Journal of Microbiological Methods 159 (2019) 34–41

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Direct detection of *Staphylococcus aureus* in positive blood cultures through molecular beacon-based fluorescence *in situ* hybridization

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ARTICLE INFO

Keywords:
Molecular beacons
Fluorescence *in situ* hybridization
Bloodstream infection
Staphylococcus aureus
Blood culture

ABSTRACT

Objective: Clinical diagnosis of bloodstream infection diseases depends on the blood culture results. Bacterial identification by traditional methods is time-consuming. This study aimed to utilize molecular beacon-based fluorescence *in situ* hybridization (MB-FISH) for rapid and direct detection of *Staphylococcus aureus* in positive blood cultures.

Methods: Three molecular beacon probes (MB1, MB2 and MB3) were designed and synthesized to target the 16S rRNA gene fragment of *S. aureus*. The MB-FISH system was optimized, and the specificity of this method in detecting *S. aureus* was evaluated. This approach was used to test 41 g-positive clinical specimens with positive blood cultures. In addition, the consistency of this method with traditional methods was evaluated.

Results: Signal-to-noise ratio (S/N) of the molecular beacon MB1 was significantly higher than that of MB2 and MB3 ($P < .001$). The S/N ratios of MB1 probe at different concentrations were all > 20. Thermal denaturation curve of the probe suggested that its hairpin structure can be opened and closed. Conditions such as deionized formamide concentration, ionic strength and temperature were optimized by monitoring the fluorescence intensity of MB1 in the presence or absence of its target sequence B1. The optimized hybridization system produced fluorescence only in *S. aureus*. The specificity and sensitivity of MB1 probe for detecting *S. aureus* in 41 specimens were 100% and 93.75%, respectively. Although sample size was small, MB-FISH appeared to be consistent with traditional culture methods (Kappa value = 0.948).

Conclusion: MB-FISH demonstrates strong specificity and high sensitivity, and can be used for direct detection of *S. aureus* in positive blood cultures.

1. Introduction

As the second most common gram-positive pathogenic bacteria (Knox et al., 2015; Musicha et al., 2017), *Staphylococcus aureus* is one of the most important factors leading to serious bloodstream infection-associated death with a mortality rate of up to 25%–30% worldwide (Kern, 2010). The rapid and precise detection of *S. aureus* in bloodstream infections is of great significance for the early control of infection, reduction of hospitalization expenses, and saving the lives of patients (Perez et al., 2013). At present, clinical identification of pathogenic bacteria infection in bloodstream mainly depends on automated blood culture system. The main procedure involves the automated culture of blood samples from patients with suspected bloodstream infection, and inoculation of bacterial solution with positive signal to solid culture medium. These procedures are usually followed by various manual or automated biochemical experiments to identify *S. aureus,* when the bacterial colonies are formed at 18–24 h after inoculation (Demiray et al., 2016). Generally, this method requires a long turnaround time of 24–72 h for the whole process (Martiny et al., 2012), which is not conducive for rapid bacterial detection. The detection time can be significantly shortened if the inoculation of positive bacterial solution to solid culture medium can be avoided.

Currently, there are several methods that help to detect pathogenic bacteria directly in the positive blood culture. These methods include matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR) (Dubourg and Raoult, 2016; Florio et al., 2018; Peker et al., 2018). However, the application of

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https://doi.org/10.1016/j.mimet.2019.02.007
Received 22 August 2018; Received in revised form 31 January 2019; Accepted 13 February 2019
Available online 15 February 2019

MALDI-TOF-MS in detecting pathogenic bacteria in positive blood cultures is limited due to low accuracy and low detection rate of gram-positive bacteria (Barberino et al., 2017; Ruiz-Aragon et al., 2018; Schubert et al., 2011; Tsuchida et al., 2018; Yonetani et al., 2016). PCR can be used to detect pathogenic bacteria in blood cultures (Rodel et al., 2017; H. Y. Wang et al., 2016; Zhu et al., 2015), but this method requires destruction of the cells for nucleic acids extraction and special precautions to prevent contamination of laboratory amplification products. Moreover, the procedures are complex and direct observation of intact bacteria *in situ* cannot be achieved. Meanwhile, it is difficult to avoid problems such as false positivity caused by contamination during DNA amplification and false negativity due to the presence of unknown enzyme inhibitors in the blood samples. Besides, if bacteria have been propagated in large amounts in blood culture bottles, other methods, rather than amplification-based PCR, might be more suitable.

Fluorescence *in situ* hybridization is a more mature method for identifying pathogenic bacteria in positive blood culture (Peters et al., 2006), especially when it is combined with peptide nucleic acid probes (Gorton et al., 2014; Harris and Hata, 2013; Stone et al., 2013). However, peptide nucleic acid probes are rarely utilized in most laboratories due to high cost and difficulty in synthesis. In addition, after completion of hybridization reaction, the commonly used linear probes must be rinsed stringently to remove the non-hybridized probes. Otherwise, they cannot be used in directly detecting the pathogenic bacteria in homogeneous solution due to high background of the fluorescence signal. A molecular beacon (MB) is a stem-loop dual-labeled oligonucleotide probe that forms a hairpin structure at the 5′ and 3′ ends. Under natural conditions, fluorescence resonance energy transfer occurs in the molecular beacons, and the fluorescence is almost completely ablated. With the existence of target sequence, the complementary region of the hairpin structure would be opened, and fluorescence of molecular beacons is restored (Tyagi and Kramer, 1996). Thus, it is unnecessary to remove the non-hybridized probes after hybridization, which simplifies the operation steps of FISH and maximally reduces the interference in experiments. Therefore, molecular beacon-based fluorescence *in situ* hybridization (MB-FISH) can be applied for direct detection of pathogenic bacteria in homogeneous solutions due to its simple operation, strong specificity and high sensitivity (Koncan et al., 2015; Leitner et al., 2013; Lenaerts et al., 2007; Wu et al., 2016).

