Infection and Drug Resistance

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METHOD

Multiplex Digital PCR-Based Development and discussion of the Detection of Genetic Association Between *Staphylococcus aureus* and *mecA*

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a predominant nosocomial infection-causing bacteria. The aim of this study was to develop a novel single-bacteria multiplex digital PCR assays (SMD-PCR), which is capable of simultaneously detecting and discriminating Methicillin-sensitive *Staphylococcus aureus* (MSSA) and MRSA. This protocol employed TaqMan probes to detect *SAOUHSC_00106* and *mecA* genes, with the latter being linked to methicillin resistance. A total of 72 samples from various specimen types were evaluated. The accuracy rates for the sputum samples, pus samples, swab samples, ear secretion samples, and catheter samples were 94.44%, 100%, 92%, 100%, and 100%, respectively. Our results showed that the clinical practicability of SMD-PCR has applicability to the rapid detection of MRSA without DNA extraction or bacterial culture, and can be utilized for the rapid detection of *Staphylococcus aureus* and the timely identification of MRSA in clinical samples, thereby providing an advanced platform for the rapid diagnosis of clinical MRSA infection.

Keywords: MSSA, MRSA, droplet digital PCR, SMD-PCR

Introduction

Staphylococcus aureus (*S. aureus*) is a common clinical pathogen that causes various infections, such as skin and soft tissue infections, pneumonia, food poisoning and sepsis.[1] In the pre-antibiotic era, invasive infections with *S. aureus* were frequently fatal. Resistance to penicillin in *S. aureus* due to the production of β-lactamase. Methicillin was developed to combat *S. aureus* infections, however, the first resistant to beta-lactam strain was found in UK within two years of clinical use, and the strains were named methicillin-resistant *Staphylococcus aureus* (MRSA), which were rapidly spread throughout the world in the 1980s.[2–4] Presently, MRSA has emerged as one of the important pathogens associated with both community-acquired and hospital-acquired infections.[1,5] The β-lactam resistance gene *mecA*, responsible for MRSA, is located in the staphylococcal cassette chromosome mec (SCCmec).[6] The *mecA* gene encodes aberrant penicillin binding protein 2A (PBP2a),[7] directly contributing to penicillin resistance. Consequently, *mecA* test has become the primary choice for MRSA detection.[8]

Rapid and accurate diagnosis of MRSA is crucial for early initiation of targeted antibiotic therapy, thereby enhancing patient clinical outcomes. Currently, MRSA mainly relies on drug sensitivity testing after cultivation. However, many culture detection methods such as chromogenic media and drug susceptibility testing require incubation for more than 12 hours, posing challenges in offering timely guidance to physicians.[9] On the other hand, the application of molecular biology-based genetic testing in clinical practice has gained widespread acceptance due to its shorter turnaround time

Received: 15 February 2024
Accepted: 2 May 2024
Published: 21 May 2024

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compared to standard methods. This approach can significantly reduce the detection time to 1–3 hours, aiding doctors to choose the appropriate treatment method.[10]

Nucleic acid amplification is the most frequently used method for nucleic acid testing, with polymerase chain reaction (PCR) emerging as the predominant technique for DNA amplification.[11,12] Although multiplex PCR testing has been applied to MRSA detection,[13] clinical samples frequently entail a mixture of *S. aureus* and less pathogenic coagulase-negative *Staphylococci* (CoNS), both potentially harboring *mecA*,[14] which could affect the accuracy of the assay. Therefore, there is an urgent requirement for a rapid and accurate method to detect MRSA. Droplet Digital PCR (ddPCR) technology utilizes droplet dispersion, where each droplet contains either one or zero DNA fragments, enabling individual PCR in each droplet and separate detection of fluorescence signal for each droplet.[15] Multiplex digital PCR (MDPCR) has become an crucial method in detecting the genes linkage on DNA fragments.[16] Moreover, it exhibits heightened tolerance to protein impurities interference, enabling the realization of the bacteria direct detection.[17] MDPCR has demonstrated high accuracy in detecting MRSA in swab samples.[18] However, this study did not assess and validate its performance on a wider range of body fluid samples, especially high background samples, such as sputum and pus.

In this study, we developed single-bacteria multiplex digital PCR assays (SMD-PCR) and applied this method to the detection of a wide range of clinical samples (Figure 1). The results showed a good detection rate for various types of complex samples, indicating that SMD-PCR have the potential to be applied in rapid clinical MRSA detection in future.

