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In silico PCR Verification and Simplex Real-Time PCR Detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from East Coast Malaysian Clinical Isolates

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Abstract

The aims of this study were to validate the primers developed for molecular-based detection and identification of Malaysian clinical isolates of methicilin-resistance *Staphylococcus aureus* (MRSA) using *in-silico* Polymerase Chain Reaction (PCR) and real-time PCR SYBR with Green I. Rapid molecular diagnostic and risk assessment of the MRSA are possible by real-time PCR SYBR Green I. However, validation of such primers for real-life samples is expensive and time consuming. Hence, development and verification of real-time PCR primers by *in-silico* PCR can be the first step in the selection of the most appropriate primers. Three species-specific markers were chosen targeting *coa* (staphylocoagulase), *nuc* (thermonuclease) and *mecA* (methicillin-resistance) and were specifically verified against 35 selected *S. aureus* strains by using *in-silico* PCR. For the actual laboratory verification, all of the 3 genes were detected with a single specific melting curve peak (T*m* at 76.16 ± 0.8 °C, 78.50 ± 0.4 °C and 74.41 ± 0.6 °C for a *coa*, *nuc* and *mecA* respectively) in 32 bacterial strains including ATCC reference strains. Thus, there is no disagreement between both *in-silico* PCR and real-time PCR verification and validation of the primers designed for the detection and identification of MRSA in this study. The potential of using a bioinformatics approach (*in-silico PCR*) before selecting primer pairs for a given study may enable researchers to accept or reject the potential primer pairs for downstream experimental (*in vitro*) PCR without wasting any chemicals as well as related cost.

Keywords: *In-silico* PCR, real-time PCR SYBR Green I, methicillin-resistant *Staphylococcus aureus* (MRSA), staphylocoagulase (*coa*), nuclease (*nuc*) and methicillin-resistant (*mecA*) genes

Introduction

Staphylococcus aureus is well known as a major pathogen, causing a variety of nosocomial and community-acquired infections [1]. Coagulase production is the principle criterion used in the clinical microbiology laboratory for the identification of *S. aureus*. The coagulase status of an isolate is not always easily established in a timely fashion, thus increasing the delay in definitive identification of *S. aureus* [2]. The identification and detection of *S. aureus* or MRSA are found to be quite troublesome in the laboratory by either conventional or molecular methods, since

both methods have their own advantages and disadvantages depending on the equipments and expertise available in the laboratory setting [3]. Usually, misidentification of *S. aureus* as coagulase-negative staphylococcus (CoNS) can result in a costly search for other pathogens or unwarranted broad-spectrum empiric antimicrobial coverage [4].

Although recent studies have shown several rapid and accurate molecular detection approaches for antimicrobial susceptibility and species identification by real-time PCR and DNA microarray [5,6], conventional microbiology based technique are still preferred among researchers. This is due to the fact that conventional antibiotic susceptibility methods can test for several antibiotics at the same time as compared to molecular methods (e.g. PCR) that requires numerous optimizations (e.g. gene identification, primer design), especially in testing for resistance involving 2 or more antibiotics. Additionally, molecular testing methods may overestimate the presence of antibiotic resistance as they do not test for expression of the relevant resistant genes [3].

Real-time PCR, known as a powerful advancement of the basic PCR, utilizes fluorescent strategies for detection of nucleic acid in a reaction without post-PCR analysis [7]. Recently, a number of real-time PCR studies based on fluorescent probes have been carried out to detect methicillin-resistance *S. aureus* (MRSA) from various clinical sources using TaqMan® probes [8,9]. However, these assays require the availability of primers and probes that must be selected according to very rigid conditions, which cannot always be easily applied. Furthermore, the development and validation of real-time PCR probes is cost-ineffective and requires expertise [10,11]. It is therefore reasoned that *in-silico* PCR verification and validation of real-time PCR primers designed for specifically targeting species-specific genes can be the first step in the selection of the appropriate chemistry for real-time PCR detection of MRSA in a clinical laboratory setting as well as limiting the usage of unwanted chemicals related with the real (*in-vitro*) PCR.

