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Multiplex real-time PCR assays to detect *Stenotrophomonas maltophilia* carrying *sul1*, *sul2*, and *sul3* genes

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ABSTRACT

Nosocomial infections caused by *Stenotrophomonas maltophilia* resistant to SXT are increasingly reported worldwide. In this study, a novel melting-curve based multiplex real-time PCR assay for the simultaneous detection of the *ssrA* and *sul1*, *sul2* and *sul3* genes was first established. The assays were performed on a Roche LightCycler® 480 II system. The results for target and non-target amplification showed that the multiplex real-time PCR assays were specific, the limit of detection for each target was 10 copies per 20 μL reaction volume, the assays were linear over six log dilutions of the target genes ($r^2 > 0.99$), and the Ct values of the coefficients of variation for intra- and interassay reproducibility were < 5%. The sensitivity for the target DNA in simulated blood samples was 10^2 CFU/mL. The multiplex real-time PCR assays showed 100% concordance with conventional PCR when tested against 20 SXT-susceptible and 20 SXT-resistant *S. maltophilia* from clinical samples. Therefore, the multiplex real-time PCR is a rapid, affordable and sensitive assay for direct detection of the *ssrA* and *sul1*, *sul2* and *sul3* genes.

1. Introduction

Stenotrophomonas maltophilia is a common aerobic Gram-negative bacillus in nature (Adegoke et al., 2017). It is increasingly emerging as a nosocomial pathogen and is the third most commonly occurring non-fermenting bacillus, after *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Spierer et al., 2018; Rizek et al., 2015). Treatment of *S. maltophilia* infections is very difficult because of the high level of natural resistance to various types of antibiotics, including β-lactams, quinolones and aminoglycosides (Consuelo et al., 2018). At present, the preferred choice of drug is sulfamethoxazole-trimethoprim (SXT) (Zhao et al., 2018). However, it has recently been reported that the resistance to SXT is increasing (Herrera-Heredia et al., 2017; Hu et al., 2016; Chung et al., 2015). The *sul* gene is known to contribute to the resistance to SXT, and some reports from Mexico (Herrera-Heredia et al., 2017), Korea (Chung et al., 2015), and the United Kingdom (Toleman et al., 2007) have determined that all high-level SXT resistance (minimum inhibitory concentration [MIC] 64–128 mg/L) is associated with the *sul* gene. For rapid and efficient detection, we established a multiplex real-time PCR assay to detect *S. maltophilia* carrying *sul1*, *sul2* and *sul3* genes.

2. Materials and methods

2.1. Primer design

The reference DNA sequences used in the assays were acquired from the GenBank homepage (http://www.ncbi.nlm.nih.gov/GenBank). We designed two sets of new primer pairs for construction of recombinant plasmids and multiplex real-time PCR using Primer premier 6.0 (Premier Biosoft, CA, USA) (Table 1). The melting temperatures of the amplicons had to be distinct enough to differentiate among the four genes. The primer pairs were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China).

2.2. Construction of recombinant plasmids

In this study, we chose SXT resistant *S. maltophilia* (MIC ≥ 4/76 μg/mL, as suggested by the Clinical and Laboratory Standards Institute), in order to amplify the *ssrA*, *sul1*, *sul2*, and *sul3* genes by PCR. The positive products of the four genes were cloned separately into the plasmid pMD18-T from pMD™18-T Vector Cloning Kit (TaKaRa Bio Inc., Dalian, China), and then sequenced. Recombinant plasmids p*ssrA*, p*sul1*, p*sul2*, and p*sul3* were constructed to optimize real-time PCR.

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Table 1
PCR primer of the multiplex real-time PCR assays.

