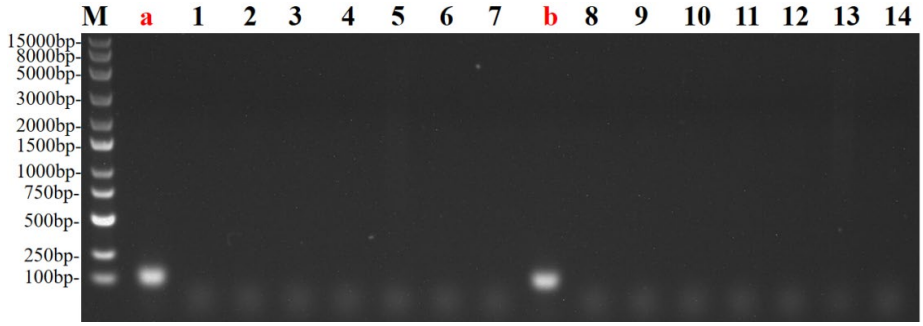

Figure 3. 3. Conventional PCR results of *recQ* gene."M" lane represents DNA marker, "a" lane represents *S. aureu-1*, "b" lane represents *S. aureu-2*, and "1~14" lanes represent respectively *P. aeruginosa, K. Pneumoniae, A. baumannii, S. pyogenes, P. mirabilis, N. meningitidis, S. salivarius, K. oxytoca, M. catarrhalis, L. pneumophila, H. influenzae, C. freundii, S. pneumoniae, M. pneumoniae*.

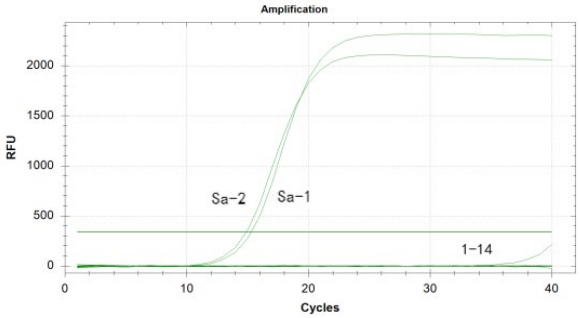

Figure 3. 4. recQ gene qPCR (SYBR Green) results.The curve "Sa-1" represents S. aureu-1, "Sa-2" represents S. aureu-2, and "1~14" respectively represent P. aeruginosa, K. Pneumoniae, A. baumannii, S. pyogenes, P. mirabilis, N. meningitidis, S. salivarius, K. oxytoca, M. catarrhalis, L. pneumophila, H. influenzae, C. freundii, S. pneumoniae, M. pneumoniae.

recQ Gene: Conventional PCR (Figure 3.3) showed a band around 100 bp only for *S. aureus-1* and *S. aureus-2*. SYBR Green qPCR (Figure 3.4) showed amplification curves only for these strains.

These results confirm the high specificity of primer pairs isdA-F/R and recQ-F/R for *S. aureus*.

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3.4. Optimization and Establishment of the Duplex Real-Time Fluorescence Quantitative PCR Method

The annealing temperature, primer concentration and probe concentration of the target gene were optimized.Optimal conditions were determined:

isdA Gene: Primer 0.9 μM, Probe 1 μM, Annealing 58 °C.

recQ Gene: Primer 12.5 μM, Probe 5 μM, Annealing 58 °C.

The optimized duplex PCR mixture (25 μL) contained: 12.5 μL Probe qPCR Mix MultiPlus (TaKaRa), 0.5 μL IsdA-qF (22.5 μM), 0.5 μL IsdA-qR (22.5 μM), 0.5 μL RecQ-qF (12.5 μM), 0.5 μL RecQ-qR (12.5 μM), 1 μL IsdA-qP (25 μM), 1 μL RecQ-qP (5 μM), 2 μL DNA template, sterile water to 25 μL.