Materials and methods

Bacterial strains used

Thirty two bacterial strains used are listed in **Table 1**. Sixteen [HN 1, HN 2, HN 3, HN 4, HN 5, HN 6, HN 7, HN 8, HN 9, HN 10, HN 11, HN 12, HN 13, HN 14, HN 15 and HN 16] of the *S. aureus* isolates were taken from an east coast public hospital, Hospital Sultanah Nur Zahirah, Kuala Terengganu, on the east coast of the Malaysian Peninsular; five [N 391, N 441, N 829, N 850 and N 1406] from Hospital Universiti Kebangsaan Malaysia (HUKM), Cheras and another 5 [UM 1, UM 2, UM 3, UM 6, and UM 7] from Universiti Malaya Medical Center (UMMC), Kuala Lumpur. Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591) and mehicillin-sensitive *S. aureus* (MSSA) (ATCC 25923 and ATCC 29213) strains were also employed as reference and *Staphylococcus epidermidis* (ATCC 12228), *Escherichia coli* (ATCC 35218) and *E. coli* (ATCC 700728) were used as negative control strains for specificity of conventional PCR amplification in this study. HUKM and UMMC are located in the Western part of the Malaysian Peninsular but the isolates from these hospitals were also included as additional PCR amplification controls. Bacterial strain isolates were maintained on Protect Bacterial Preservers (Technical Service Consultants, Heywood, and Lancashire, England) and cultured in tripticase soy broth (TSB) (Difco Laboratories, Detroit, USA) at 37 °C.

Genomic DNA extraction

Whole genomic DNA extractions of *S. aureus* isolate and reference strains were done using the Wizard Genomic DNA extraction kit (Promega Inc., Madison, WI, USA) without using lysostaphin as previously published by Suhaili *et al.* [12]. Colonies were grown overnight at 37 °C in TSB and centrifuged at 13,000 × g for 2 min. Cell pellets were resuspended in 500 µl of distilled water with 700 µl of lysozyme (30 mg/ml) and the mixture were incubated at 37 °C for 1 h. Other prepared solutions from the extraction kit were added according to the manufacturer's recommendations. Extracted DNA was stored at –20 °C until PCR was performed.

Primer design

The *nuc* primer set was as described by Saiful *et al.* [13] with an expected product of 279 bp nuclease gene of *Staphylococcus aureus*. Meanwhile, for staphylocoagulase (*coa*) and methicillin resistance (*mecA*), gene sequences are

referred to accession numbers BA000017 and AP009324 in the GenBank data base (www.ncbi.nlm.nih.gov) respectively. The designing of the primers was performed using the Primers3 software free access on-line bioinformatics tool (http://www-genome.wi.mit.edu/cgi-bin/primer/ primer3_ www.cgi). The parameters for designing all oligonucleotide primers were as follows: length of primer 18 - 20 nt for both *coa* and *mecA* genes except for the *nuc* gene; primer annealing temperature in the range between 50 - 60 °C. The BLAST-N v.2.2.6 tool (National Biotechnology Information, NCBI www.ncbi.nlm.nih.gov) was used to confirm that none of the selected oligonucleotides recognized any registered DNA sequences other than the target. The oligonucleotides used in this study are listed in **Table 2**.

***In-silico* PCR verification**

In-silico PCR is a theoretical method used to calculate PCR products by using up-to-date sequenced genomes that allows the amplification of specific DNA sequences [14]. Verification was carried out according to http://insilico.ehu.es before the actual experiment to predict the outcome of the amplification product and its DNA sequences. All primers used for species-specific identification of MRSA from Malaysian clinical isolates were verified by using *in-silico* PCR against 35 published genomes of *S. aureus* strains available. The primer pairs that were newly designed in this study were *coa* and *mecA* genes, except the *nuc* gene which was adapted from Saiful *et al.* [13]. The results for this assay were illustrated as electrophoresis mobility simulations.

Real-time PCR verification and melting curve analysis

Real-time PCR amplification were carried out with SYBR Green I dye by using a RotorGene® RG6000 cycler platform (Qiagen, Germany) with a 36-carousel rotor. Amplification conditions were optimized for each of the primers before proceeding to the further detection assay. The cycling parameter conditions were as follows; an initial hold at 95 °C for 3 min, followed by 40 cycles consisting of 95 °C for 40 s, annealing temperature as listed in **Table 2** for 40 s, and 72 °C for 50 s (**Table 2**). All isolates including positive and negative samples were tested in triplicates. A melting curve was acquired using a ramping rate of 1 °C/60 s for 65 - 99 °C. The differentiated data were analysed using RotorGene® RG6000 software V6.0. The T*m* of each sample was compared to the T*m* of the positive control (MRSA ATCC 33591).