Primer		Sequence(5′–3′)	position	Aplicom size	Annealing T_m	T_m value	Reference
Multiplex real-time PCR	msul1-F	CGCGACACCGAGACCAAT	209–228	302	58	91.5	This study
	msul1-R	CGCTCTATCCCGATATTGCT	493–514				
	msul2-F	GTGGCCTATCTCAATGATATTCG	298–322	105		83.3	
	msul2-R	CCCGTCTTGCACCGAATG	385–404				
	msul3-F	CTGAAGTGGGCGTTGTGG	161–180	232		79.9	
	msul3-R	ATTCGCTGAACGGAGTGC	375–394				
	mssrA-F	TCAGTCCGGCACTAGAACAC	206–227	119		87.6	
	mssrA-R	CGAAGGCACTCCATCCC	307–325				
Construction of Recombinant plasmids	sul1-F-w	ATGGTGACGGTGTTCGG	1–17	840	55	–	
	sul1-R-w	CTAGGCATGATCTAACCCTCG	818–840				
	sul2-F-w	ATGAATAAATCGCTCATCATTTTC	1–24	816	59		
	sul2-R-w	TTAACGAATTCTTGCGGTTTC	796–816				
	sul3-F-w	ATGAGCAAGATTTTTGGAATCG	1–22	792	53		
	sul3-R-w	CTAACCTAGGGCTTTGGATATTTTC	768–792				
	ssrA-F-w	GGTGGAGGTGGGCGG	1–15	353	60		
	ssrA-R-w	GGGGGTGCATTGGTTTCG	336–353				
Conventional PCR	sul1-F	GCGAGGGTTTCCGAGAAGGTG	50–72	790	62		(Hu et al., 2016)
	sul1-R	TGGTGACGGTGTTCGGCATTC	819–841				
	sul2-F	CGGCATCGTCAACATAACC	24–44	722	60		
	sul2-R	GTGTGCGGATGAAGTCAG	728–747				
	sul3-F	CGAGATTTCACATCGGTTCC	119–140	206	52		(Chung et al., 2015)
	sul3-R	TTGCTGCTTTAGTTGAGGCT	307–328				
	ssrA-F	CGAAACTGCTTGTGTCCAT	138–158	163	58		(Toleman et al., 2007)
	ssrA-R	TACATGCTTAGCTCACCGT	282–302				

2.3. Singleplex real-time PCR and melting curve analysis

Singleplex real-time PCR was performed with four recombinant plasmids on a Roche LightCycler® 480 II system (Roche Ltd., Basel, Switzerland) using the SYBR Premix Ex Taq™ kit (TaKaRa Bio Inc.) to ensure that the amplicons showed the expected T_m values. To confirm the specificities of the primers, singleplex real-time PCR assays were performed by using a DNA mixture from other species of bacteria that do not carry *sul* genes. The amplicons were analyzed by electrophoresis on a 2% agarose gel.

2.4. Multiplex real-time PCR

Multiplex real-time PCR was performed with primer mixes for all four genes. The real-time PCR mixture contained 1 μL of extracted DNA, 10 μL of SYBR Premix Ex Taq qPCR (2×), pairs of primers (optimized to final concentrations of 0.25 μM for *sul1*, *sul2*, and *sul3* genes, or 0.05 μM for the *ssrA* gene), and sterile water to a final reaction volume of 20 μL. Each run was performed with four recombinant plasmids and a water blank as the negative control. The optimal cycling conditions were 10 min at 95 °C; 35 cycles of 10 s at 95 °C, 15 s at 58 °C, and 20 s at 72 °C; and a melting curve step, gradually increasing from 65 °C by 0.11 °C/s to 99 °C (with fluorescence data acquisition every 1 s). All real-time reactions were performed in triplicate.

2.5. Evaluation of multiplex real-time PCR

To determine the specificity of the multiplex real-time PCR assays, several non-target species, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Staphylococcus haemolyticus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Candida albicans*, *Candida tropicalis*, and *Aspergillus flavus*, as well as human leukocytes and blank control were evaluated.

To determine the sensitivity of the multiplex real-time PCR assays, the C_T values obtained from a series of template DNA dilutions were graphed on the y axis versus the log of the dilution on the x axis. The slope of this line was used to determine the efficiency (E) according to the equation: $E = 10^{(-1/slope)}$. A standard curve was generated with serial 10-fold dilutions of the recombinant plasmids p*ssrA*, p*sul1*, p*sul2*, and p*sul3*. The linearity of the calibration curve was expressed as the coefficient of correlation (R^2).

To evaluate the repeatability of the multiplex real-time PCR assay, data generated from sensitivity assays using three replicates of 10^3 copies/μL were used to determine the mean value and the percentage coefficients of variation (% CV).

2.6. Detection in simulated blood samples

To assess the feasibility of the multiplex real-time PCR assays in detecting the target genes in blood samples, serial dilutions of cultured *S. maltophilia* 27,375 (*sul2*-carrying) cells (10^1–10^8 CFU/mL) were prepared in 0.3 mL and added to 2.7 mL of blood for dilution. The samples were allowed to precipitate by standing for 2 min, and 1.8 mL of the supernatants were used to determine the final concentrations of cells (10^0–10^7 CFU/mL). DNA was extracted from these samples directly with a Mag-MK Bacterial Genomic DNA extraction kit (Sangon Biotech Co. Ltd.), according to the manufacturer's instructions.

