Original Article

A simple STAR Element Repetitive-PCR (SER-PCR) typing method for the rapid characterization of *Staphylococcus aureus*

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Abstract

Introduction: *Staphylococcus aureus* repeat (STAR) elements are abundant repeat sequences that are highly variable in numbers and locations within the genome of *S. aureus*. The evolutionary variation of these repeats occurs rapidly over time and may correlate with their evolutionary lineage. Therefore, the aim of this study is to analyze STAR elements to develop a simple PCR-based typing technique that can be used routinely in clinical laboratories.

Methodology: The genomes of 10 reference strains of *S. aureus* were analyzed to identify the number and location of STAR elements. One pair of PCR primers was designed to target the variable region of these elements. The designed primers were first evaluated against 13 well-known reference strains of *S. aureus* and eventually evaluated against 40 well-characterized clinical isolates.

Results: STAR element repetitive-PCR (SER-PCR) showed good typeability with acceptable discriminatory power against the reference and clinical strains. The new method clearly differentiated between community-associated methicillin-resistant *S. aureus* (CA-MRSA) and hospital-associated (HA-MRSA) strains. Phylogenetic analysis of the SER-PCR patterns and multilocus sequence typing (MLST) data showed good correlation between the two methods.

Conclusions: This study provides a simple genotyping method for *S. aureus* based on the variation of the amplicon size of a single set of primers and rapidly extracted DNA. The method is rapid with acceptable discriminatory power and does not require a sophisticated apparatus or special expertise. The results suggest that SER-PCR could be routinely useful in any clinical microbiology laboratory, particularly in developing countries.

Key words: Molecular typing; genotyping; STAR element; *S. aureus*.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important nosocomial pathogens. For the management of MRSA infections, it is important to understand the epidemiological behavior of *S. aureus* and to identify potential sources of infections. This can partially be accomplished by using the most appropriate MRSA typing technology [1,2]. The typing of MRSA is also an essential component for establishing an effective surveillance program to monitor the prevalence of MRSA.

There are many phenotypic and genotypic typing techniques, which have various typeability and ease of interpretation. In the last two decades, genotyping techniques have replaced the phenotypic methods and include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), Staphylococcal protein A (*spa*) typing, repetitive element sequence-based PCR (rep-PCR), and *Sma*I-multiplex typing (SMT). Most of these methods have been extensively evaluated against each other and have showed various discriminatory power, stability, reproducibility, cost, and ease of use [3-8]. However, several technical limitations hinder the routine use of many molecular typing techniques in clinical laboratories, such as the time required, high cost, and the need for specialist equipment and specific expertise [9,10].

PCR-based techniques such as SMT and rep-PCR seem to be more attractive for routine use due to their simplicity and rapidity. Rep-PCR methods have been introduced as a means of typing in many studies with a variety target repetitive sequences, such as Rep MP3 [11,5], InterIS256 [12], and *Tn*916 [13]. These elements were evaluated against *S. aureus* isolates and were shown to have a good reproducibility with acceptable discriminatory power [11,5,14]. Rep-PCR has been

commercially adapted to an automated high-throughput system known as the DiversiLab (DL) system. In general, DL shows good discriminatory power and reproducibility compared with other methods such as PFGE. However, DL shows poor discriminatory power with some bacterial species that show clonality and low genetic variability, such as *S. aureus* [15,2]. Moreover, DL is still relatively expensive, particularly in developing countries.

S. aureus repeat (STAR) elements are short repeat sequences that are CG-rich and located in intergenic regions (IGRs) of the *S. aureus* genome [16]. The numbers of STAR elements at different locations are highly variable, which allows complementary primers to produce specific DNA fragment patterns for individual *S. aureus* strains [17,11,16]. In 2011, Al-Zahrani *et al*. showed that the presence of STAR elements can improve the discriminatory power of SMT at the *Sma*I-group 5 site, at which they have identified 5 amplicon size variations (a-e) [8]. The aim of the current study is to analyze the STAR elements of *S. aureus* genomes to develop a simple rep-PCR-based typing technique with a single set of primers that can be used routinely in any clinical laboratory.

Methodology

Strains and growth conditions

All reference strains and clinical isolates of *S. aureus* were kept in brain heart infusion (BHI) broth containing 20% (v/v) glycerol and maintained at -80ºC for long-term storage. For molecular characterization, bacterial isolates stored at -80˚C were thawed, sub-cultured on fresh blood agar, and incubated at 37˚C overnight.