Results

***In-silico* PCR verification of designed primers**

In this study, the *in-silico* PCR amplification was used as a verification tool for the designed primers targeting the 3 selected genes nuclease (*nuc*), staphylocoagulase (*coa*), and methicillin-resistance (*mecA*) genes. The *in-silico* PCR amplification results showed that the product sizes were correctly amplified according to the expected results during the designing of the primers as summarised in **Table 3**. **Figures 1a-c** represent electrophoresis mobility simulations for the *nuc*, *coa*, and *mecA* genes for the primer verifications against 35 selected strains available in the database (Updated May 2012). For the virtual amplification of *nuc* and *coa* genes, all 26 *S. aureus* species, as well as other strains, were successfully amplified and the product sizes were 279 bp and 117 bp for *nuc* and *coa* genes respectively. This *in-silico* amplification also revealed that there were no bands amplified using both set of primers against all 9 negative controls as described in **Table 3**. For the verification of *mecA* primers, 17 out of 26 *S. aureus* strains were positively identified as Methicillin-resistant *S. aureus* (MRSA) with amplification products of 147 bp in size. There was no cross amplification of the *mecA* gene against negative control strains.

Actual real-time PCR amplification of selected primers

Representative real-time PCR amplification melting curve analysis obtained by real-time PCR SYBR Green I for the clinical isolates as well as ATCC references strains for *nuc*, *coa*, and *mecA* genes are shown in **Figures 2a-c**, respectively. Actual primer amplification for screening 32 selected clinical isolates of *S. aureus* including ATCC reference strains, as well as 2 non-staphylococci, are summarized in **Table 1**. MRSA strains were unequivocally detected within less than 2 h using real-time PCR targeting the staphylocoagulase (*coa*), nuclease (*nuc*), and

methicillin resistance (*mecA*) gene-specific oligonucleotides. For *coa* and *nuc* (species-specific) amplification, all *S. aureus* clinical isolates, including ATCC reference strains, produced single melting peak profiles with T_m at 76.16 ± 0.8 °C and 78.5 ± 0.4 °C, respectively. Meanwhile, for *mecA* amplification, 19 out of 27 clinical isolates, including 8 positive MRSA isolates from UMMC and HUKM, as well as MRSA ATCC 33591, were identified as MRSA by a single melting peak with T_m at 74.7 ± 0.6 °C. No melting peaks were observed for the 3 ATCC negative control strains as was observed for *nuc* and *coa* genes amplification. Both primers for *nuc* and *mecA* genes used were in total agreement with duplex real-time PCR amplification, which produced consistent melting temperatures (T_m) for all MRSA tested, as described by Suhaili *et al.* [12].

Table 1 Real-time PCR amplification of bacterial isolates used in this study.

Isolates	Real-time PCR amplification		
	***coa* gene**	***nuc* gene**	***mecA* gene**
Reference strains			
ATCC 25293 (MSSA)*	+	+	-
ATCC 29213 (MSSA)	+	+	-
ATCC 33591 (MRSA)**	+	+	+
ATCC 12228 (MSSE)***	-	-	-
ATCC 35218 (*E. coli*)	-	-	-
ATCC 700728 (*E. coli*)	-	-	-
Clinical isolates			
HN 1	+	+	+
HN 2	+	+	+
HN 3	+	+	+
HN 4	+	+	+
HN 5	+	+	+
HN 6	+	+	+
HN 7	+	+	-
HN 8	+	+	-
HN 9	+	+	-
HN 10	+	+	-
HN 11	+	+	-
HN 12	+	+	+
HN 13	+	+	+
HN 14	+	+	+
HN 15	+	+	+
HN 16	+	+	+
N 391	+	+	+
N 441	+	+	+
N 829	+	+	+
N 850	+	+	+
N 1405	+	+	+
UM 1	+	+	+
UM 2	+	+	+
UM 3	+	+	+
UM 6	+	+	-
UM 7	+	+	-

Degree of amplification: Positive amplified (+); Negative amplified (-)
*Methicillin-sensitive *S. aureus*;
**Methicillin-resistance *S. aureus*;
***Methicillin-sensitive *S. epidermidis*

Table 2 Primer and annealing temperature used in this study.