Materials and Methods

Sample Isolation

The strains used in this study were clinical isolated, and the clinical samples were provided by Hangzhou First People's Hospital of Zhejiang Province. All samples were stored at 4 °C. The studies involving human samples were reviewed and approved by Hangzhou First People's Hospital local ethics committee (ZN-20230315-0041-01).

Screening and Reaction Optimization of Wall-Breaking Enzymes

MRSA single-colony bacteria were selected, 1 mL of 4% paraformaldehyde fixative (Biosharp) was added for overnight fixation.[19] After centrifugation at 12,000 rpm for 5 min, the supernatant was discarded and the pellet was resuspended in PBS (Shanghai Sangon). After centrifugation at 12,000 rpm for another 5 min, the supernatant was discarded and resuspended in 1 mL 100 mM Tris-HCL (pH=7.0, Phygene). Lysozyme (Solarbio) at 2.5 mg/mL and lysostaphin (Nanjing Duly Biotech Co., Ltd) at 1.0 mg/mL were added, followed by a water bath at 37 °C for 15 min. Then, 1

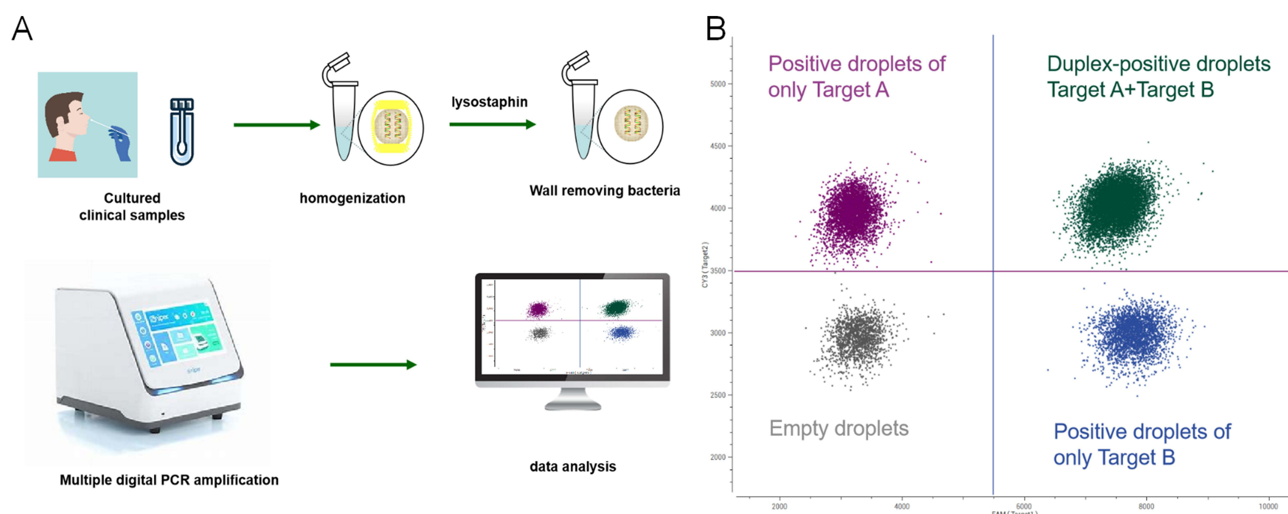

Figure 1 ddPCR detection flow diagram. (**A**)The lysostaphin-treated sample was dispersed by PCR reaction into 20,000 droplets containing target and background DNA. (**B**) Data output from a MDPCR experiment. The droplets form four clusters, arranged orthogonally to each other. Gray: empty droplet, negative for both targets; Purplish red: only Target A positive; Blue: only positive for Target B; Green: duplex-positive droplets.

mL cold PBS was added. Based on the results, the reaction time was optimized via a time gradient. qPCR was employed to assess the cell wall disruption effect of the optimized enzyme on *S. aureus*, MRSA, and MRcoNS.

qPCR Analysis

The primer and probe sequences used in this experiment are shown in Table 1. qPCR analysis was performed using the StepOne real-time PCR system. The final volume of qPCR for each detection panel was 20 μL and contained 1x qPCR mix, 0.5 μM forward and reverse primers, 300 nM each probe, 1 μL suspension bacterial solution or DNA fragment and DNA-free water. Data were performed by the instrument program.