DNA extraction

The bacterial DNA was extracted using a rapid extraction method as follows. From pure culture, two bacterial colonies were suspended in a microcentrifuge tube containing 200 µL of sterile distilled water with 5% Chelex 100 (HiMedia, Mumbai , India) and mixed thoroughly. The bacterial suspension was boiled at 100˚C for 10 minutes, and the suspension was then spun down at 13,000 *g* for 5 minutes. The PCR reaction was done using 3 µL of the resulting supernatant.

SER-PCR primers and DNA amplification

PCR was performed in a reaction mixture volume of 25 µL, which contained 1X TopTaq DNA polymerase buffer, 1 mM $MgCl_2$ (Qiagen, Hilden, Germany), 400 µM dNTP (ThermoFisher, Schwerte, Germany), 8X bovine serum albumin (BSA; New

England BioLabs, Ipswich, Massachusetes, USA), 2 µM of each primer (SER-PCR-F, 5'-GTTATATTGGCAGTAGTTGAC-3' and SER-PCR-R, 5'-GCCAGCTTCTATGTTG-3') (Macrogen, South Korea), 5 U/µL TopTaq DNA polymerase (Qiagen, Hilden, Germany), and 3 µL of chromosomal DNA. The PCR was carried out using the following conditions: 5 minutes of initial denaturation at 94˚C; 30 cycles of 94˚C for 1 minute, 54˚C for 1 minute, and 72˚C for 1 minute; and a final extension for 5 minutes at 72˚C.

Gel electrophoresis

The resulting amplicons were separated on 1.7% agarose (in 1X TBE with 0.5 µg/mL ethidium bromide) at 100 V for 70 minutes and then visualized using a gel documentation system (Syngene, Cambridge, UK).

Bioinformatical analysis

The genome sequences of 10 reference strains of *S. aureus* (NCTC8325, COL, MW2, MSSA476, MRSA252, N315, Mu50, USA300TCH1516, EMRSA-15 (HO 5096 0412), and NRS 484 (USA1100)) were obtained from the national Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and European Bioinformatics Institute (EMBL-EBI; http: //www.ebi.ac.uk/genomes/bacteria.html). The sequences were analyzed using Tandem Repeats Finder software (https://tandem.bu.edu/trf/trf.html) [18]. The Primer-BLAST tool from the NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to determine the numbers and locations of PCR amplicons of the designed primers in genomes of *S. aureus*.

Data analysis

SER-PCR gel images were analyzed based on the differences in DNA band patterns. A clustering dendrogram was created by the unweighted-pair group method with arithmetic average (UPGMA) using open-source Python-based software. A neighbor-joining MLST tree was constructed using TART2 software.

Results

Bioinformatics analysis

A bioinformatic analysis was first carried out to determine whether STAR elements can be used for epidemiological typing. The number and location of STAR elements within the genomes of the current sequences of *S. aureus* strains were determined using Tandem Repeats Finder software. The analysis showed that the STAR elements were highly variable in number

and location among the 10 sequenced genomes of *S. aureus* (Table 1). The distinct feature of these elements is that they contain a signature sequence (GGGGCCCC) that can be present in many copies at a single locus flanked by invariable sequences. The invariable sequences on either side of the STAR elements can be targeted by PCR primers to generate amplicons of various sizes.
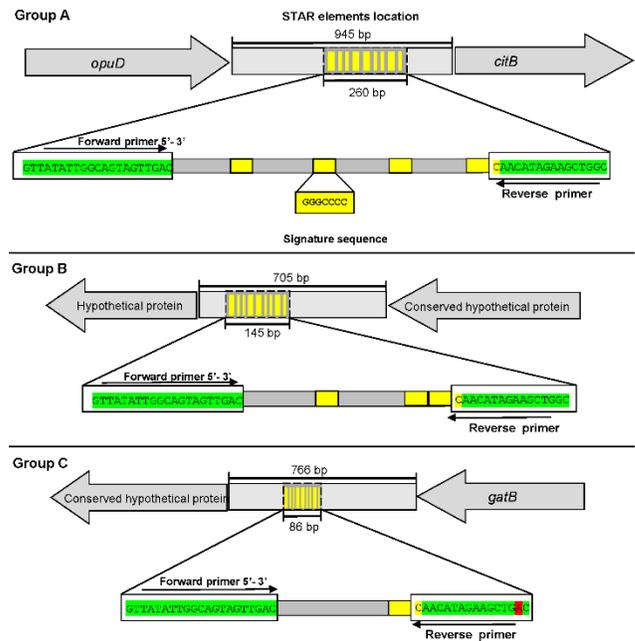
Primer design strategy

The STAR elements located downstream of *opu*D and upstream of the *citB* locus seemed to be more variable among the *S. aureus* strains. Thus, one pair of primers was designed to target an intergenic region upstream of the *citB* locus. The forward primer was designed from a conserved region, and the reverse primers contained the signature sequence (GGGGCCCC) at its 5' end (Figure 1).