Genes	Prime sequences (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	*T_m °C	Primer conc. (µM)	Ref.
mecA	F (5'- AAA ACT AGG TGT TGG TGA AGA TAT ACC -3') R (5'-GAA AGG ATC TGT ACT GGG TTA ATC AG-3')	147	56	74.41 ± 0.6	0.50 0.50	Suhaili *et al.* [12]
nuc	F (5'- GCG ATT GAT GGT GAT ACG GTT-3') R (5'- AGC CAA GCC TTG ACG AAC TAA AGC-3')	279	56	78.5 ± 0.4	0.65 0.65	Saiful *et al.* [13]
Coa	F (5'-GTA GAT TGG GCA ATT ACA TTT TGG AGG -3') R (5'-CGC ATC AGC TTT GTT ATC CCA TGT -3')	117	55	76.16 ± 0.8	0.45 0.54	This study

*T_m Melting temperature obtained from melting curve analysis in triplicate

Table 3 *In-silico* PCR amplification results for primers verification.

No.	Staphylococci species and strains (Accession number)	Amplification results		
		Nuc	*coa*	*mecA*
1	*Staphylococcus aureus* RF122 (AJ938182.1)	+	+	-
2	*Staphylococcus aureus* strain Mu50 (BA000017.4)	+	+	+
3	*Staphylococcus aureus* subsp. *aureus* 11819-97 (CP003194.1)	+	+	+
4	*Staphylococcus aureus* subsp. *aureus* 71193 (CP003045.1)	+	+	-
5	*Staphylococcus aureus* subsp. *aureus COL* (CP000046.1)	+	+	+
6	*Staphylococcus aureus* subsp. *aureus* ECT-R 2 (FR714927.1)	+	+	-
7	*Staphylococcus aureus* subsp. *aureus* ED133 (CP001996.1)	+	+	-
8	*Staphylococcus aureus* subsp. *aureus* ED98 (CP001781.1)	+	+	-
9	*Staphylococcus aureus* subsp. *aureus* JH1 (CP000736.1)	+	+	+
10	*Staphylococcus aureus* subsp. *aureus* JH9 (CP000703.1)	+	+	+
11	*Staphylococcus aureus* subsp. *aureus* JKD6159 (CP002114.2)	+	+	+
12	*Staphylococcus aureus* subsp. *aureus* M013 (CP003166.1)	+	+	+
13	*Staphylococcus aureus* subsp. *aureus* MRSA252 (NC002952.2)	+	+	+
14	*Staphylococcus aureus* subsp. *aureus* MSHR1132 (NC016941.1)	+	+	+
15	*Staphylococcus aureus* subsp. *aureus* MSSA476 (BX571857.1)	+	+	-
16	*Staphylococcus aureus* subsp. *aureus* MW2 (NC003923.1)	+	+	+
17	*Staphylococcus aureus* subsp. *aureus* Mu3 (NC009782.1)	+	+	+
18	*Staphylococcus aureus* subsp. *aureus* N315 (NC002745.2)	+	+	+
19	*Staphylococcus aureus* subsp. *aureus* NCTC 8325 (JN571546.1)	+	+	-
20	*Staphylococcus aureus* subsp. *aureus* T0131 (CP002643.1)	+	+	+
21	*Staphylococcus aureus* subsp. *aureus* TCH60 (NC017342.1)	+	+	+
22	*Staphylococcus aureus* subsp. *aureus* USA300_FPR3757 (CP000255.1)	+	+	+
23	*Staphylococcus aureus* subsp. *aureus* USA300_TCH1516 (CP000730.1)	+	+	+
24	*Staphylococcus aureus* subsp. *aureus* VC40 (CP003033.1)	+	+	-
25	*Staphylococcus aureus* subsp. *aureus* str. JKD6008 (NC_017341.1)	+	+	+
26	*Staphylococcus aureus* subsp. *aureus* str. Newman (NC_009641.1)	+	+	-
27	*Staphylococcus carnosus* subsp. *carnosus* TM300 (AM295250.1)	-	-	-
28	*Staphylococcus epidermidis* ATCC 12228 (NC004461.1)	-	-	-
29	*Staphylococcus epidermidis* RP62A (NC004461.1)	-	-	-
30	*Staphylococcus haemolyticus* JCSC1435 (NC007168.1)	-	-	-
31	*Staphylococcus lugdunensis* HKU09-01 (NC007168.1)	-	-	-
32	*Staphylococcus lugdunensis* N920143 (NC017353.1)	-	-	-
33	*Staphylococcus pseudintermedius* ED99 (NC017568.1)	-	-	-
34	*Staphylococcus pseudintermedius* HKU10-03 (NC014925.1)	-	-	-
35	*Staphylococcus saprophyticus* subsp. *Saprophyticus* (AP008934.1)	-	-	-