Specificity and Sensitivity Testing

Genomic DNA from 4 strains of *staphylococci* species were analyzed using the ddPCR assay to examine the specificity of the SMD-PCR assay. The strains were *Staphylococcus warneri, Staphylococcus haemolyticus, Staphylococcus hominis* and *Staphylococcus cohnii*.

For the sensitivity testing, the bacterial suspension was prepared by *S. aureus* and Methicillin-resistant coagulase-negative *staphylococci* (MRcoNS), and quantified by ddPCR. *S. aureus* and MRcoNS were mixed at various concentration ratios (the concentration ratio can be found in Table 2) and detected as the above experimental scheme.

ddPCR Analysis

The sequences of primers and probes used in this experiment are shown in Table 1. ddPCR analysis was performed using a Sniper Droplet ddPCR system according to the manufacturer's protocol. Briefly, the final volume of the ddPCR master mixture for each assay panel was 22 μL and contained 1-fold ddPCR premix, 1 μM forward and reverse primers, 300 nM each probe, 2 μL suspension bacterial solution, and DNA-free water. Data analysis was performed automatically by the instrument.

The distribution of the samples in the droplets follows a Poisson distribution, allowing for the possibility that a droplet may contain more than one bacterium. In MRSA samples, interference from miscellaneous bacteria is common. After fluorescence detection of each droplet, we will observe four orthogonal clusters in the ddPCR instrument, where each droplet is either blank fluorescence, containing *SAOUHSC_00106* gene and(or) *mecA* gene. Therefore, we used the following formula to correct the ddPCR results.

In the reaction system without MRSA, the ratio of *SAOUHSC_00106* signal obtained by detection alone was a1.

$$a1 = P(x = x1) = \frac{\lambda^{x1}}{x1!} e^{-\lambda} \tag{1}$$

The ratio of the *mecA* signal alone is a2,

$$a2 = P(x = x2) = \frac{\lambda^{x2}}{x2!} e^{-\lambda} \tag{2}$$

Then the relationship between the proportion of duplex-positive signals and the proportion of single positive signals is consistent with:

$$b = a1 * a2 + P(x = x3) - P(x = x3) * a1 * a2 \tag{3}$$

$$P(x = x3) = \frac{b - a1 * a2}{1 - a1 * a2} \tag{4}$$

a1 represents the proportion of *SAOUHSC_00106* single positive region, a2 represents the proportion of *mecA* single positive region, and b represents the proportion of duplex-positive region. When P(x=x3) <0, no MRSA strains were considered to be present.

Table 1 Primer and Probe Sequences

Name	Sequence	Target Sequence	Product Length (bp)
SAOUHSC_00106-F	TCAAACGGTTGGTGTGATAGGTT	TGGGATTGTTACTAGCGAATCATGTTATAGAACAAGACAGAAGGCAGTATGA	182
SAOUHSC_00106-R	TGCTCTGGTTGTTCCCAATG	CCAAAGTTTTAAAATAGATAATGGTGATTTTTTGCAAGGGTCACCATTTTG	
SAOUHSC_00106-P	[5TAMRA]-AACGACACAATTTATTCC-[MGB]	TAATTACTTAATCGCGCATAGCGGCAGTAGCCAGCCTTTAGTTGATTTTTAT	
		AATCGAATGGCATTCGACTTTGGTACG	
mecA-F	AAAACTAGGTGTTGGTGAAGATATACC	GAAAGGATCTGTACTGGGTTAATCAGTATTTCACCTTGTCCGTAACCTGAA	147
mecA-R	GAAAGGATCTGTACTGGGTTAATCAG	TCAGCTAATAATATTTCATTATCTAAATTTTTGTTTGAAATTTGAGCATTAT	
mecA-P	[6FAM]-TTCACCTTGTCCGTAACCTGAATCAGCT-[BHQ1]	AAAATGGATAATCACTTGGTATATCTTCACCAACACCTAGTTTT	

https://doi.org/10.2147/IDR.S464213

Table 2 Comparison Table of the Concentration of Mixed Bacterial Solution

S. aureus	MRcoNS
11,760	60
11,760	70
11,760	84
11,760	105
11,760	140
11,760	210
11,760	840
11,760	1260
11,760	1680
11,760	2100
11,760	2520
11,760	2940

Testing of Clinical Sample

In order to prove the detection efficiency of SMD-PCR on different types of samples, a large number of different types of clinical samples have been collected, including 1 case of catheterization, 1 case of ear discharge, 9 cases of purulent material, 36 cases of sputum, and 25 cases of throat swabs. All samples were collected from hospital labs after routine testing.