In this study, we designed and synthesized molecular beacon probes for specific segments of 16S rRNA gene in *S. aureus*. After optimizing the parameters including signal/noise ratio, hybridization buffer and temperature in our system, we tested the specificity and sensitivity of selected molecular beacon in *in situ* hybridization for direct detection of *S. aureus* in positive blood cultures.

2. Materials and methods

2.1. Bacterial strains and blood culture specimens

The bacterial strains used in this study were isolated and stored by the microbiology laboratory of Xinjiang Production & Construction Corps Hospital, Urumqi, China. The reference strains including *Staphylococcus aureus* (ATCC29213), *Staphylococcus epidermidis* (ATCC12228), *Streptococcus pneumoniae* (ATCC49619) and *Enterococcus faecalis* (ATCC29212), were purchased from American Type Culture Collection (ATCC, USA). The clinically isolated strains included *Staphylococcus hominis*, *Streptococcus viridans*, *Staphylococcus haemolyticus*, *Enterococcus faecium*, *Streptococcus agalactiae,* and *Streptococcus pyogenes*. A total of 41 g-positive specimens of positive blood cultures were selected from 41 patients and submitted to the Clinical Laboratory of Xinjiang Production and Construction Corps Hospital between December 2017 and April 2018.

2.2. Instruments and reagents

The following instruments and reagents were used in this study: DA7600 spectrofluorometric thermal cycler (DAAN GENE of Sun Yat-sen University, Guangzhou, China); ultra-low temperature and high-speed centrifuge (Eppendorf AG, Hamburg, Germany); air-bath drying oven at constant temperature (Changzhou Guoyu Instrument, Changzhou, China); Leica DM2500 fluorescence microscope (Leica, Germany), BacT/ALERT 3D automatic blood culture system (bioMérieux, France); VITEK-Compact automatic bacterial identification and drug sensitivity analysis system (bioMérieux, France); Biochemical analysis pure reagents (Sangon Biotech Shanghai Co., Ltd., Shanghai, China).

2.3. Design and synthesis of molecular beacons

A detailed protocol for molecular beacon design is available at http://www.molecular-beacons.org. The sequences of 16S rRNA gene of *S. aureus* with different subtypes were downloaded from the US NCBI website. Homology comparison was performed on Clustal Omega to find out the conserved sequences among subtypes. Multiple molecular beacon sequences were selected using the Beacon Designer 8.14 software based on the design requirements for molecular beacons (Mhlanga et al., 2005). Additionally, the sequences were compared online by BLAST to identify gene sequences relatively more specific to *S. aureus*. The molecular beacons MB1 and MB2 were specific for gene sequences (GenBank No. L37597.1), while MB3 was designed by referring to the study of Kempf VA et al. (Kempf et al., 2000). Sequences of molecular beacons and their complementary sequences are shown in Table 1. For all molecular beacons, the 5′ end was labeled with fluorescent agent FAM (Carboxyfluorescein), and the 3′ end was labeled with quencher DABCYL. DNA sequences (D1, D2, D2, D3, D4, D5, D6, D7 and D8) that partially mismatched the molecular beacon MB1 were synthesized simultaneously (Table 2). Molecular beacons and their target DNA sequences were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.

2.4. Determination of signal-to-noise ratios of molecular beacons and target DNA sequences

A spectrofluorometric thermal cycler was used to assess the response characteristics by calculating the signal-to-noise ratios (S/N) of molecular beacons to target sequences by using the formula, $S/N = (F_{open} - F_{buffer})/(F_{close} - F_{buffer})$. In this formula, F_{buffer} represented the baseline fluorescence intensity of 50 μL hybridization buffer (pH 8.0, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 2.5 mmol/L $MgCl_2$, 10% deionized formamide); F_{open} represented the fluorescence intensity of 50 μL hybridization buffer supplemented with 5 μL of 10 μmol/L target sequences and different concentrations of molecular beacon solutions (the final concentrations ranged from 0.2 μmol/L to 2 μmol/L), and

Table 1
The DNA sequences of molecular beacons and their targets used in this study.

Name	Sequence(5′ → 3′)
MB1	cgacgTCCGCCGCTAACATCAGAGAAcgtcg
MB2	cgacgAGAAGCAAGCTTCTCGTCCGTcgtcg
MB3	cgacgGAAGCAAGCTTCTCGTCCGcgtcg
B1	TTCTCTGATGTTAGCGGCGGA
B2	ACGGACGAGAAGCTTGCTTCT
B3	CGGACGAGAAGCTTGCTTC

Note: The underscored parts represent the stem sequences complementary to the molecular beacon. Fully complementary relationships are present between the molecular beacon MB1 and B2, the molecular beacon MB2 and B2 and the molecular beacon MB3 and B3.

Table 2
The DNA sequences of multiple targets partially mismatched with the molecular beacon MB1.