2.7. Detection of S. maltophilia from clinical samples

Twenty SXT-susceptible and twenty SXT-resistant *S. maltophilia* were collected from clinical isolates from the Nanfang Hospital, Southern Medical University, and identified using the BD Phoenix 100 Automated Microbiology System (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). All the isolates were screened for the presence of *ssrA*, *sul1*, *sul2*, and *sul3* genes using multiplex real-time PCR and conventional PCR, followed by sequencing. The primers for the conventional PCR are shown in Table 1 (Ombarak et al., 2018; Yu et al., 2017; Minogue et al., 2015).

3. Results

3.1. Singleplex real-time PCR and melting curve analysis

The primer pairs were validated initially by using positive control strains carrying the target genes, identified by their expected sizes (Fig. 1). Furthermore, three genome sequences of *Stenotrophomonas*

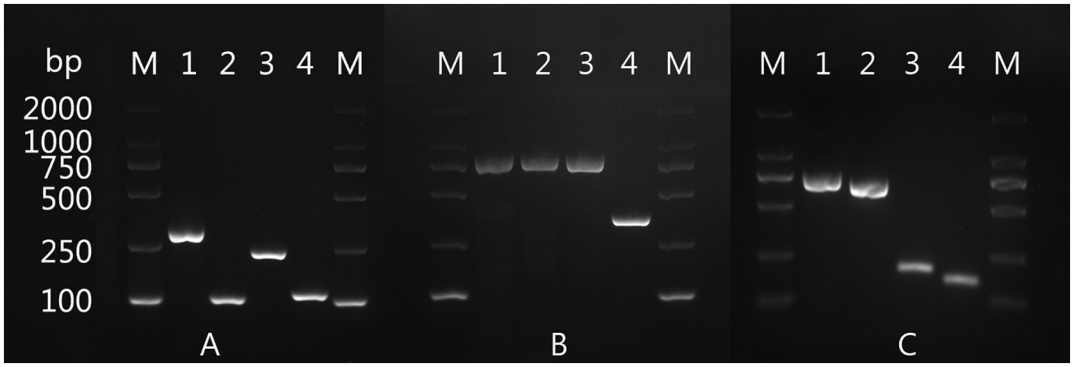

Fig. 1. Agarose gel electrophoresis of products. A: Multiplex real-time PCR; B: Recombinant plasmids; C: Conventional PCR. Primers are shown in Table 1 respectively. Positive control for target gene wss used as template. M: DL2,000 DNA marker; 1: *sul1*; 2: *sul2*; 3: *sul3*; 4: *ssrA*.

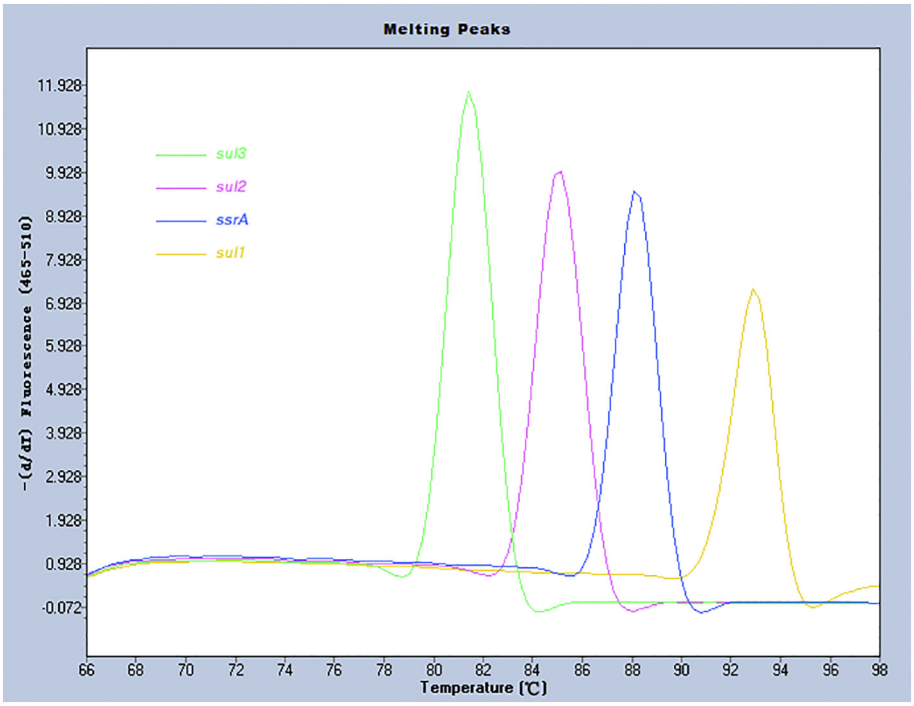

Fig. 2. T_m values corresponding to the amplicons of four DNA targets in real-time PCR.

rhizophila (GenBank ID: CP007597, CP031729, CP016294, respectively) were downloaded to confirm the specificity of the primers. By using Oligo 7 Primer Analysis Software (version 7.0.6.0), the primers did not match any target sequence indicating that the primers are predicted to be specific. Equivalent T_m values for each gene were detected when the positive control strains were tested with simplex real-time PCR assays. The T_m analysis of the amplicons provided the following results: *ssrA*, T_m 88.2 °C; *sul1*, T_m 92.9 °C; *sul2*, T_m 85.1 °C; and *sul3*, T_m 81.4 °C (Fig. 2).