Amplification used a CFX96 Real-Time PCR system (Bio-Rad): 95 °C for 20 s; 40 cycles of 95 °C for 1 s, 58 °C for 20 s (fluorescence collection).

3.5. Standard Curves for Duplex Real-Time Fluorescence Quantitative PCR

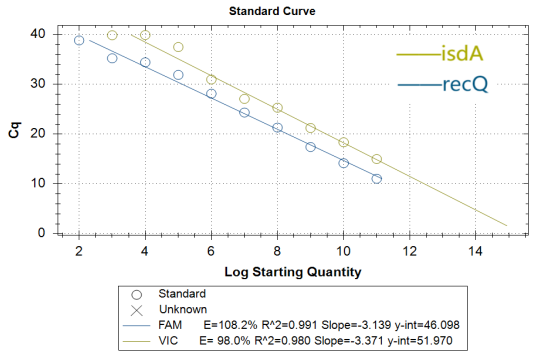

Figure 4. Double fluorescence quantitative PCR standard curve.

Standard plasmids pMD-18T-isdA (33.5 ng/μL) and pMD-18T-recQ (63.7 ng/μL) were constructed. Copy numbers were calculated as 1.335×10^{11} copies/μL (*isdA*) and 7.265×10^{11} copies/μL (*recQ*).

Equal volumes were mixed, serially diluted (10^{0} to 10^{-10}), and amplified under optimized conditions (Section 3.4). The standard curves (Figure 4) showed:

pMD-18T-isdA: y = -3.371x + 51.970, R^2 = 0.980, Efficiency = 98.0%.

pMD-18T-recQ: y = -3.139x + 46.098, R^2 = 0.991, Efficiency = 108.2%.

This demonstrates a strong linear relationship between Ct values and log copy number concentration, with high amplification efficiency, confirming assay robustness and stability.

3.6. Specificity Analysis of the Duplex Real-Time Fluorescence Quantitative PCR Assay

Testing genomic DNA from *S. aureus* and other pathogens under optimized conditions showed amplification curves only for *S. aureus* genomic DNA with all primer-probe sets (Figure 5). No amplification occurred with other pathogens or the negative control, confirming high specificity.

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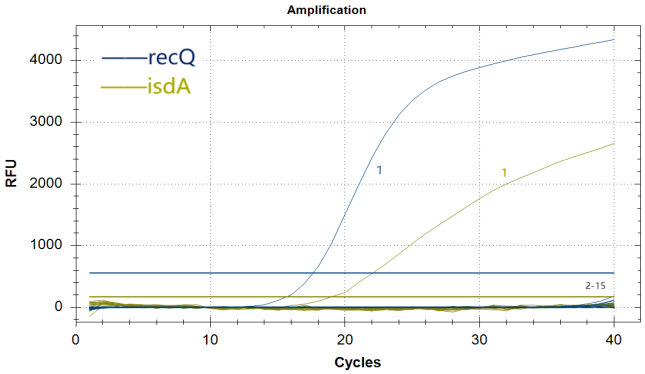

Figure 5. Specificity analysis of double fluorescence quantitative PCR detection.Curve "1" represents *S. aureus*, and "2~15" respectively represent *P. aeruginosa, K. Pneumoniae, A. baumannii, S. pyogenes, P. mirabilis, N. meningitidis, S. salivarius, K. oxytoca, M. catarrhalis, L. pneumophila, H. influenzae, C. freundii, S. pneumoniae, M. pneumoniae.*

3.7. Reproducibility Analysis of the Duplex Real-Time Fluorescence Quantitative PCR Assay

Testing three mixed plasmid concentrations (10^{-1}, 10^{-2}, 10^{-3}) yielded intra-assay and inter-assay CVs for Ct values below 1.86% and 2.87%, respectively (Table 3), demonstrating excellent accuracy and reproducibility.

Table 3. Reproducibility analysis of double fluorescence quantitative PCR detection.