Clinical sample pretreatment: (1) Swab: Add 1 mL PBS and shake vigorously for 2 min. (2) Sputum: an equal volume of sputum digestion solution (PERFEMIKER) was added, shaken for 30 seconds, heated in 37 °C water bath for 30 min, then PBS buffer was added to 50 mL, centrifuged at 3000 rpm for 15 min, and the supernatant was discarded and resuspended in the original volume of sputum PBS. (3) Pus: clear pus can be used directly; cloudy pus and sputum are treated in the same way. After that, the processed sample was diluted 10 times. The stock solution and diluent were subjected to a lysostaphin reaction at 37 °C for 2 min.

Results

Verification of Primer and Probe Design

The primers and probes used in this study were designed based on the *SAOUHSC_00106* (Gene ID: 3919815) gene and *mecA* gene. The reference sequence for the *SAOUHSC_00106* gene was provided by KaShonda et al,[20] however, the detection efficiency of the primers and probes were not as expected, so the primers and probes were redesigned (Table 1). For the *mecA* gene, the primers and probes were used as our published paper.[21] We employed qPCR to conduct five gradient dilutions of the MRSA DNA to determine the amplification efficiency, as depicted in Figure 2. The amplification efficiencies of *SAOUHSC_00106* (Gene ID: 3919815) gene and *mecA* were found to be 94% and 90%, respectively, which are suitable for subsequent experiments.

Screening of Reaction Enzymes and Reaction Optimization

In this experiment, ddPCR was employed for the detection of samples. Due to the presence of bacterial cell walls and their poor susceptibility to thermal lysis, direct detection of the original samples was not feasible. Therefore, enzymatic treatment was used to selectively remove the cell wall while preserving the intact morphology of the cell membrane. After the cell wall is completely removed, the intact bacteria are dispersed into the droplets to ensure that their whole genomes can be detected in the subsequent ddPCR reaction.

In this study, lysozyme and lysostaphin for bacterial cell removal were employed. After the fixed bacteria were treated with lysostaphin and lysozyme, as shown in Figure 3A, compared to lysozyme treatment, lysostaphin treatment showed the more significant cell wall breaking effect. Based on the results, lysostaphin was selected for subsequent experiments. Then, by taking different concentrations of lysostaphin (1, 10, 100 μg/mL), as shown in Figure 3B, Lysostaphin can

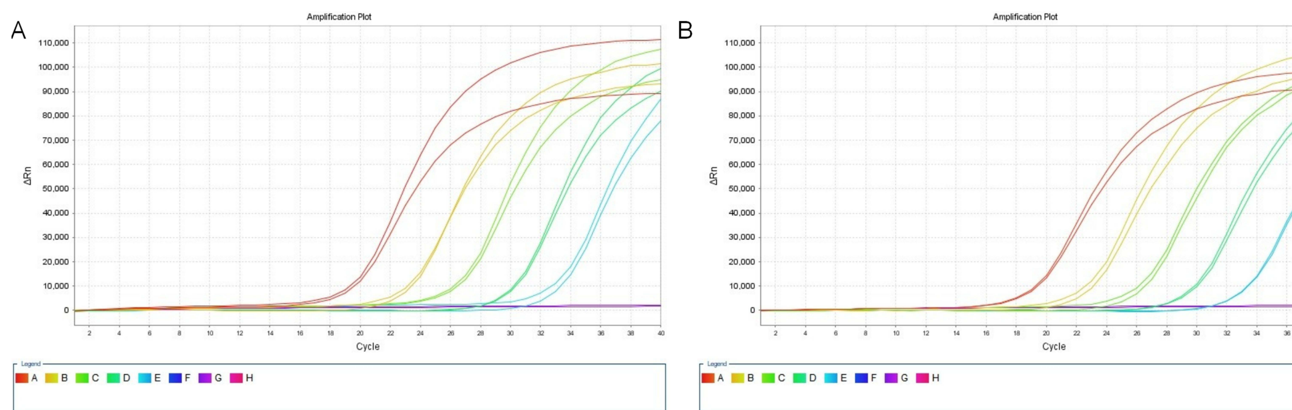

Figure 2 Primer amplification efficiency. (**A**) *SAOUHSC_00106*; (**B**) *mecA*.