3.2. Multiplex real-time PCR

Multiplex real-time PCR was performed using DNA from the recombinant plasmids with primer mixes for all four genes. The melting temperatures of the amplicons from each template were similar to those from the singleplex assays. For the negative control (NC), no amplification was observed (Fig. 3A). The melting curve profile of the NC (below 77 °C), revealed a peak height lower and peak shape broader than those observed for the positive peaks; this peak seems to have resulted from primer dimer formation. We also simulated the condition of co-existing genes by adding the corresponding recombinant plasmid, and the results showed good agreement with the experimental measurements (Fig. 3B, C, D).

3.3. Evaluate of multiplex real-time PCR

The melting curve analysis showed an irregular shape, with no peaks, with non-target species or gene samples. The linearity and limits of detection of the assays were determined with serial 10-fold dilutions of each recombinant plasmid from 10 to 10^6 copies/μL (Fig. 4). The limit of detection for the target DNA was ten copies per 20 μL reaction volume. The assays correlated well for p*ssrA* (R^2 = 0.996), p*sul1* (R^2 = 0.996), p*sul2* (R^2 = 0.994), and p*sul3* (R^2 = 0.991) over the entire copy number range, with efficiencies of 1.944, 1.869, 1.878, and 1.777, respectively. We also showed that the assays were highly stable and precise, based on the performance of the recombinant plasmids. Intra- and interassay repeatability was tested in triplicate for 10^3 copies/μL within the same run and repeated three different times to assess the reproducibility of the multiplex real-time PCR assays (Table 2).