In the first stage, the primers and their amplicons were bioinformatically examined to determine the number, location, and size of the expected amplicons using primer-BLAST from the NCBI. The amplicons at different locations were significantly variable in number and size among *S. aureus* genomes (Figure 2). Some single nucleotide polymorphisms (SNPs) were observed within the binding sites of the forward and reverse primers, which seemed to have no effects on the stability of oligonucleotide binding and target specificity (Figure 1).

Validity of SER-PCR primers and their discriminatory power against reference S. aureus strains

The discriminatory power of the designed primers was examined against 13 reference strains of *S. aureus* (NCTC8325, COL, MW2, MSSA476, MRSA252, N315, Mu3, Mu50, USA300TCH1516, EMRSA-15 (HO 5096 0412), USA1000, CA-629, and NRS 484 (USA1100)). All strains were typeable, and the primers showed good resolving power (Figure 3). SER-PCR distinguished between nosocomial epidemic strains

Figure 1. The principle behind the design of SER-PCR primers.

The sequences highlighted with yellow are the signature sequences of the STAR elements. Groups A, B, and C illustrate how the designed primer pair generates PCR products with various sizes due to the presence of STAR sequences in the genome of *S. aureus* (*Mu50 strain*). The red highlights in group C show the SNP in the sequence of the reverse primer.

MRSA252(EMRSA-16) and EMRSA-15 and between community-associated MRSA strains CA-629, USA1000, and USA1100 (Figure 3A). The designed primers were not able to differentiate between closely related community-associated strains MSSA476 and MW2 or the closely related nosocomial strains N315, Mu50, and Mu3 (Figure 3). The sequence types (STs) of these reference strains were previously established, and nine STs were found among the 13 *S. aureus* strains. ST5 was found in three strains (Mu50, Mu3, and N315), while strains MW2 and MSSA476 belong to the same ST (ST1). ST8 was also reported in two strains (NCTC8325 and USA300), and the rest of the strains showed different STs (Figure 3B).

Table 1. The number of STAR elements among the sequenced *S. aureus* strains.

S. aureus strains	Type	Origin	Year	STAR element no.
NCTC 8325	MSSA	Colindale, UK	1949	62
COL	HA-MRSA	Colindale, UK	1961	76
MW2	CA-MRSA	Bacteremia, USA	1998	80
MSSA476	CA-MRSA	Osteomyelitis, UK	1998	74
Mu50	HA-VISA	Wound, Japan	1997	76
N315	HA-VISA	Pharynx, Japan	1982	83
EMRSA-15 (HO 5096 0412)	HA-MRSA	Sanger, UK	2009	65
MRSA252	HA-MRSA	Bacteremia, UK	1997	72
USA300_TCH1516	CA-MRSA	Sepsis, USA	2007	71
USA1100	CA-MRSA	USA	N/A	79

Discriminatory power of SER-PCR primers against clinical isolates

Forty clinical isolates of *S. aureus* with known sequence types were selected to be tested by SER-PCR. These isolates belong to 26 STs and were previously described by Al-Zahrani *et al*. [19]. All 40 isolates were typeable by SER-PCR, which discriminated the isolates into 27 distinct SER-PCR profiles (Figures 4 and 5). Six isolates (1, 7, 39, 47, 52, and 74) had identical SER-PCR profiles and were grouped in one cluster. Isolates 39 and 74 shared ST97, whereas isolates 1, 7, 47, and 52 showed different sequence types (ST789, ST1292, ST15, and ST96, respectively) (Figure 5).

Of the ST728 isolates (n=4), only isolates 23 and 84 shared identical SER-PCR profiles (Figure 5). The ST5 isolates (50 and 66) shared the same SER-PCR profile, but the SER-PCR profile of isolate 9, which also had

ST5, was closely related to those of isolates 50 and 66 (Figure 5). Isolates 11, 38, and 45 all had ST217 but showed different SER-PCR profiles. Isolates 60 and 22 had identical SER-PCR profiles and both shared ST3303 (Figure 5). Similarly, isolates 31 and 27 exhibited identical SER-PCR profiles and were identified previously as ST88 (Figure 5).