Yang plasmid copy number/ (copies/μL)		Intra-assay Ct values			Inter-assay Ct values		
		average value	standard deviation	coefficient of variation/%	average value	standard deviation	coefficient of variation /%
isdA	1.335×10^{8}	24.55	0.325	1.32	24.62	0.330	1.34
	1.335×10^{9}	20.71	0.386	1.86	20.64	0.592	2.87
	1.335×10^{10}	17.36	0.162	0.93	17.45	0.421	2.42
recQ	7.265×10^{8}	22.21	0.072	0.32	22.24	0.078	0.35
	7.265×10^{9}	18.62	0.232	1.24	18.66	0.290	1.55
	7.265×10^{10}	15.08	0.220	1.46	15.10	0.428	2.83

3.8. Sensitivity Analysis of the Duplex Real-Time Fluorescence Quantitative PCR Assay

Plasmid Sensitivity: Serial dilutions of the mixed plasmids yielded limits of detection of 1.335×10^3 copies/μL for isdA and 7.265×10^2 copies/μL for recQ (Figure 6).

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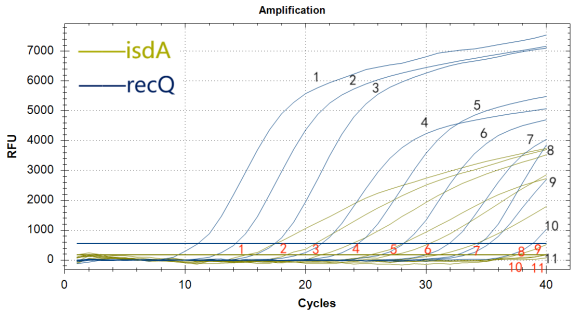

Figure 6. Sensitivity analysis of double fluorescence quantitative PCR detection. The curves "1~11" represent the amplification curves of the template 2μL diluted by 1×10^{0},1×10^{-1} to 1×10^{-10} of the mixed plasmids.

Culture Sensitivity: Testing serial dilutions of genomic DNA from a *S. aureus* culture (3.59×10^{9} CFU/mL) by single-gene qPCR yielded limits of detection of 3.59×10^{2} CFU/mL for *isdA* and 3.59×10^{3} CFU/mL for *recQ* (Figure 7).

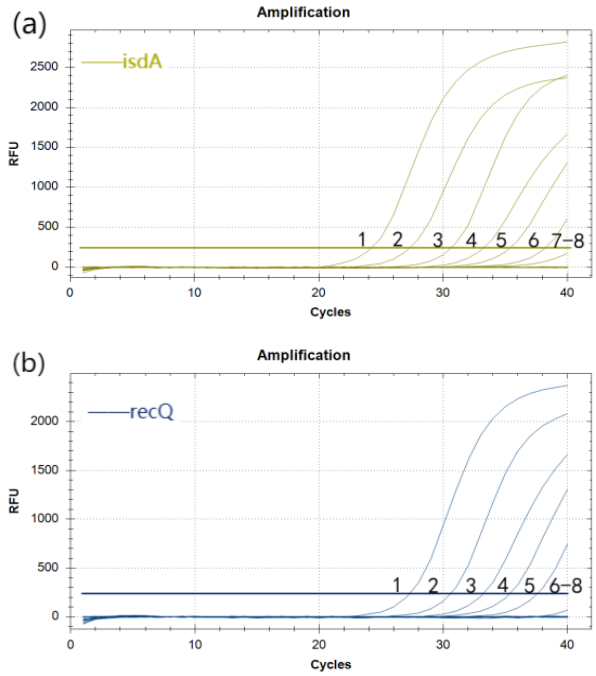

Figure 7. (a) Sensitivity analysis of *isdA gene to* qPCR in *S. aureus* cultures. (b) Sensitivity analysis of *recQ gene to* qPCR in *S. aureus* cultures. Curves "1~7" represent the amplification curves of DNA from *S. aureus* cultures with templates of 2μL 3.59×10^{7}, 3.59×10^{6} to 3.59×10^{1}CFU/mL, respectively; curve "8" represents the negative control.