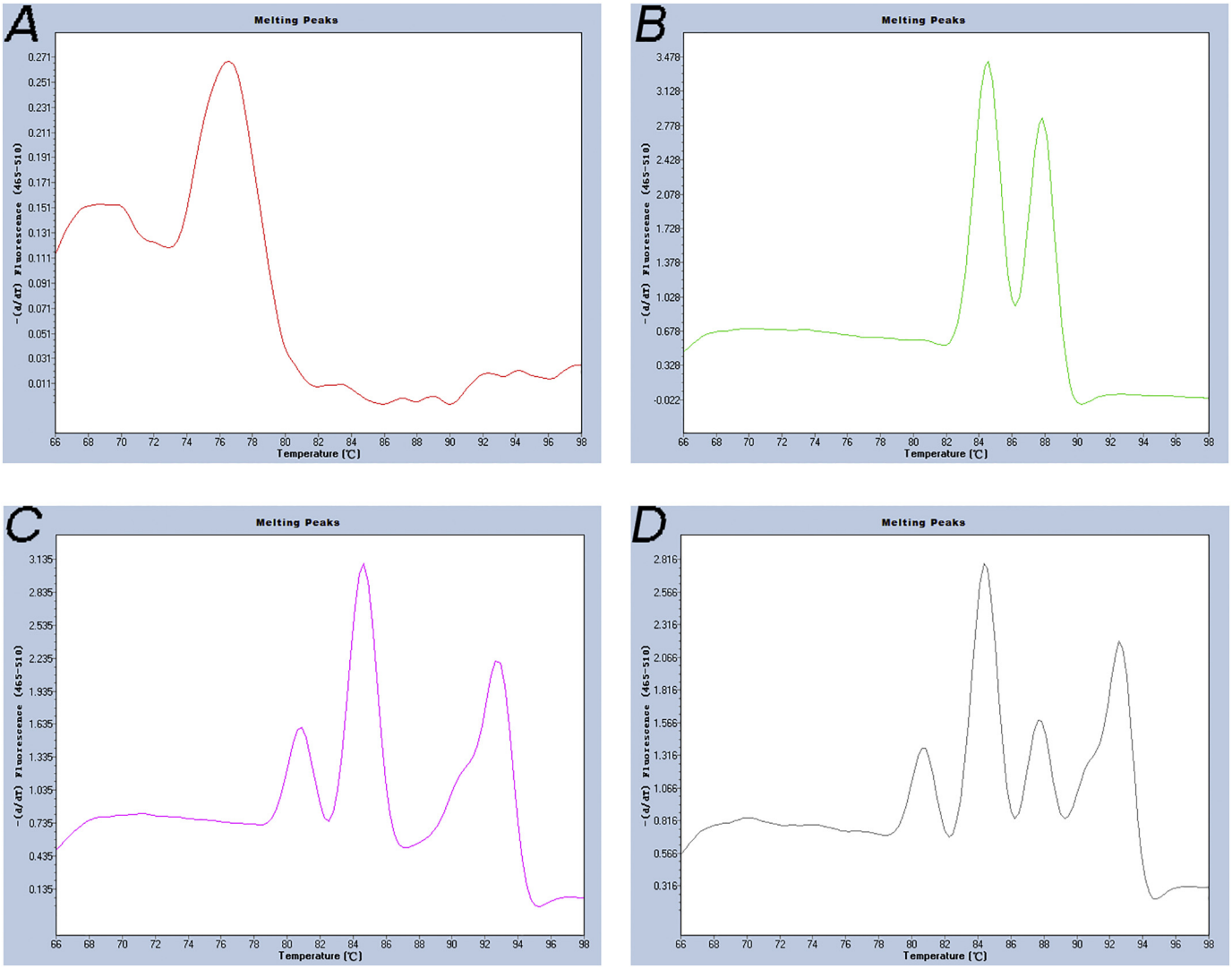

Fig. 3. Representative results of the multiplex real-time PCR assays for recombinant plasmids. A: NC. B: co-detection of *ssrA* and *sul2* genes. C: co-detection of *sul1*, *sul2* and *sul3* genes. D: co-detection of *ssrA*, *sul1*, *sul2* and *sul3* genes.

3.4. Detection in simulated blood samples

We evaluated the sensitivity of the multiplex real-time PCR assays in directly detecting the *ssrA* and *sul2* genes in blood samples. The sensitivity was 10^2 CFU/mL (Fig. 5).

3.5. Detection of S. maltophilia from clinical samples

The results are shown in Table 3. The multiplex real-time PCR assays showed 100% concordance with conventional PCR. We can rapidly differentiate *S. maltophilia* from non-*S. maltophilia* species, and the *sul* genes carried by them can be detected by reading the T_m values from the melting curve (Fig. 6).

4. Discussion

The incidence of nosocomial infections of *S. maltophilia* is on the rise (Steinmann et al., 2018). Due to intrinsic or acquired resistance of *S. maltophilia*, it is very difficult to treat its infection via drugs including all carbapenem antibiotics (Herrera-Heredia et al., 2017). Traditionally, SXT is the preferred treatment for *S. maltophilia* infection because of its low drug resistance (Adegoke et al., 2017). However, the treatment of *S. maltophilia* infection has more obstacles with the intensification of acquired SXT resistance (Chung et al., 2015). It is known that the *sul* and *dfr* gene contribute to SXT resistance, which has been reported to be related to class 1 integron and the insertion sequence common region (Li-Fen et al., 2011). Four mobile sulfonamide resistance genes (*sul1*, *sul2*, *sul3* and *sul4*) have previously been identified. The *sul4* gene (GenBank: MG649393) was reported by Razavi (Razavi et al., 2017) in 2017 as its ability to provide sulfonamide resistance, its mobile character, as demonstrated by its presence in integrons, and the homology to previously known sulfonamide resistance genes. The homology is evaluated by DNAMAN (version 1.0.051), the *sul4* has only 41.34%, 42.07% and 36.89% identity to *sul1*, *sul2* and *sul3*, respectively.

In the past few decades, conventional PCR, real-time quantitative PCR and loop mediated isothermal amplification (LAMP) have been widely used for the rapid identification of many pathogens. However, conventional PCR requires opening the cap to detect the result by agarose gel electrophoresis, which may result in environmental contamination and false-positive results (Czurda et al., 2016; Tetzner, 2009). LAMP requires six primers to detect one target sequence, thus the LAMP method is unsuitable to multiplex analyses (Wong et al., 2018). Multiplex real-time fluorescence quantitative PCR detection based on probes and dyes has been applied for simultaneous detection of multiplex targets, but fluorescence-labeled target-specific probes have the main drawback of being expensive (Kamboj et al., 2014). A