Discussion

The currently available molecular technologies for MRSA typing, such as PFGE, MLST, and *spa* typing, provide valuable information and have been preferred methods for decades. Whole genome sequencing (WGS) as a typing method provides the whole image of any pathogen of interest with all information that is necessary for both epidemiological and treatment purposes [20].

Figure 2. The location of SER-PCR primers and the number of their expected amplicons (red rectangles) in genomes of S. aureus. Mb: Megabase pair
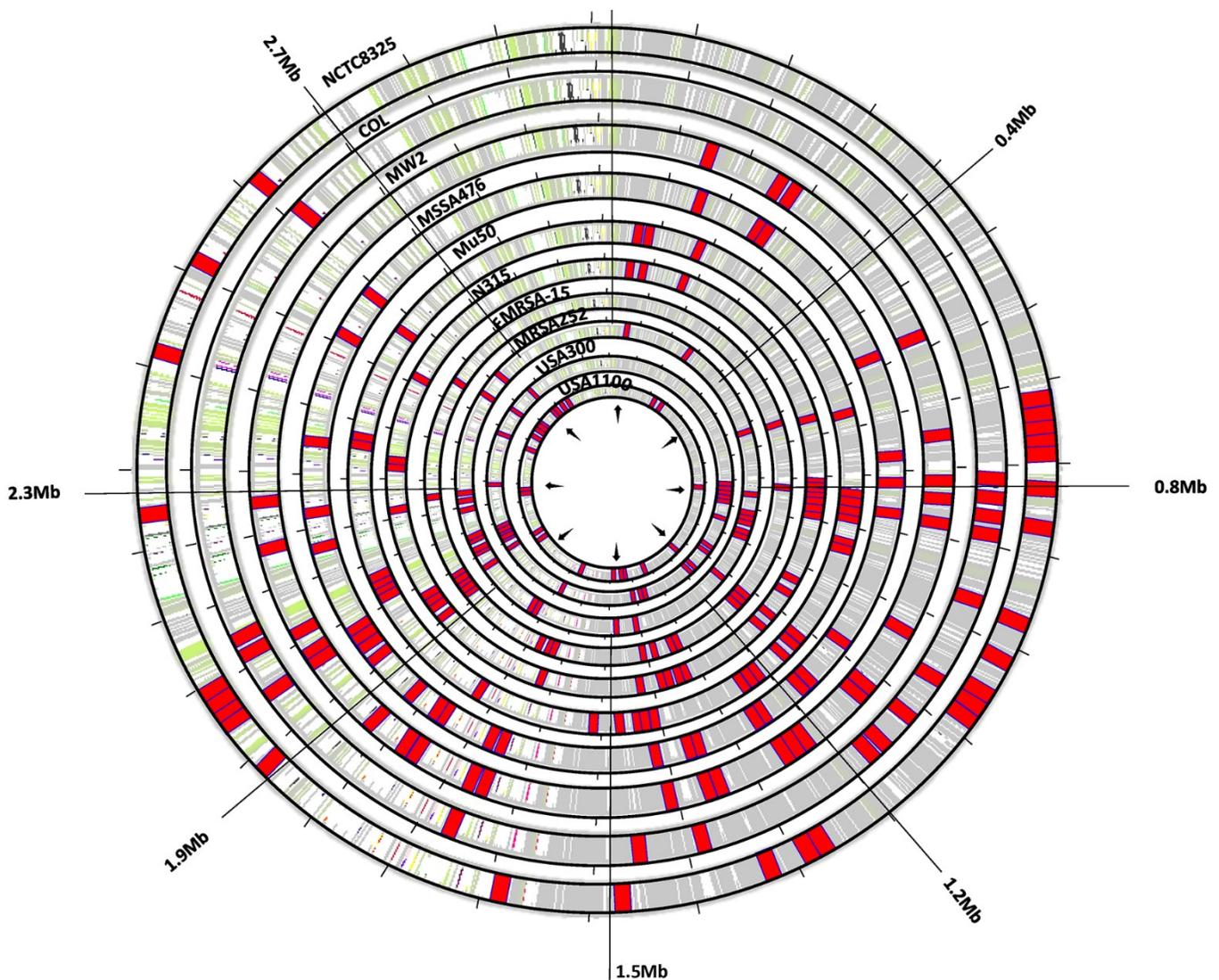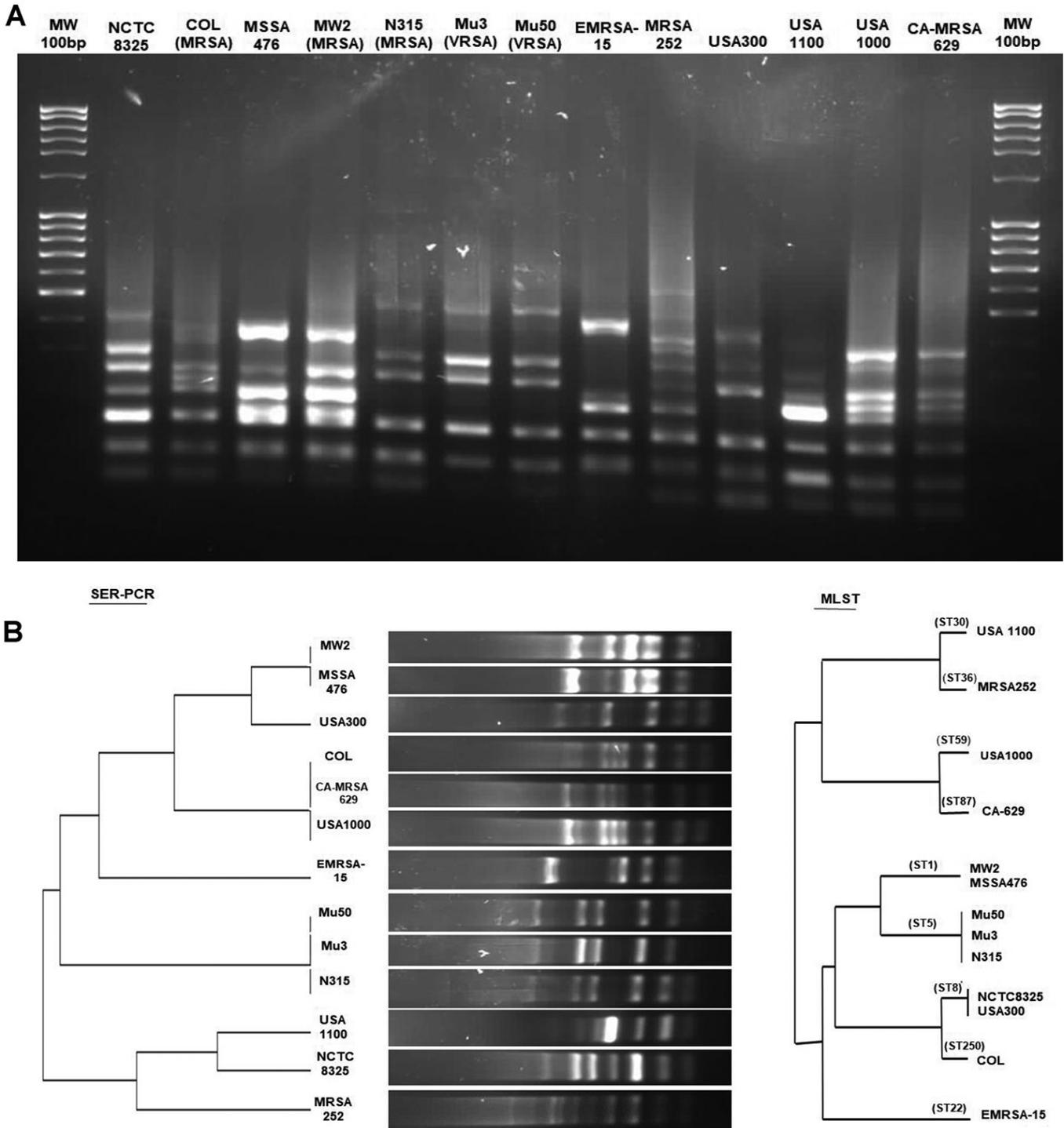
Figure 3. SER-PCR patterns and UPGMA dendrogram.

(A) SER-PCR patterns of 13 reference strains of *S. aureus*. **(B)** The UPGMA dendrogram of SER-PCR patterns and the neighbor-joining MLST phylogenetic tree showing the genetic relationship of the 13 strains. MW 100bp: 100-bp molecular size markers.