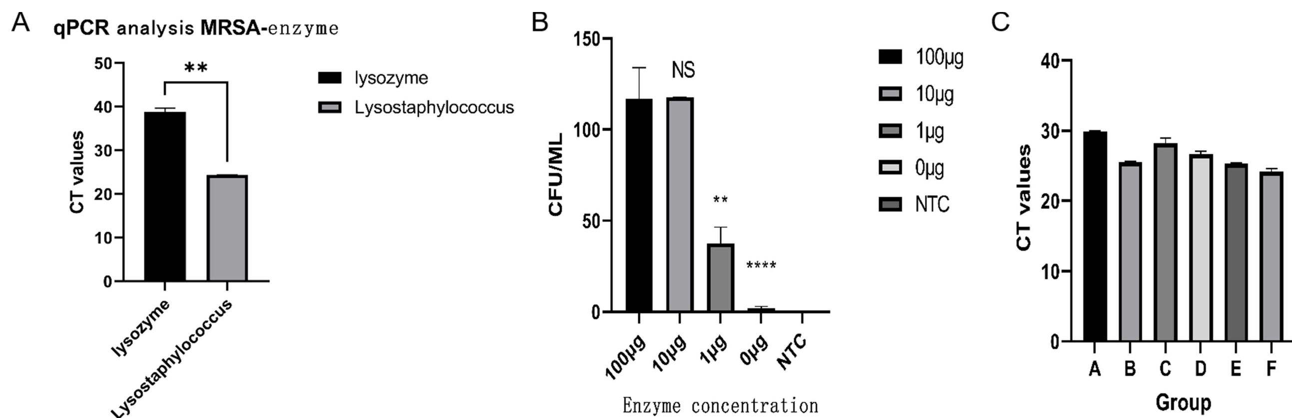

Figure 3 Enzyme selection and optimization of reaction conditions. (**A**) The disparity in MRSA lysis between lysostaphin and lysozyme was assessed using qPCR.; (**B**) The disparity in enzyme concentration and cleavage efficacy remains consistent for the duration of the same action time (5 min). The symbols on the column represent differences with the first group, NS represents no difference, ** represents significant difference with the first group, **** represents significant difference with the first group and significant difference with the ** group. (**C**) Reaction time optimization, the test was performed in the presence of 10μg/mL lysostaphin. A: MRcoNS, reaction time 2min; B: MRcoNS, reaction time 5min; C: MRSA, reaction time 2min; D: MRSA, reaction time 5min; E: *S. aureus*, reaction time 2min; F: *S. aureus*, reaction time 5min.

effectively degrade the cell wall of MRSA. In addition, concentration gradient experiments showed that the wall-breaking efficiency of 10 μg/mL was significantly higher than that of 1 μg/mL, but there was no difference between 10 μg/mL and 100 μg/mL, so 10 μg/mL concentration was selected in the following experiments.

Considering that lysostaphin may have different effects on different species of *Staphylococcus*, we need to find the most effective reaction conditions for *S. aureus*. Therefore, we designed a reaction efficiency test for MRcoNS and *S. aureus*. In these experiments, *S. aureus*, MRSA, and MRcoNS were treated with 10 μg/mL lysostaphin for different reaction time (2 and 5 min) firstly, followed by qPCR analysis. As shown in Figure 3C, when the threshold value was set as 5000, for S. aureus and MRSA, the detection sensitivity of lysostaphin treatment for 5 minutes is 2.9 and 2.1 times higher than that of treatment for 2 minutes, respectively, however, for MRcoNS showed 20.32 times. As results, the reaction time for 2 min at a concentration of 10μg/mL was selected, because it is more favorable for detect of *S. aureus*.