Name	Sequences(5'→3')
D1	TTCTCTGATGATAGCGGCGGA
D2	TTCTCTGATGTTAGCGGCCGA
D3	TTCACTGATGTTAGCGGCGGA
D4	TTCTCTGATCTTAGCGGCGGA
D5	TTCTCTCATGTTACCGGCGGA
D6	TTCTCTGAAGTTAGCCGCGGA
D7	TTCACTGCTGTTAGCGGCGGA
D8	TTCTCTGATGTTATCGGCAGA

Note: The red marks represent mismatched locations between targets sequences and the bases of molecular beacon probes.

F_{close} was the fluorescence intensity of 50 μL hybridization buffer with different concentrations of molecular beacons but without target sequences.

2.5. Determination of thermal denaturation curve of molecular beacons hybridized with target DNA sequences

A spectrofluorometric thermal cycler was used to obtain the melting curve. The temperature was increased from 30 °C to 90 °C. Fluorescence intensity was collected at every 1 °C increment of the temperature to observe thermal denaturation of molecular beacons hybridized with DNA oligonucleotides of their target sequences. In the reaction system, the final concentrations of molecular beacon at 1.4 μmol/L, target sequence B1 at 1.0 μmol/L, $MgCl_2$ at 2.5 mmol/L, and deionized formamide at 10% were used.

2.6. Optimization of the constituent concentration in the hybridization buffer

The hybridization buffer with varied concentrations of deionized formamide and $MgCl_2$ was prepared. In experiment groups, molecular beacon MB1 was hybridized with target sequence B1 in the hybridization buffer with different constituent concentrations. In the control groups, MB1 was added alone in the hybridization buffer with different constituent concentrations. A spectrofluorometric thermal cycler was used to detect the fluorescence intensity every 1 min, and a total of 90 cycles at 30 °C were performed. The differences in term of the fluorescence intensities between the experimental group and the control group were calculated.

2.7. Optimization of the hybridization temperature

Multiple DNA oligonucleotides of target sequences were hybridized with molecular beacons in optimized hybridization mixture with specified concentrations of deionized formamide and $MgCl_2$. A spectrofluorometric thermal cycler was used to detect the fluorescence intensity every 1 min, and data on melting curves were acquired. The temperature was elevated from 30 °C to 90 °C, and fluorescence intensities were collected at every 1 °C increment of temperature.

2.8. Preprocessing of bacteria samples for molecular beacon-based fluorescence in situ hybridization

1 McFarland (3×10^8 CFU/mL) bacterial solution at logarithmic growth was prepared using sterile saline. 1 mL bacterial solution was placed into a 1.5 mL Eppendorf tube for centrifugation (12,000 rpm, 4 °C, 5 min). After removing supernatant, 100 μL PBS buffer (pH 7.2) along with 300 μL of 4% paraformaldehyde was added to the solution to fix bacterial samples at room temperature for 1 h. Paraformaldehyde was removed by centrifugation, and the solution was washed once with 200 μL of PBS buffer. 40 μL of 2 mg/mL lysozyme was added to the centrifuged precipitate (37 °C, 10 min). After centrifugation, 50 μL of 5 U/mL lysostaphin was added and samples were centrifuged once at 37 °C for 5 min for later use.

2.9. Molecular beacon-based fluorescence in situ hybridization

The processed bacteria were mixed with 7 μL of 10 μmol/L molecular beacon MB1 probe, 10 μL of 25 mmol/L $MgCl_2$ and 25 μL of deionized formamide (final concentration of 25%). The reaction system was then supplemented with TE buffer to reach the volume of 100 μL and samples were mixed well. Avoiding light, the bacteria-molecular beacon solution was hybridized in a 50 °C water bath for 2 h. 10 μL of the hybridized bacterial solution was dropped onto the polylysine-treated slide, and then the slide was stained using a non-specific nucleic acid stain DAPI after drying slightly, which was followed by addition of an anti-fluorescence quenching sealer. The slide was sealed with a cover slip and observed under a fluorescence microscope.

2.10. Detection of S. aureus in blood culture bottles

Blood of patients with suspected blood infection was cultured using the BacT/ALERT 3D automated blood culture system. After positive culture was indicated by the system, potentially positive bacterial solution was used for smear and gram staining. If there were positive cocci arranged in clusters, then 2 mL of positive blood was collected using a syringe and placed in a tube for centrifugation (700 rpm, 4 °C, 10 min) to remove the erythrocytes. The supernatant was collected for centrifugation (12,000 rpm, 4 °C, 10 min). 1 mL of distilled water was added into the precipitate. After being mixed well, sample tubes were placed at room temperature for 5 min, followed by centrifugation and resuspension using 100 μL of PBS buffer. Molecular beacon-based fluorescence *in situ* hybridization was performed following the method described above. Meanwhile, the positive blood culture was identified by the traditional laboratory method, where the positive bacterial solution was transferred to the blood agar medium for culturing for 24 h. After a single bacterial colony was formed, identification was performed by the microbiological laboratory automated VITEK-Compact bacteria identification and drug sensitivity analysis system.