Figure 4. SER-PCR profiles of 19 representative clinical S. aureus isolates. MW 100bp: 100-bp molecular size markers.
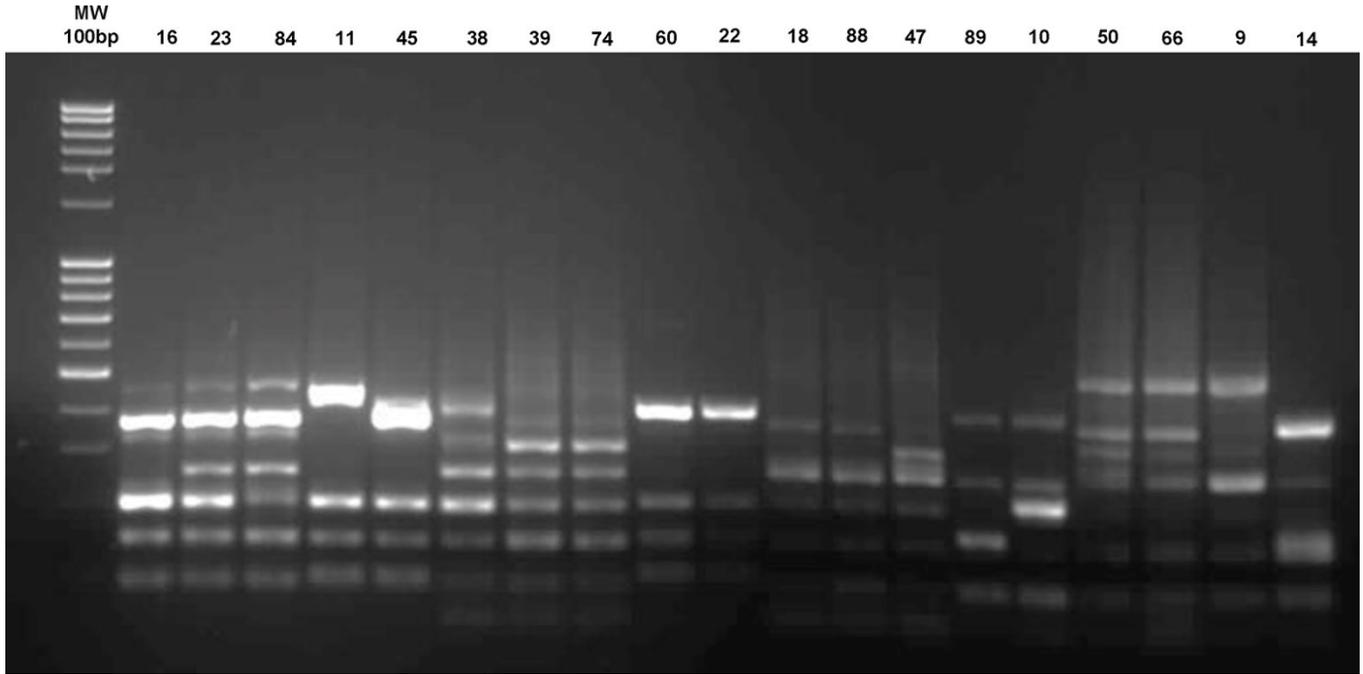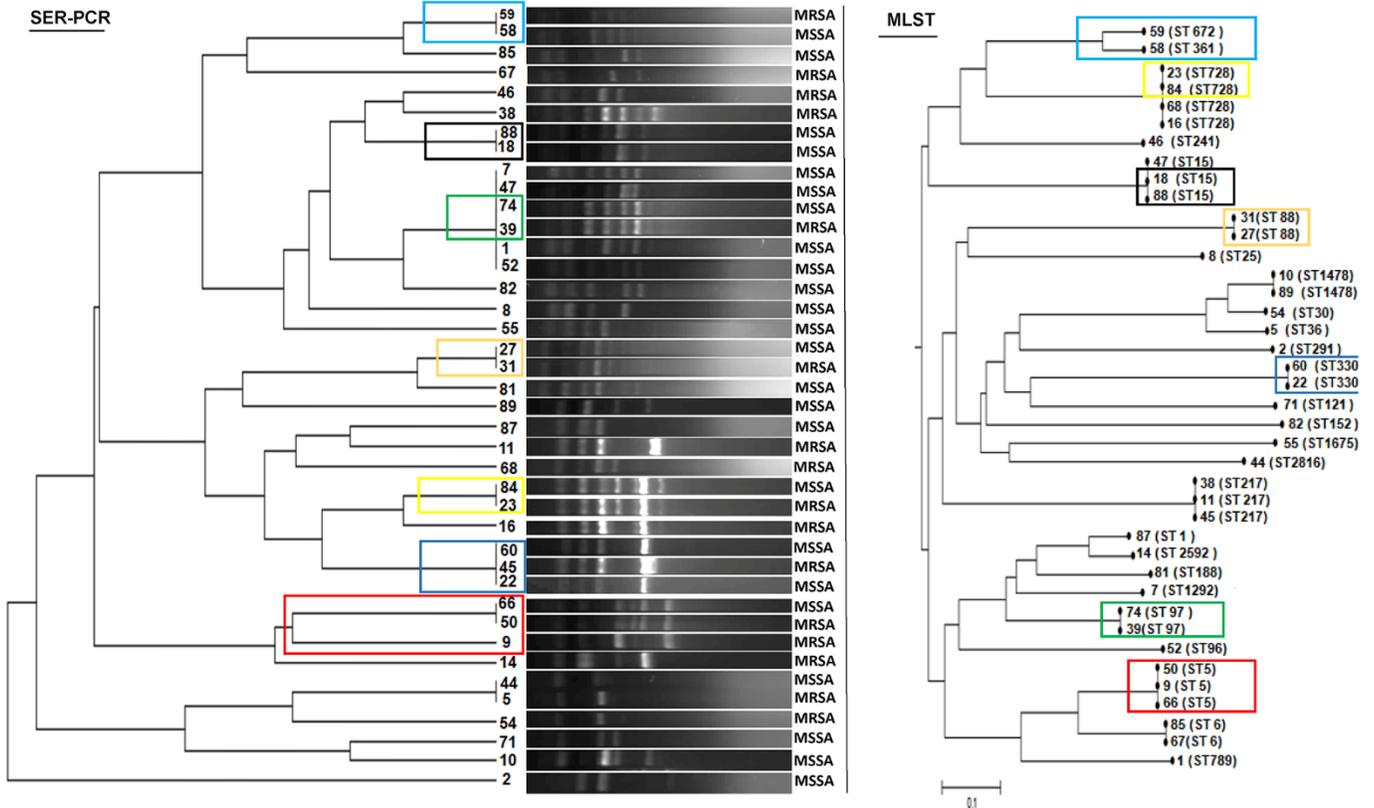