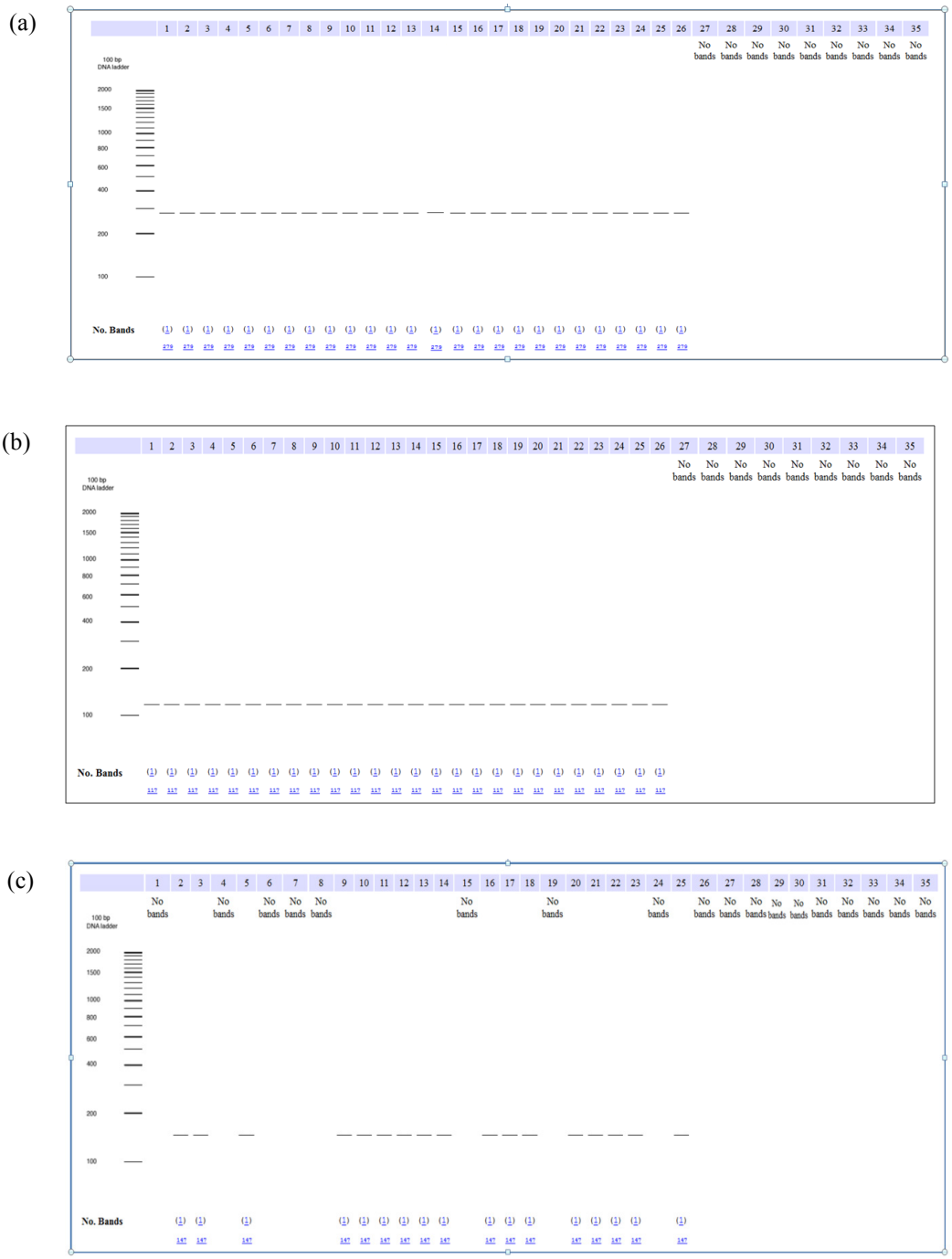

Figure 1 Electrophoresis mobility simulation results for amplification of (a) thermonuclease (*nuc*) genes against 35 selected *Staphylococci* currently available in database, (b) Staphylocoagulase (*coa*) genes against 35 selected *Staphylococci* currently available in database, (c) Methicillin-resistance (*mecA*) genes against 35 selected *Staphylococci* currently available in database.