Specificity and Sensitivity Testing

Several *Staphylococcus* species were used to test the specificity of SMD-PCR assay, these strains included MRSA, *S. warneri, S. haemolyticus, S. hominis* and *S. cohnii*. As expected, SMD-PCR shows no false positive or false negative results were observed in this study (Table 3). The presence of mixed bacterial infections in clinical samples often leads to significant interference with test results. Therefore, to assess the protocol's ability to handle false positive samples, we utilized a mixture of bacterial liquid for testing purposes. The *S. aureus* and MRcoNS mixed bacterial solution were detected by SMD-PCR, and

Table 3 Validation of Specificity

Strain	P (x=3)	Results
Staphylococcus aureus	0	–
Methicillin-resistant *Staphylococcus aureus*	0.277086883	+
Methicillin-resistant *coagulase-negative staphylococci*	0	-
Staphylococcus warneri	0	-
Staphylococcus haemolyticus	0	-
Staphylococcus hominis	0	-
Staphylococcus cohnii	0	-

Notes: + was positive; - is negative.

the acquired single signal frequency was employed to calculate the theoretical probability value of duplex-positive, which was compared with our actual detection value. In order to present the results more intuitively, we used the *SAOUHSC_00106* signal ratio as the benchmark, calculated the theoretical value at this concentration, and compared it with the actual value (Figure 4). All the results were below the theoretical value. These results showed that at different concentration ratios of *S. aureus* and MRcoNS, SMD-PCR was observed to be well tolerated.

Validation of Clinical Samples

In order to verify the effectiveness of SMD-PCR in clinical detection, we selected 72 different types of clinical samples, comprising 27 MRSA samples and 45*S. aureus* samples (Table 4). Compared with the results of drug sensitivity testing, the accuracy of SMD-PCR in detecting MRSA samples was 94.44%, with positive sample accuracy was 100% and negative sample accuracy was 88.89%. Except for 2 sputum samples and 2 swab samples, all other results were consistent with the drug sensitivity test results (Figure 5).

Discussion

MRSA can be spread between infected patients and health care workers. Beke et al's study indicates that implementing active surveillance for colonization, followed by contact prophylaxis, has shown promising results in controlling outbreaks in neonatal intensive care units and reducing endemism.[22] Rapid detection of MRSA has been demonstrated to reduce the length of hospital stay and costs.[23] The historical drug of choice for the treatment of severe MRSA infections is vancomycin, often serving as a last resort option, offering both empirical coverage and definitive treatment. However, the increased usage of antibiotic has led to the emergence of vancomycin intermediate *Staphylococcus aureus*

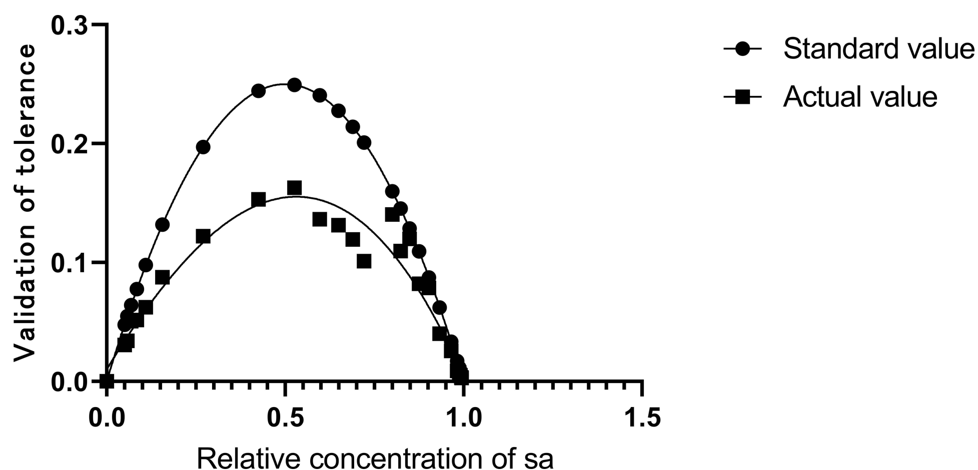

Figure 4 The mixed bacterial solution of S. aureus and MRcoNS was utilized to validate the robustness of the protocol, with the X-axis representing the relative concentration of SAOUHSC_00106 signal and the Y-axis indicating the ratio of duplex-positive concentration to total signal.The circle (●) is the theoretical value, and the square (■) is the actual detection value.

Table 4 Differences in Results of ddPCR and Drug Susceptibility Testing Clinical Samples

Sample Types	Number of Samples	Results of ddPCR Assay	Results of Drug Susceptibility Testing
Pus	9	*S. aureus*:9	*S. aureus*:9
Swab	25	*S. aureus*:13 MRSA:12	*S. aureus*:15 MRSA:10
Sputum	36	*S. aureus*:20 MRSA:16	*S. aureus*:22 MRSA:14
Catheter	1	*S. aureus*:1	*S. aureus*:1
Ear secretion fluid	1	*S. aureus*:1	*S. aureus*:1

and vancomycin-resistant *Staphylococcus aureus*.[24] Early identification of *S. aureus* and MRSA can mitigate the abuse of broad-spectrum antibiotics, reducing the selection pressure that leads to the emergence and spread of bacterial resistance.