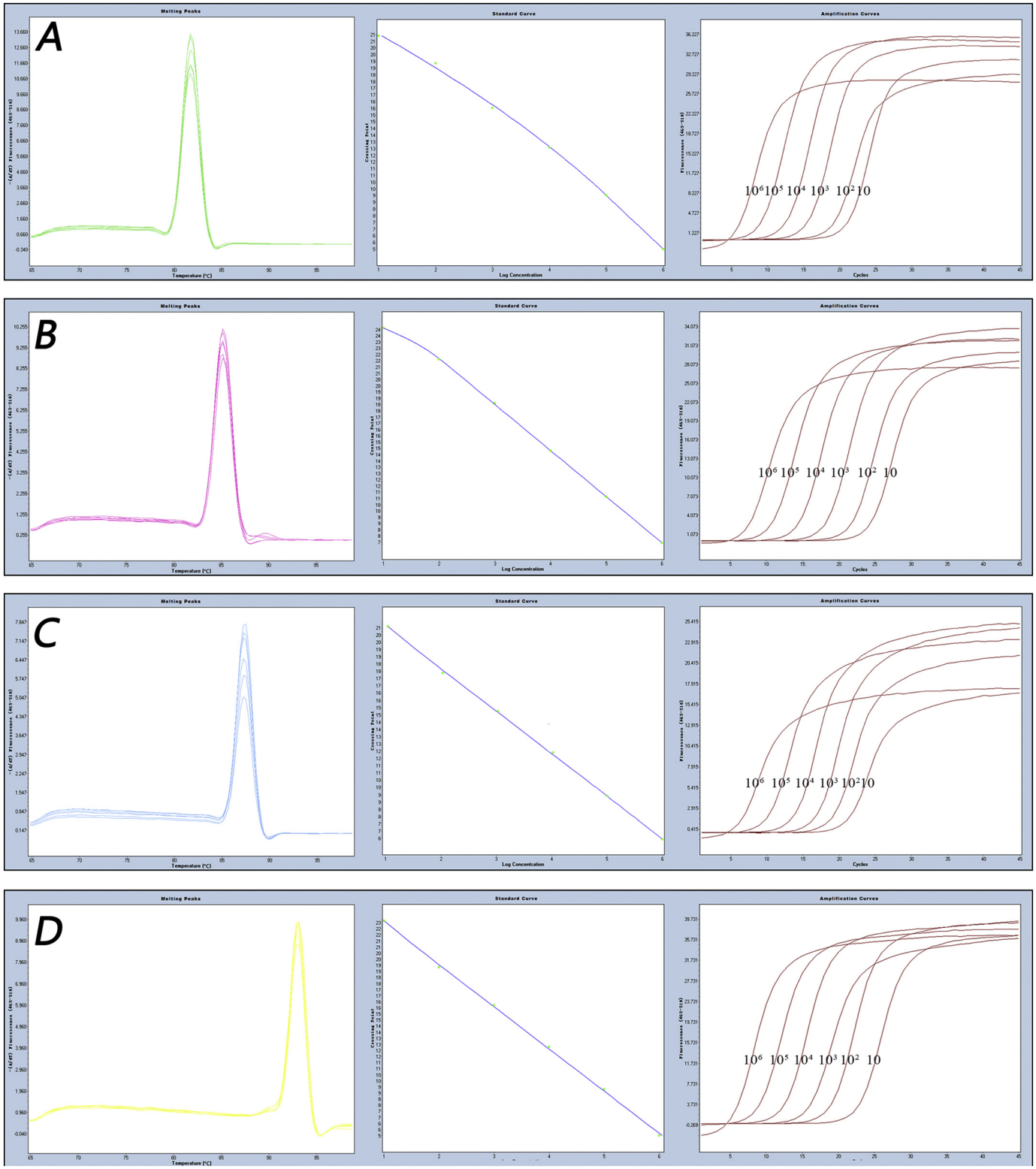

Fig. 4. Standard curves and amplification plots of the multiplex real-time PCR assays for recombinant plasmids. A: *sul3*; B: *sul2*; C: *ssrA*; D:*sul1*.

new method, allowing differentiation of genes based on the melting temperature of a PCR product, has been reported for multiplex real-time PCR systems. It permits fast and effective detection of genes with low cost. The result is completely reliable, from a completely closed system.