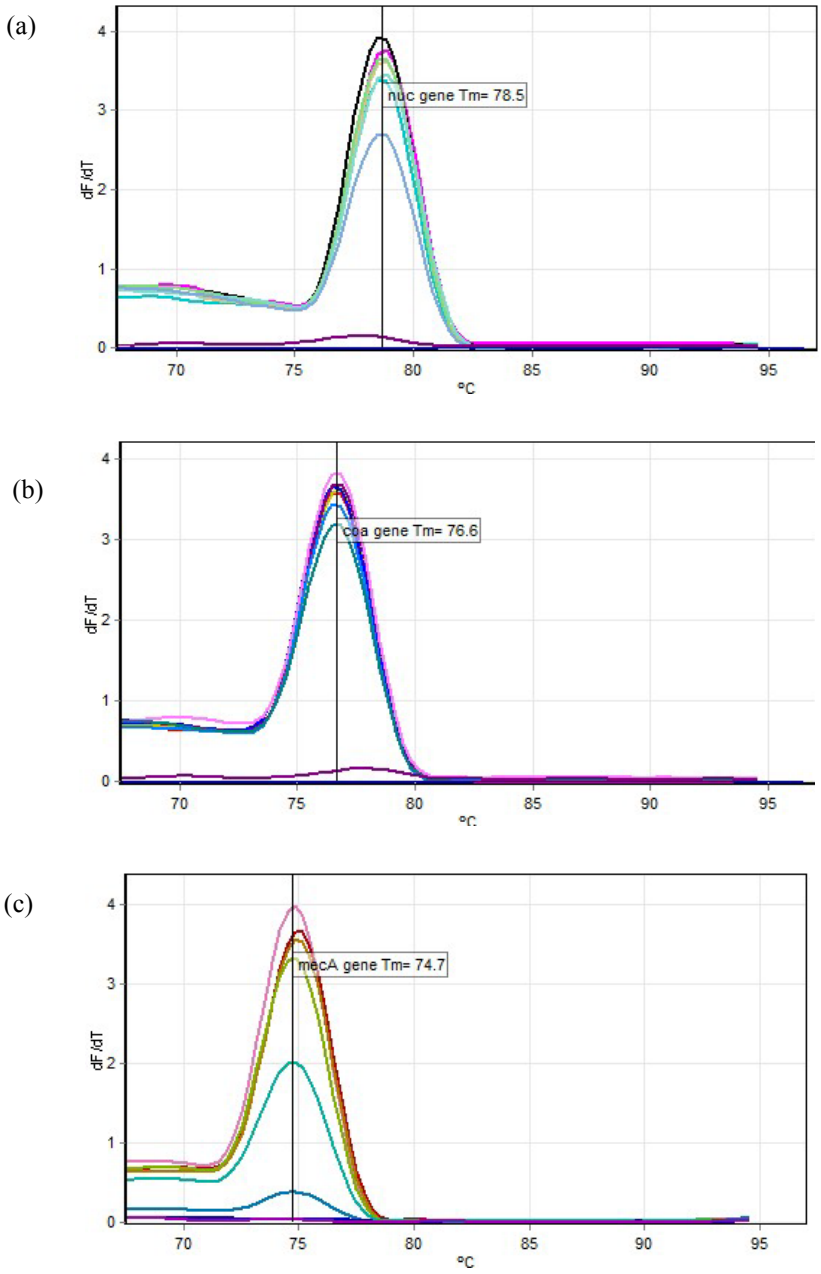

Figure 2 A representative melting curve analysis of real-time PCR assays targeting (a) the *nuc* (thermonuclease) gene from selected *S. aureus* as well as ATCC strains control. Melting peak profile at 78.5 ± 0.4 °C (b) *coa* (staphylocoagulase) gene from selected *S. aureus* as well as ATCC strains control. Melting peak profile at 76.16 ± 0.8 °C (c) *mecA* (methicillin-resistance) gene from selected *S. aureus* as well as ATCC strains control. Melting peak profile at 74.7 ± 0.6 °C.