2.11. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2007 and SPSS19.0 statistical software. Data were expressed as mean ± SD (standard deviation). Comparison among multiple groups was done by one-way ANOVA, and followed by Tukey-Kramer HSD test. $P < .05$ was considered to be statistically significant. Methodological comparisons were performed using the Kappa consistency test. The degree of consistency was referred to the study of Landis et al. (Landis and Koch, 1977) and judgment was made based on the Kappa coefficient (if the value < 0, poor; 0 and 0.2, slightly weak; 0.21 and 0.40, weak; 0.41

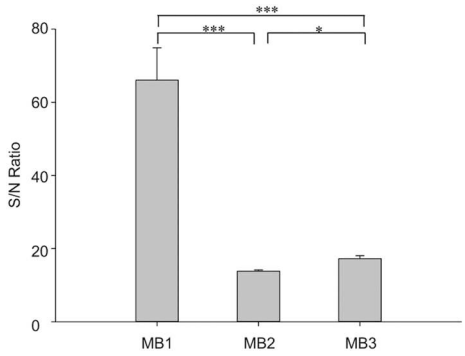

Fig. 1. The S/N ratios of three molecular beacons designed in this study.
The fluorescence intensities of molecular beacons MB1, MB2 and MB3 in the hybridization buffer (pH 8.0, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 2.5 mmol/L $MgCl_2$, 10% deionized formamide) at 30 °C in the presence or absence of their corresponding target sequences B1, B2 and B3 were detected by a spectrofluorometric thermal cycler. The concentration of 1.0 μmol/L was used for both molecular beacons and target sequences. The signal-to-noise ratios (S/N) of molecular beacons to target sequences were calculated using the formula described in the Materials and methods part. $n = 13$ for each group; $^{*}P < .05$; $^{***}P < .001$.

and 0.60, moderate; 0.61–0.80, strong; 0.81–1.0, extremely strong).

3. Results

3.1. Screening of molecular beacons

Three molecular beacon probes (MB1, MB2 and MB3) specific for the 16S rRNA gene sequence of *S. aureus* were designed and synthesized in this study. The molecular beacons were hybridized with their corresponding target sequences (B1, B2 and B3 with final concentrations of 1.0 μmol/L each). The S/N ratios in the initial screening process were calculated to select the optimal molecular beacon(s) (Fig. 1). The S/N ratio of the molecular beacon MB1 (66.07 ± 8.85) was significantly ($P < .001$) higher than that of MB2 (13.82 ± 0.35) and MB3 (17.25 ± 0.80). Our secondary structure analysis indicated that MB1 could form a hairpin structure with an intact stem, while both MB2 and MB3 could form structures of self-hybridization to variable extents (Supplemental Fig. 1) and they largely overlap in sequences (Table 1). The self-hybridization structures probably accounted for the low S/N ratios in MB2 and MB3. Therefore, the molecular beacon MB1 with a lower hybridization background in the homogeneous solution was chosen to be used in further optimization of hybridization parameters to obtain higher detection sensitivity.

3.2. Response characteristics of molecular beacon MB1 and its target sequence

The ratio of molecular beacon to the target sequence was optimized according to their response characteristics when they were mixed at various concentrations. In addition, whether the molecular beacon MB1 can satisfy the laboratory requirements in terms of stem-loop structure and fluorescence resonance energy transfer (Tyagi and Kramer, 1996; K. Wang et al., 2009) was also evaluated. Fluorescence intensity of the molecular beacon reached the maximum when the final concentration of MB1 reached 1.4 μmol/L (the ratio to its target sequence B1 was 7:5) (Fig. 2A). When the concentration of MB1 was > 1.4 μmol/L, its fluorescence intensity was basically unchanged (Fig. 2A), while the S/N ratio of MB1 decreased along with the elevation of concentration. However, the S/N ratio was > 20, even when MB1 reached 2 μmol/L (Fig. 2B). Therefore, when the concentration of MB1 was 1.4 μmol/L (the ratio to the target sequence B1 was 7:5), its fluorescence intensity was the highest and the S/N was still > 20, indicating that the molecular beacon MB1 can meet the experimental requirements.

3.3. Determination of thermal denaturation curve of MB1 hybridized with the target sequence

The quality of synthesized molecular beacons can be evaluated by determining the thermal denaturation curves. As shown in Fig. 2C, When the temperature was below 50 °C, the fluorescence intensity of MB1 without target sequence in the solution was maintained at a low level (close to the background). When the temperature was between 50 °C and 65 °C, the fluorescence intensity of MB1 without target sequence in the solution increased gradually. When the fluorescence intensity of MB1 hybridized with its target sequence B1 decreased, the hairpin structure of the MB1 was opened gradually. When the temperature was higher than 65 °C, the fluorescence intensity was basically unchanged, indicating that the hairpin structure of MB1 had been completely opened. Therefore, the hairpin structure of the molecular beacon MB1 can be opened and closed depending on temperature, which satisfied the design requirements of molecular beacons.

3.4. Optimization of the constituent concentration of hybridization buffer

We detected the fluorescence intensity of the molecular beacon MB1 with or without target sequences in the reaction system, when different concentrations of deionized formamide were used. As shown in Fig. 3A, when the concentration of deionized formamide was 25% to 40%, the fluorescence intensity of MB1 hybridized with the target sequence B1 was the highest. When the concentration of deionized formamide was > 40%, the fluorescence intensity of MB1 without target sequence B1 in the reaction system was increased significantly. Therefore, when the concentration of deionized formamide was 25% to 40%, the hybridization of MB1 can obtain better fluorescence intensity and lower background. The effects of different concentrations of $MgCl_2$ in the hybridization system on fluorescence intensity are shown in Fig. 3B. When there was no $MgCl_2$ present in the system, fluorescence intensities of MB1 with or without target sequence B1 were basically the same, suggesting that the hairpin structure of MB1 was open in the absence of $MgCl_2$. When the concentration of $MgCl_2$ was further changed (from 1 mmol/L to 7 mmol/L), the fluorescence intensity of MB1 without the target sequence B1 remained low, while MB1 with the target sequence B1 had largely unchanged fluorescence intensity (Fig. 3B). This suggested that the change of $MgCl_2$ concentration from 1 mmol/L to 7 mmol/L demonstrated negligible effects on the fluorescence intensity of MB1.