This multiplex Real-Time PCR assays is depended upon the melting temperatures (Tm) of the amplicons to differentiate the genes. Furthermore when the difference in melting temperatures exceeds 1.5 °C, the curves can be distinguished each other (Souza et al., 2013). The Tm is defined as the temperature at which half of the DNA strands are in the random coil or single-stranded state. It depends on the length of the DNA molecule and its specific nucleotide sequence (such as GC%). High-resolution melting has been shown to be suitable in principle for the detection of single nucleotide polymorphisms because of its

Table 2
Reproducibility of the multiplex real-time PCR assays.

target	Intra-assay					Inter-assay				
	Ct of amplicons			Mean	CV%	Ct of amplicons			Mean	CV%
*ssr*A	22.04	22.61	22.07	22.24	1.44	22.24	22.13	21.88	22.08	0.84
sul1	24.00	24.63	24.20	24.28	1.32	24.28	24.29	25.06	24.54	1.82
sul2	26.47	26.52	26.69	26.56	0.43	26.56	26.91	25.95	26.47	1.84
sul3	23.16	23.43	23.23	23.27	0.60	23.27	22.98	22.47	22.91	1.77

high sensitivity. But this is exactly the disadvantage of this method that cannot detect too many genes at once. For all we know, *S. maltophilia* resistance to SXT mediated by acquisition of *sul* and *dfrA* genes (Hu et al., 2011) but there are many subtypes of *dfrA* gene, which reported from *dfrA*1 to *dfrA*32 now. If several *dfrA* genes co-exist, it is difficult to differentiate them by curve that are likely to fuse together.

Our multiplex real-time fluorescent PCR can simultaneously detect specific genes '*ssrA*' of *S. maltophilia* and the *sul* genes (*sul1*, *sul2*, *sul3*). The multiplex real-time PCR assays showed good specificity, sensitivity (the limit of detection for each target was 10 copies per 20 μL reaction volume) and repeatability (the Ct values of the coefficients of variation for intra- and interassay reproducibility were < 5%). The sensitivity for the target DNA in simulated blood samples was 10^2 CFU/mL. Twenty SXT-susceptible and 20 SXT-resistant *S. maltophilia* strains were selected to compare multiplex real-time PCR with conventional PCR, as the comparative method. Their results showed 100% consistency.

In conclusion, we have first established multiplex real-time PCR assays that provide low-cost, sensitive and specific characterization and differentiation for *S. maltophilia* and the *sul* gene. The test has potential for direct detection of the pathogen in clinical samples.

Ethics approval and consent to participate

Not required.

Consent for publication

Not Applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Table 3
Results of 40 *S. maltophilia* via multiplex real-time PCR assays.

Gene types	SXT-susceptible *S. maltophilia* (n = 20)		SXT-resistant *S. maltophilia* (n = 20)*	
	Multiplex Real-Time PCR	Conventional PCR	Multiplex Real-Time PCR	Conventional PCR
*ssr*A	20	20	20	20
sul1	2	2	17	17
sul2	0	0	3	3
sul3	0	0	2	2

* With regard to the 20 isolates, of with 2 have contained both *sul1* and *sul2*.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Si Li carried out the experiments, data organization and analysis, and contributed to writing and annotating the results. Yuan Peng has participated in correction and modification, and contributed to collecting references. Yongyu Rui contributed to the design of the study and assisted in the drafting of the manuscript. All authors have read and approved the manuscript.

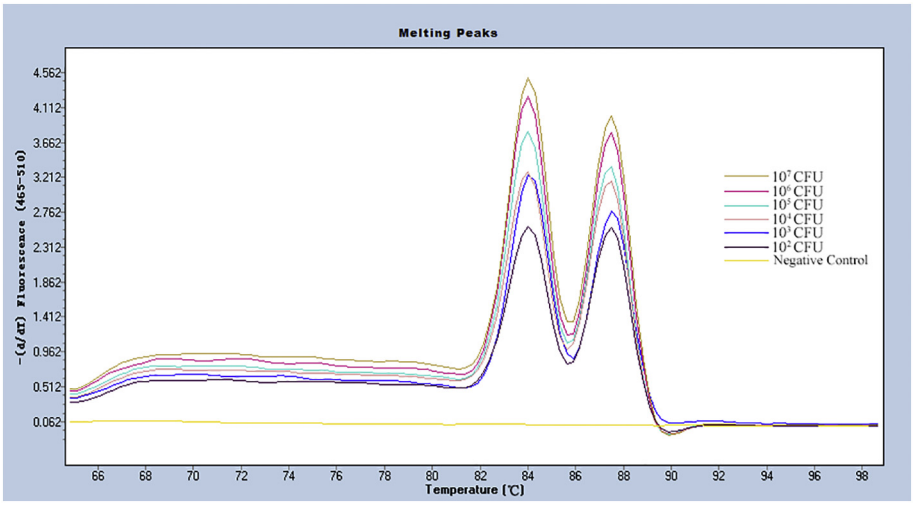

Fig. 5. Results of the multiplex real-time PCR assays for simulated blood samples.