Discussions

The aim of on-line accessible *in silico* PCR is to predict and calculate theoretical PCR amplification results by using up-to-date sequenced bacterial genomes, a technique which allows virtual amplification of specific DNA sequences [14]. After choosing the selected bacterial genome to be amplified, the program will show the length, and a simulation of the electrophoretic mobility of the amplicons. *In-silico* PCR amplification in this research also provided a systemic approach to prescreen custom-designed primers before purchasing the given templates. This will reduce the cost for purchasing unwanted primer pairs as well as decrease the time spent on trial and error of the primer selection. It also elucidates the prediction of probable PCR products and the search for potential mismatching location(s) of the specific primer(s). The output of these programs allows us to make quick and easy changes to the primer design or chosen template. Thus, all of the primers designed here can be conclusively defined as species-specific for *S. aureus* and suitable for real-time PCR SYBR Green I amplification in real laboratory sessions. The use of software in biotechnology applications has given a new dimension to the field of bioinformatics. There are many different programs for the designs of primers that are now available on-line and freely accessible. Freeware is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences [10,15]. In recent years a plethora of software programs has been developed to aid in primer design, for examples, Primer3 [16], OligoArray [17], FindGDPs [18,19], and VPCR [20]. Detection of the *mecA* gene for MRSA has been considered to be the 'gold standard' method because of its simplicity and reproducibility [12,13,21,22]. The result from the real-time PCR amplification of samples from HUKM (N391, N441, N829, N850 and N1406); UMMC (UM1, UM2, UM3, UM6 and UM7) as well as HSNZ (HN 7, HN 8, HN 9, HN 10 and HN 11) is in agreement with the oxacillin disc diffusion and duplex real-time PCR targeting both *nuc* and *mecA* genes carried out previously [12]. Although conventional phenotypic methods of detecting methicillin-resistance in staphylococci have always been claimed to be burdened with difficulties [3], no dissimilarity or inconsistency of results was observed in this study. Low correlation between phenotypic and genotypic methods in methicillin-resistance detection has been observed in coagulase-negative staphylococci [23]. Therefore, it is important to rule out coagulase-negative isolates before using any detection method for MRSA since it is one of the main causes of identification problems. Stringent isolation and identification protocol should be carried out to avoid cross contamination. The amplification of *nuc*-specific primers was in complete agreement with species determination of *S. aureus*, as described previously by Fluit *et al* [21]. The detection of both *nuc*, and *mecA* genes in an isolate would confirm the identity of the isolate as an MRSA [8].

During the last decade, many researchers have demonstrated the extremely high capacity of PCR for specifically detecting bacteria and genes of interest [8,24]. That capability has revealed PCR as a powerful tool in clinical microbiology studies [8,24]. Several authors have already shown the feasibility of the PCR methodology for the identification of *S. aureus* strains and for the detection of antibiotic resistance genes [9,13]. Usually PCR identification of *S. aureus* has been based on the detection of different species-specific target sequences such as *nuc* [13,24] and *coaA* [25] or factors essential for methicillin resistance such as *femA* or *femB* [8,9,21]. On the other hand, different studies have also shown the applicability of PCR to the detection of staphylococcal antibiotic resistance genes [8,9]. Based on our data, all MRSA clinical isolates harboured all the three genes (*coa*, *nuc* and *mecA*) and showed consistent amplified products with *in-silico* PCR verification as well as positive control ATCC 33591, which was absent in susceptible strains. This is supported by the work which used modified PCR analysis (Multiplex PCR) for detection of *mecA* and *nuc* genes in MDR (multidrug resistant) and NMDR (non-multidrug resistant) MRSA [13,24,26].

Conclusions

In summary, there was a great correlation between *in-silico* PCR verification together with actual real-time PCR detection targeting *coa*, *nuc* and *mecA* genes towards identification of methicillin-resistant *S. aureus* (MRSA). Also, all 3

primers designed to target staphylocoagulase (*coa*), nuclease (*nuc*) and methicillin resistance (*mecA*) genes gave high specificity and sensitivity for *in-silico* PCR and real-time PCR SYBR Green I assays. The increasing use of information from the World Wide Web and the sequences held in gene databases are practical starting points when validating primers and reaction conditions for the conventional and real-time PCR. Hence, it can be considered that it represents a rapid, simple, and cost-effective method, and could be systematically applied in clinical microbiology laboratories for the identification of coagulase positive MRSA, bringing insights into antibiotic therapy design and helping treatment to be initiated without delay.

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