3.5. Optimization of hybridization temperature

We tested the hybridization temperature by detecting the melting curve and fluorescence derivative curve of the molecular beacon MB1 hybridized with different DNA sequences (B1/D1/D2/D3/D4/D5/D6/D7/D8; Table 2). As shown in Fig. 4, MB1 better distinguished the target sequence B1 with single mismatched base. When the temperature was < 45 °C, MB1 did not hybridize with D5, D6, D7 and D8, indicating that MB1 can only distinguish these gene sequences with at least two mismatched bases under this thermal condition. When the temperature was between 45 °C and 50 °C, MB1 was able to distinguish the gene sequences with single mismatched base (D1, D2, D3, and D4). When the temperature was > 50 °C, the stability of molecular beacon hybridization was affected as the temperature exceeded the melting temperature (Tm value). Through conducting online alignments of the sequences of MB1 and multiple bacterial 16S rRNA genes in the SILVA website (https://www.arb-silva.de), we found that MB1 had a 2-base mismatch with *S. epidermidis*, a 4-base mismatch with *S. hominis*, a 3-base mismatch with *S. hemolyticus*, and mismatches of > 3 bases with other bacteria. Therefore, hybridization of MB1 at a temperature between 45 °C and 50 °C can obtain better specificity.

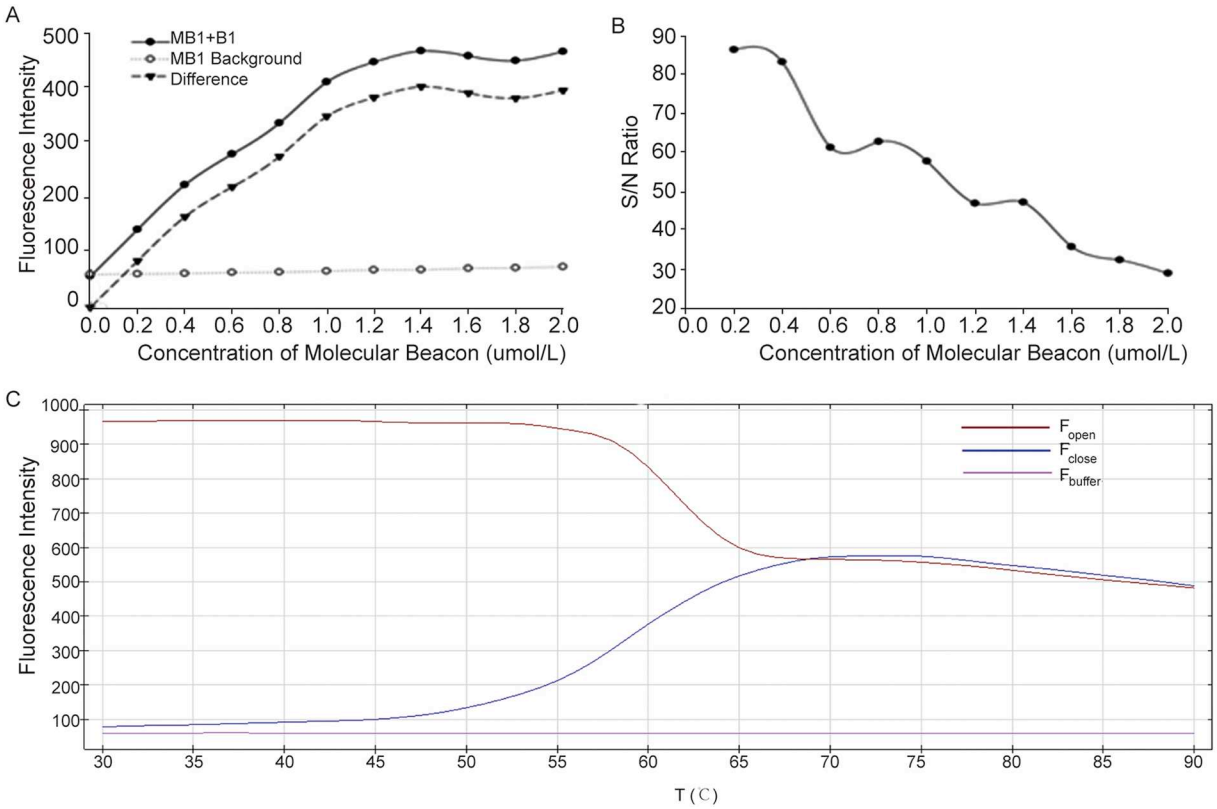

Fig. 2. Response characteristics of molecular beacons and their target sequences.
(A) The fluorescence intensity of different concentrations of molecular beacon MB1 hybridized with its target sequence B1. (B) The S/N ratio of different concentrations of molecular beacon MB1 hybridized with its target sequence B1. (C) The thermal denaturation curves of molecular beacon MB1 hybridized with its target sequence B1.