Figure 5. The UPGMA dendrogram of SER-PCR patterns and the neighbor-joining MLST phylogenetic tree illustrating the genetic relationship among 40 clinical isolates of *S. aureus*.

Although WGS has become more accessible in some developed countries, WGS platforms are still relatively expensive and not affordable for many developing countries. Furthermore, the interpretation of WGS data is still a major limitation that restricts the routine use of WGS in many clinical laboratories [20,21]. In contrast, PCR-based typing techniques are still more attractive for routine practice because they do not require a sophisticated apparatus or special expertise.

Tandemly repeated sequences have been exploited to develop many typing methods for many bacterial pathogens, including *S. aureus* [11]. These methods first started with gel-based fingerprinting analysis of coagulase gene polymorphisms [22], followed by protein A tandem repeats (TRs) [23] and the simultaneous analysis of TRs within individual genes, such as multiple-locus variable numbers of tandem repeats (MLVNTRs) [24]. Some of these approaches were later adapted to sequence-based repeat alleles (*e.g., spa* typing and MLVTR) [25]. These methods have been evaluated extensively and showed comparable results to standard typing methods (*e.g*., MLST and PFGE) [26-28]. The limited accessibility and relatively high cost have hindered the routine use of these methods in many clinical microbiology laboratories.

STAR elements are abundant repeat sequences that are highly variable in number and location within the genomes of *S. aureus* [16]. The evolutionary variation of these repeats occurs rapidly over time and may correlate with their evolutionary lineage [16,29]. This suggests that the variation of STAR elements may be useful for *S. aureus* typing [16]. In 2003, Quelle *et al*. proposed the first method called STAR repetitive element PCR (STAR-RP PCR) to explore the efficacy of STAR-RP as a typing method. They performed the amplification of STAR elements within *uvrA* and *hprk* loci using the primers designated by Cramton *et al*. (2000), followed by a digestion step with two restriction enzymes (*Rsa*I and *Alu*I). STAR-RP was rapid with good discriminatory power and reproducibility [16,14]. In 2011, Al-Zahrani *et al*. proved that these elements can show good discriminatory power if they are properly targeted by PCR primers [8].