4. Discussion

Significant genetic variation exists among strains of pathogenic bacteria. Pangenome analysis identifies core genes shared by all strains and accessory genes present in subsets, providing a comprehensive view of genetic diversity. Core genes are typically associated with fundamental biological functions and pathogenesis. Identifying highly conserved core genes through this analysis enables the development of diagnostic assays targeting multiple genes, enhancing accuracy and reliability.

This study performed pan-genome analysis on 200 *S. aureus* genomes. BLAST analysis identified *isdA* and *recQ* as candidate target genes. Both genes were present in all 200 genomes with intraspecies sequence similarity exceeding 90%, indicating high conservation and species specificity. These

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characteristics make them suitable molecular diagnostic targets. Targeting both genes reduces the risk of false negatives due to sequence variability among strains.

This study selected the highly specific and conserved core genes *isdA* and *recQ* as detection targets. We systematically investigated the effects of primer concentration, probe concentration, and annealing temperature on duplex qPCR performance[23], determined optimal reaction conditions, and successfully established a duplex qPCR assay[24]. The method demonstrated excellent specificity, reproducibility, and sensitivity. Simultaneous detection of two targets in a single reaction significantly reduces the risk of false positives and false negatives during *S. aureus* detection, eliminates the need for repeated testing, and saves time and cost[25].

Wang Yan et al. (2024) employed traditional culture methods as the gold standard for *S. aureus* detection, improving accuracy but requiring 3~5 days, making it unsuitable for rapid diagnosis[26]. Sun Xiuxiu (2024) and Wang Xuerong (2025) applied qPCR targeting single *S. aureus*-specific genes, increasing the likelihood of false negatives and reducing accuracy[27,28]. Current qPCR studies for *S. aureus* mainly target genes like *nuc*, *Sa442*, and *pvl*, often without systematic evaluation of gene specificity and conservation. The duplex qPCR method developed here is the first to select two conserved genes as detection targets, effectively reducing false-positive and false-negative results. This provides new molecular targets for rapid *S. aureus* detection and supports early, accurate diagnosis, with significant potential for disease prevention and control[29].

Recently, global antibiotic resistance rates in *S. aureus* have risen, posing a serious challenge for clinical treatment. Resistance analysis reveals *S. aureus* employs multiple resistance mechanisms. The emergence of methicillin-resistant *S. aureus* (MRSA) has further complicated treatment[30]. Identified resistance genes include *mecA* (encoding penicillin-binding protein 2A, PBP2a, conferring β-lactam resistance)[31] and *erm* genes (conferring macrolide resistance via ribosomal methylation)[32]. Based on this study's findings, we aim to develop multiplex detection methods targeting *S. aureus* resistance genes to support precise antibiotic use and provide new strategies for effective treatment.

5. Conclusions

In this study, we conducted a pan-genome analysis of *S. aureus* and identified two highly conserved and specific core genes, *isdA* and *recQ*, as detection targets. Based on these targets, we developed a TaqMan probe-based duplex real-time PCR assay for the detection of *S. aureus*, enabling simultaneous detection of both genes in a single reaction.

The established method demonstrated a detection sensitivity of 1.335×10^3 copies/μL for *isdA* and 7.265×10^2copies/μL for *recQ*. The intra-assay and inter-assay coefficients of variation (CVs) for the amplification Ct values were below 1.86% and 2.87%, respectively, indicating high reproducibility. Specificity testing confirmed no cross-reactivity with common pathogenic bacteria, including Pseudomonas aeruginosa, Streptococcus pneumoniae, and 12 other respiratory pathogens.This assay provides a rapid, accurate, and highly specific diagnostic tool for the early detection of *S. aureus* infections.

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