3.6. Verification of the specificity of the molecular beacon-based fluorescence in situ hybridization

To validate the specificity of our optimized MB-FISH-based reaction system, we tested this method using samples from the clinically isolated bacteria strains and reference bacteria strains. As shown in Fig. 5, *S. aureus* specific MB1 can only detect *S. aureus* but not other strains like *S. epidermidis*, as green fluorescence can only be observed in *S. aureus*. Although the reference *S. epidermidis* sample on the slide was confirmed to be positive for nucleic acid as evidenced by DAPI staining, MB1 was not able to display green fluorescence. Similarly, MB1 also exhibited negative results when tested with other bacteria, including *S. pneumoniae, E. faecalis, S. hominis, S. viridans, S. haemolyticus, E. faecium, S. agalactiae,* and *S. pyogenes* (data not shown), which further strengthened the specificity of MB1-based fluorescence *in situ* hybridization in identifying *S. aureus*.

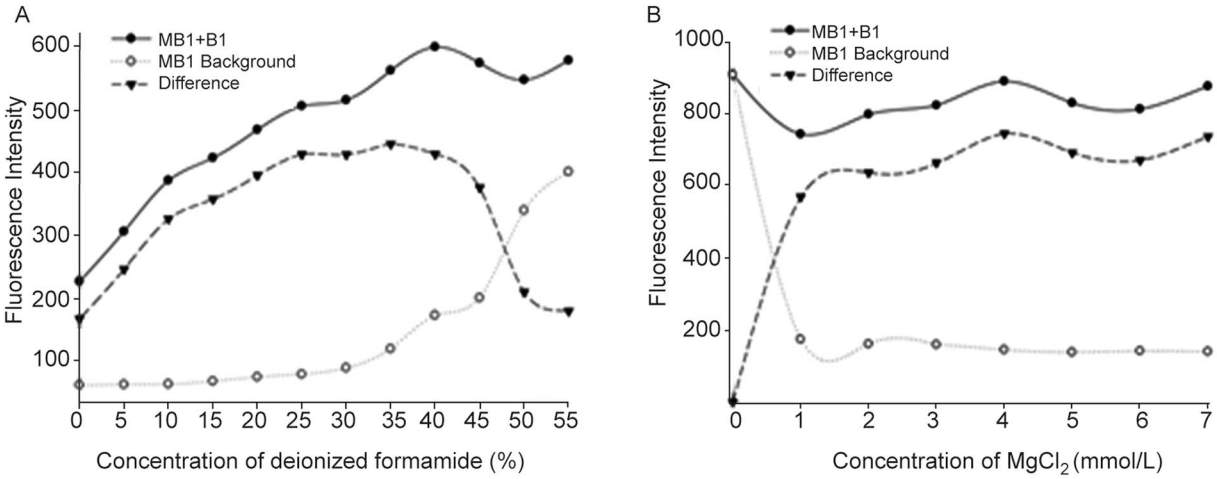

Fig. 3. Optimizing the constituent concentration of hybridization buffer.
(A-B) Curves showing the effects of different concentrations of deionized formamide (A) and $MgCl_2$ (B) on the fluorescence intensity of the molecular beacon MB1 in the presence or absence of target sequence B1. The concentrations for MB1 and B1 were 1.4 μmol/L and 1.0 μmol/L, respectively. $MgCl_2$ at the concentration of 2.5 mmol/L was used in A, while deionized formamide at the concentration of 25% was used in B.