In the current study, the STAR elements of the 10 reference strains analyzed were highly variable in number and location. It is interesting to note that a signature sequence (GGGGCCCC) was found within those elements, which can be present in many copies at a single locus. The STAR elements in the *opuD-citB* intergenic region seemed more variable, and it was selected to be targeted by PCR primers. The forward primer was designed from the conserved sequence of the *opuD-citB* intergenic region, while the portion of the signature sequence was included at the 5' end of the reverse primer (Figure 1). This was mainly done to increase the chance of producing many PCR amplicons with different sizes. To make sure this strategy works, a bioinformatic analysis using Primer-BLAST software was carried out to determine the size and expected number of amplicons in the genomes of the 10 reference strains. The results indicated that the primer pair generated many amplicons that are variable in location, number, and size (Figure 2).

The resolving power is an essential parameter for any typing technology, and therefore, an experimental evaluation of the primer pair was carried out against 13 well-characterized reference strains of *S. aureus*. The typeability of SER-PCR was good with acceptable discriminatory power. The distinction between CA-MRSA and HA-MRSA is also an important process for treatment and infection control, and SER-PCR clearly differentiated between CA-MRSA and HA-MRSA strains (Figure 3). One interesting finding is that SER-PCR distinguished between a highly virulent strain of community-associated MRSA (USA300) and other CAMRSA strains (USA1100 and USA1000), and each strain showed unique SER-PCR profiles (Figure 3).

The strains EMRSA-16 (MRSA252) and EMRSA-15 were firstly identified in the UK and have become predominant clones in all UK hospitals. These clones have been reported in many countries, including those in Europe, North America, and the Middle East [30]. Expectedly, SER-PCR discriminated between both epidemic clones (EMRSA-16 and EMRSA-15). Moreover, NCTC8325 and USA300 belonged to the same ST (ST8), but each strain showed distinguishable SER-PCR profiles (Figure 3). However, SER-PCR was unable to differentiate between the closely related nosocomial strains N315, Mu3, and Mu50. These strains are also indistinguishable by MLST, and all three strains have the same MLST type (ST5). Similarly, the ST1 strains (MW2 and MSSA476) showed identical SER-PCR profiles (Figure 3).

SER-PCR was further evaluated using 40 clinical isolates that were previously characterized by SMT and MLST. These isolates were previously shown to have 40 distinct SMT profiles, while only 26 STs were found among the isolates [19]. In the current study, all 40 isolates were typeable by SER-PCR and showed 27 SER-PCR patterns. There was good correlation between the two techniques according to the UPGMA dendrogram of SER-PCR patterns and the neighbor-

joining MLST phylogenic tree (Figure 5). Another important finding is that the cluster analysis revealed that the majority of isolates were grouped by SER-PCR into the same clusters as MLST, although other isolates showed some differences in the relationships. For example, ST5 isolates (MRSA-50 and MSSA-66) showed an identical SER-PCR profile (Figure 5) that is closely related to the SER-PCR profiles of hospital-acquired VISA strains (Mu3, Mu50, and N315) (Figure 3).

　　MRSA-9 also has ST5 but exhibited a different SER-PCR profile and was tightly clustered with ST5 isolates by SER-PCR (Figure 5). Similarly, MRSA-39 and MSSA-74 showed identical SER-PCR profiles, and both isolates had the same ST97 (Figures 3 and 5). It is interesting to note that ST3303 is a novel sequence type that has been recently identified in two isolates of *S. aureus* recovered from Saudi Arabia [19]. Consistent with the MLST, these two isolates showed the same SER-PCR profile (Figures 4 and 5).

Conclusion

　　In conclusion, this study has provided a simple genotyping method for *S. aureus* (MRSA and MSSA) based on the variation of the amplicon size of a single set of primers and rapidly extracted DNA. Although SER-PCR has less discriminatory power than other multiplex-PCR techniques (e.g., SMT), it is able to predict MLST types with good concordance with other methods. The new method is rapid with acceptable discriminatory power and does not require a sophisticated apparatus or special expertise. The results suggest that SER-PCR could be routinely useful in any clinical microbiology laboratory, particularly in developing countries. However, SER-PCR still needs more evaluations against a large number of isolates, including *S. aureus* isolates recovered from hospital outbreaks.

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