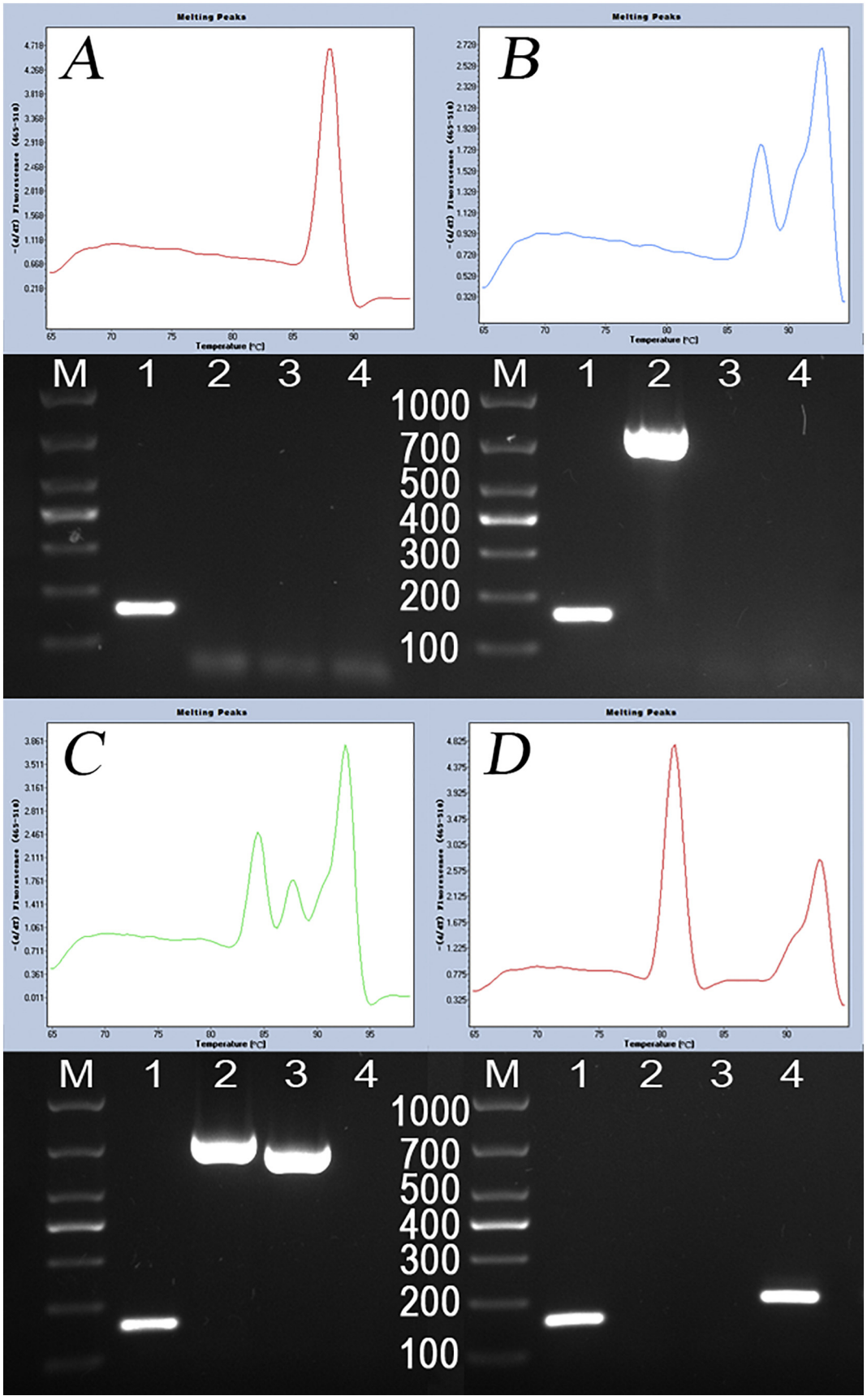

Fig. 6. Representative results of the multiplex real-time PCR assays and conventional PCR for clinical samples. M: DL1,000 DNA marker; 1: *ssrA*; 2: *sul1*; 3: *sul2*; 4: *sul3*; A: *S. maltophilia*. B: *S. maltophilia* carrying *sul1* (co-detection of *ssrA* and *sul1* genes). C: *S. maltophilia* carrying *sul1* and *sul2* (co-detection of *ssrA*, *sul1* and *sul2* genes). D: *S. maltophilia* carrying *sul3* (co-detection of *ssrA* and *sul3* genes). Samples A and B were SXT-susceptible *S. maltophilia*, while samples C and D were SXT-resistant *S. maltophilia*.

Acknowledgements

None to declare.

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