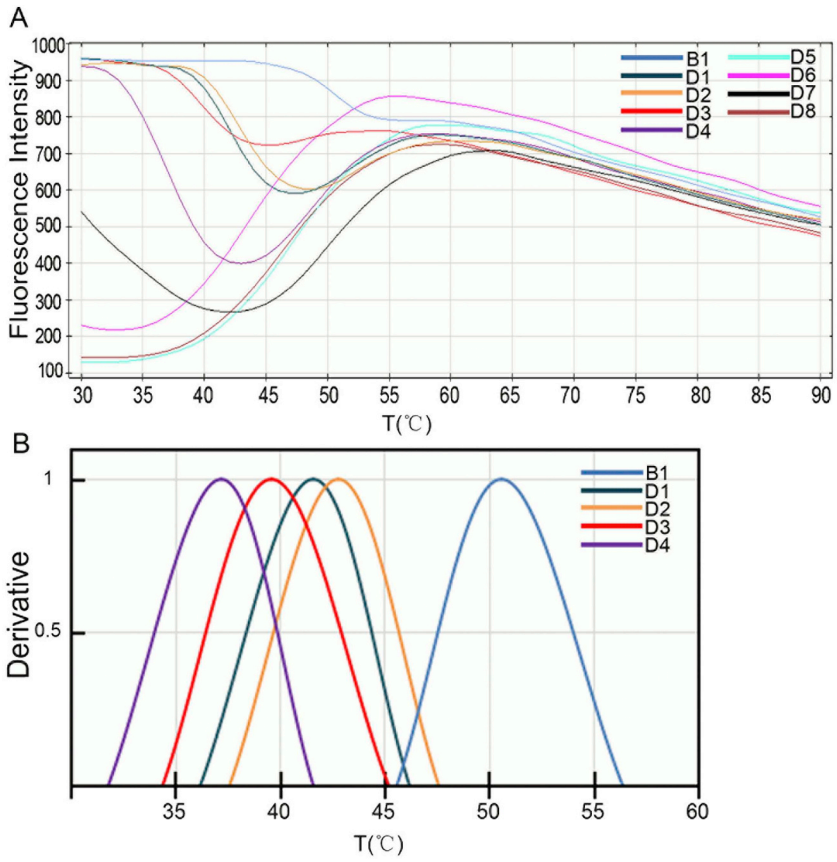

Fig. 4. Verification of the specificity of the optimized reaction system of molecular beacon-based fluorescence *in situ* hybridization.
(A-B) The melting curves (A) and fluorescence derivative curves (B) of the molecular beacon MB1 hybridized with different target sequences under optimized experimental conditions (concentrations for MB1 and target sequences were 1.4 μmol/L and 1.0 μmol/L, respectively, while 2.5 mmol/L $MgCl_2$ and 25% deionized formamide were used).

3.7. Detection of *S. aureus* in blood culture bottles

A total of 41 g-positive cocci from blood culture bottles with positive bacterial solution were collected. *S. aureus* was detected by the traditional culture method and our molecular beacon-based fluorescence *in situ* hybridization system. The traditional culture method detected 16 strains of *S. aureus* and 25 strains of other gram-positive cocci. Molecular beacon-based fluorescence *in situ* hybridization detected 15 strains of *S. aureus* and distinguished 26 strains of other gram-positive cocci from *S. aureus*. Thus, the specificity and sensitivity of molecular beacon-based fluorescence *in situ* hybridization in detecting *S. aureus* were 100% (25/25) and 93.75% (15/16), respectively (Table 3). Kappa consistency test of the two methods suggested that the consistency was extremely strong, with a Kappa value of 0.948. Therefore, the specificity and sensitivity of molecular beacon-based fluorescence *in situ* hybridization were high in detecting *S. aureus* in blood culture, and

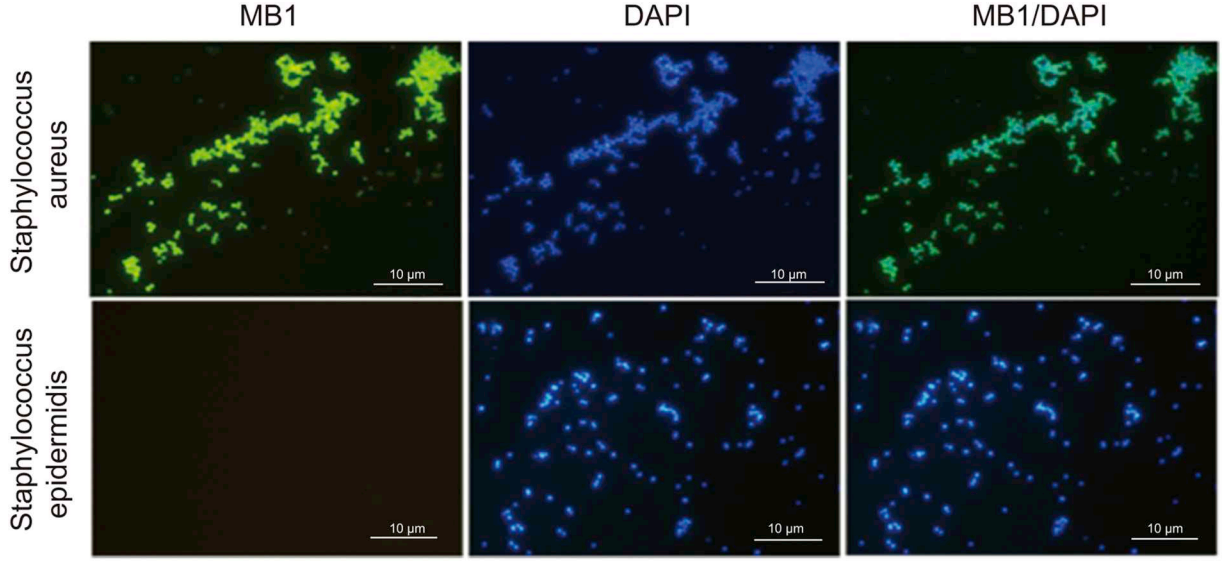

Fig. 5. Representative images demonstrating the specificity of MB-FISH in detection of *S. aureus*.
The molecular beacon MB1 was hybridized with *S. aureus* (the first row) and *S. epidermidis* (the second row) under the optimized condition, and green fluorescence (green) indicated positive signal. DAPI staining (blue) indicated the existence of bacterial nucleus. The images of MB1/DAPI demonstrate the merge of MB1 signal and DAPI signal at the same location. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of 41 g-positive clinical specimens detected using molecular beacon-based fluorescence *in situ* hybridization.

Fluorescence *in situ* hybridization	Traditional culture (gold standard)		Sum
	Positive	Negative	
Positive	15	0	15
Negative	1	25	26
Sum	16	25	41

Note: Positive means that the bacterium was detected as *S. aureus*; negative means that the bacterium was detected as not *S. aureus*.

showed a strong consistency with the traditional culture method in clinic.