PCR technology has long been used in MRSA detection, and multiplex PCR has emerged as a crucial method for MRSA detection.[25] Eliezer M et al have established a protocol for MRSA detection by multiplex PCR. However, the sample contains impurities such as proteins that can inhibit the PCR reaction, and the multiplex PCR test also requires the extraction of DNA, which cannot directly detect the sample.[26] In clinical samples, the accuracy of detection rate of by multiplex PCR is very low, which would lead to false negative or false positive results.[27,28] In our previous study, we observed a significant false positive rate of 50% for MRSA detection using qRT-PCR (Quantitative Real-time PCR),[21] severely limiting the practical utility of this method. Compared with qRT-PCR, ddPCR has stronger tolerance, with weak inhibition by inhibitors such as proteins, enabling directly detect clinical samples without DNA extraction.[29] However, the detection of MRSA by MDPCR remains an unresolved challenge. We proposed a method to directly detect the presence of MRSA in clinical samples. The presence of MRSA in samples can be calculated by the distribution of *SAOUHSC_00106* gene and *mecA* gene in the samples. This provides the possibility to directly detect MRSA in high background samples. The development and validation of SMD-PCR were conducted on various clinical samples, including swabs, pus, sputum, ear secretions, and catheters. Compared with the results of drug susceptibility test, the consistency rate of SMD-PCR test and drug susceptibility test were 94.4%. Except for false positive results from sputum and swab samples, all other findings were consistent with drug susceptibility outcomes. In future research, the accuracy of the detection scheme in high background samples needs to be further improved. Moreover, the efficacy of this regimen in blood specimens also would be detected.

Another potential limitation of multiplex qRT-PCR is the detection of infections with other colonized bacterial species containing the *mecA* gene contained in the sample.[30] It is worth noting that if patients have nasal colonization of *mecA* dropout MSSA and *mecA*-harboring CoNS, those assays could still lead to false positive detection of MRSA.[31] The presence of multiplex bacterial strains often contributes to the development of infections in certain diseases such as diabetic foot.[32] Thus, the co-occurrence of *SAOUHSC_00106* and *mecA* could potentially be attributed to a mixture of

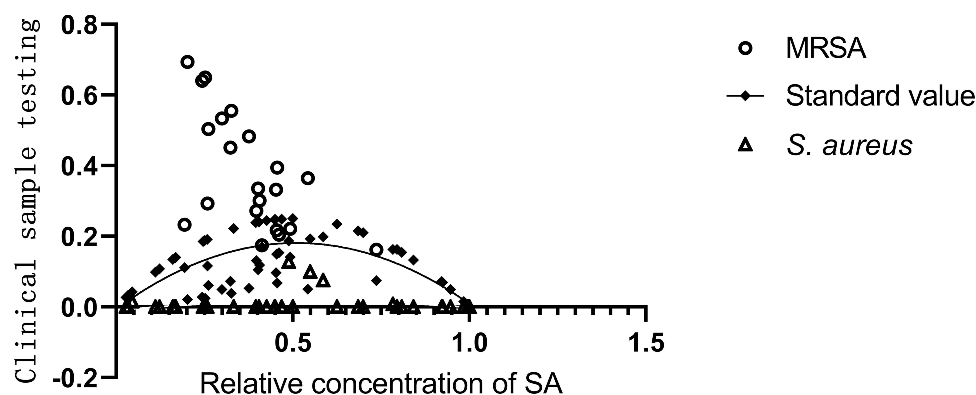

Figure 5 The results in 72 clinical samples were detected using single-bacterium MDPCR. The X-axis representing the relative concentration of *SAOUHSC_00106* signal and the Y-axis indicating the ratio of duplex-positive concentration to total signal. The diamond (◆) is the theoretical value of the duplex-positive area, the circle (○) is the test result of MRSA samples, and the triangle (Δ) is the test result of *S. aureus* samples.