4. Discussion

Rapid detection of pathogenic bacteria in positive blood cultures is critical for diagnosing and treatment of diseases. Molecular beacon-based fluorescence *in situ* hybridization is a relatively new technology for direct detection of pathogenic bacteria in positive blood culture, which speeds up the diagnosis and treatment of bloodstream infection (Han et al., 2013; Leitner et al., 2013; Makristathis et al., 2014; Sakarikou et al., 2014). In this study, molecular beacon probes were designed and synthesized to target 16S rRNA gene sequences of *S. aureus*. We performed systematical optimization on multiple parameters of the reaction system, including signal/noise ratio, hybridization buffer components and temperature, and established the molecular beacon-based fluorescence *in situ* hybridization method for direct detection of *S. aureus* in positive blood cultures. We validated the specificity of this method in detecting *S. aureus* from both reference bacteria strains and clinical strains. This detection method demonstrated high specificity and sensitivity, and was highly consistent with the clinically widely used traditional culture method.

The inherent quality of molecular beacons probably is the key for successful establishment of the MB-FISH detection system. The S/N ratio can be used to evaluate the quality of molecular beacon probes. To obtain high detection sensitivity, the S/N value should be > 20 (Xi et al., 2003). It was found that the S/N ratio of the molecular beacon MB1 was significantly higher than that of the other two probes according to the determined S/N ratios of the three synthesized molecular beacons. The response characteristics and thermal denaturation curve of MB1 hybridized with DNA of target sequence B1 were further determined. Our results showed that the S/N ratios of different concentrations of molecular beacons were > 20, and the hairpin structure of molecular beacons can be opened and closed under a certain temperature. These facts suggest that the designed and synthesized molecular beacon MB1 satisfied the requirements in sensitivity and controllability.

Stringent hybridization conditions were beneficial to reduce non-specific hybridization and background fluorescence. Therefore, this study optimized conditions such as deionized formamide concentration, ionic strength and reaction temperature. The results showed that the differences in fluorescence intensity of molecular beacons with or without target sequences was basically unchanged with the deionized formamide concentration of 25%~40%, which was basically consistent with the previous literature report (Vet and Marras, 2005). During the optimization of $MgCl_2$ concentration, we found that a certain ionic strength can stabilize the stem hairpin structure of the molecular beacon MB1. When there is no $MgCl_2$ in the buffer, the molecular beacon stem hairpin structure is opened, which may be related to the deionization of formamide in the buffer. There was no significant change in the fluorescence intensity of molecular beacon hybridization when there was a certain concentration of $MgCl_2$, which was similar to the previous study (Bouvier and Del Giorgio, 2003; Yilmaz et al., 2006). However, different ionic strengths will affect the melting temperature of molecular beacons, and hence it is necessary to optimize the hybridization temperature of molecular beacons. El-Hajj *et al* in their study have demonstrated that the probes can be hybridized with target gene sequences even if there are a certain number of mismatched bases between target gene sequences and probes (El-Hajj et al., 2009). In this experiment, gene sequences that partially mismatched with molecular beacons were designed and synthesized, and our results indicated that non-specific hybridization can be reduced at 50 °C by the melting curve of molecular beacon hybridization.

This study utilized similar process to detect the laboratory reference strains and clinically isolated strains directly. The molecular beacon probe MB1 only hybridized with *S. aureus*, thus confirming the specificity of this method. The MB-FISH method was applied to examine 41 clinical blood culture specimens, and misidentification occurred in only one strain of *S. aureus*. This might be due to certain improper experimental operations, such as too long time permeabilization of the bacterial cell wall with lysostaphin, which could dissolve *S. aureus*. Some studies used FISH combined with flow cytometry to detect pathogenic bacteria in the blood culture directly (Hartmann et al., 2005; Shrestha et al., 2011; Xue et al., 2016). Rather than flow cytometry, our MB-FISH method adopted fluorescence microscopy observation as the readout to determine *S. aureus* infection in the positive blood culture directly. However, since flow cytometry and fluorescence microscopy are both fluorescence-based technologies, this study also provided experimental basis for direct detection of pathogenic bacteria in the blood culture using flow cytometry.

By introducing detailed procedures on systematic optimization of the MB-FISH system, our work also provides a feasible and less-expensive framework for design and evaluation of molecular beacon probes for rapid detection of pathogenic bacteria. However, our study has certain limitations. When optimizing the conditions of MB-FISH, hybridization using the complementary target sequences always had ideal hybridization signals. Therefore, the optimal hybridization conditions need to be further explored during the direct detection of *S. aureus* in homogeneous solution, just in order to obtain stronger fluorescence intensity and avoid non-specific hybridization. Secondly, the specimens used for the direct detection in positive blood cultures were relatively few, and the consistency of this method with the traditional culture method should be further verified by utilizing a larger amount of specimens from patients.

5. Conclusions

In summary, we designed and synthesized molecular beacon probes specific for the conserved sequences of 16S rRNA genes from *S. aureus*. After optimization of multiple parameters in the reaction system, we successfully established the approach for detecting *S. aureus* in the positive blood cultures, which had high specificity and sensitivity. This study provided a new method for direct identification of *S. aureus* in positive blood cultures, and also laid a foundation for further direct detection of pathogenic bacteria in positive blood cultures by flow cytometry.

Declarations of interest

None.

Acknowledgments

Not applicable.

Funding

This work was supported by grants from Clinical Key Speciality Construction Project of Xinjiang Production and Construction Corps

(Bingweifa [2013] 24), and National Key Speciality Construction Project (Bingweifa [2011] 24).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.02.007.

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