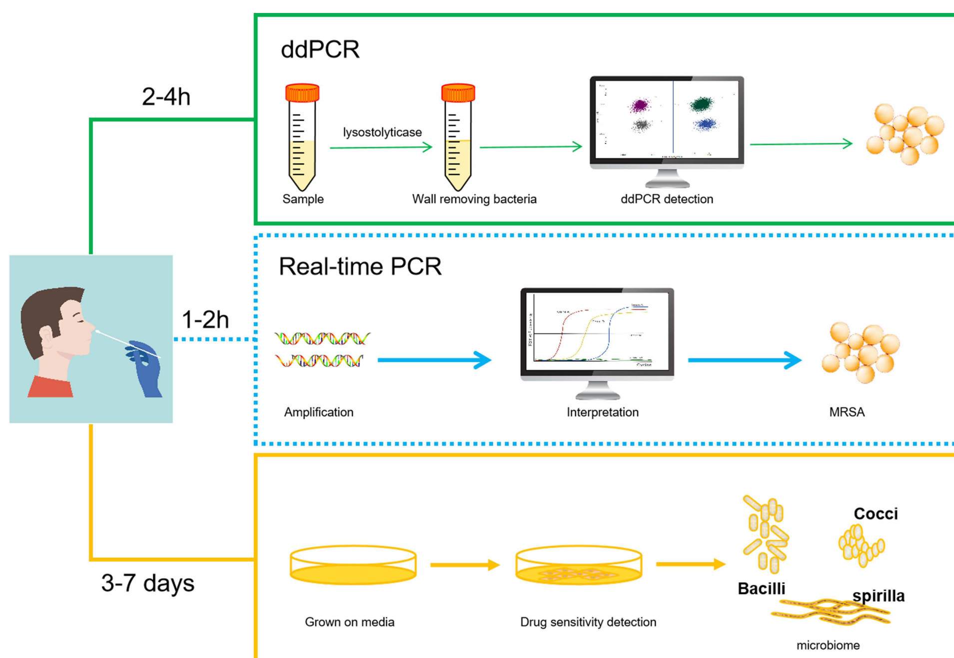

Figure 6 SMD-PCR, real-time fluorescence quantitative PCR and drug sensitivity detection of three detection schemes.

Staphylococcal isolates rather than solely MRSA. In this study, such an occurrence was observed in one sample. By utilizing SMD-PCR capable of encompassing genomes from a singular strain, including *SAOUHSC_00106* and *mecA*, it becomes feasible to distinctly identify MRSA. Meanwhile, since theoretically calculated values will produce large errors and false positives at extremely low and extremely high concentrations, which indicates that a single miscellaneous bacterium will cause extremely large errors when the concentration is extremely different, therefore, we plan to exclude samples with *SAOUHSC_00106* signal proportion lower than 8% or higher than 92%. However, in actual testing, there was no composite exclusion criterion for any sample. We believe SMD-PCR will address the limitations of current molecular testing and improve accuracy.

Subsequently, we compared SMD-PCR with qRT-PCR and drug susceptibility testing (Figure 6). In contrast to other diagnostic techniques, the SMD-PCR technique effectively eliminates false positive results of *S. aureus* and CoNS, thereby enhancing the accuracy of detection results while inheriting the advantages of rapid qRT-PCR detection. SMD-PCR assays play a crucial role in aiding clinicians to select targeted antibiotics and effectively combating the transmission of multidrug-resistant organisms. The implementation of SMD-PCR assays has significantly enhanced the early treatment of MRSA infections, thereby complementing the existing clinical diagnostic paradigm.

Conclusion

In this study, we have successfully established a direct detection method for MRSA in clinical samples using ddPCR. SMD-PCR is rapid, well-tolerated and specific. Although more expensive than conventional protocols, this molecular diagnostic technique can reduce the diagnostic time to 3–4 hours, providing important implications for clinical medication guidance. Therefore, the direct and rapid detection of MRSA by ddPCR can provide information to accelerate treatment decisions for early treatment of MRSA infection and antibiotic treatment.

Acknowledgments

This work was supported by "Leading Goose" R&D programs of Zhejiang Province (2023C03045), the Major Science and Technology Medicine and Healthcare in Zhejiang (WKJ-ZJ-2309), Public Welfare Project of Jinhua city (2021-4-359), the National Key Research and Development Program of China (2021FY100901).

Disclosure

The authors report no conflicts of interest in this work.

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