Journal of Microbiology, Immunology and Infection (2014) 47, 484–490

Available online at www.sciencedirect.com

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ORIGINAL ARTICLE

Rapid detection of methicillin-resistant *Staphylococcus aureus* by a newly developed dry reagent-based polymerase chain reaction assay

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Received 22 February 2013; received in revised form 28 May 2013; accepted 20 June 2013
Available online 6 August 2013

KEYWORDS
Methicillin-resistant *Staphylococcus aureus*;
Nasal carriers;
Nosocomial infection;
Thermostabilized polymerase chain reaction

Background/Purpose: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major pathogen responsible for significant numbers of nosocomial and community-acquired infections worldwide. Molecular diagnosis for MRSA nasal carriers is increasingly important for rapid detection and screening of MRSA colonization because the conventional methods are time consuming and labor intensive. However, conventional polymerase chain reaction (PCR) tests still require cold-chain storage as well as trained personnel, which makes them unsuitable for rapid high-throughput analysis. The aim of this study was to develop a thermostabilized PCR assay for MRSA in a ready-to-use form that requires no cold chain.
Methods: The thermostabilized PCR assay detects the following targets simultaneously: (1) 16S rRNA of the *Staphylococcus* genus; (2) *fem*A gene specific for *S. aureus*; (3) *mec*A gene conferring methicillin resistance; and (4) *luk*S gene, which encodes the virulent toxin. The thermostabilized PCR incorporates an internal amplification control that helps to verify the presence of PCR inhibitors in samples. PCR reagents and specific primers were lyophilized into a pellet form with an enzyme stabilizer.

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http://dx.doi.org/10.1016/j.jmii.2013.06.004

Results: The PCR was validated with 235 nasal swabs specimens and was found to be 100% sensitive and specific. The stability of the thermostabilized PCR was evaluated using the Q_{10} method and it was found to be stable for approximately 6 months at 24°C. The limit of detection of thermostabilized PCR assay was determined by probit regression (95% confidence interval) was 10^6 colony forming units at the bacterial cell level and 10 ng of DNA at the genomic DNA level, which is comparable with conventional PCR methods.
Conclusion: A rapid thermostabilized PCR assay that requires minimal pipetting steps and is cold chain-free was developed for detecting MRSA nasal carriers.

Introduction

Staphylococcus aureus is a major pathogen responsible for nosocomial and community-acquired infections. Methicillin resistance in *S. aureus* (MRSA) is mediated primarily by the *mec*A gene, which encodes the modified penicillin-binding protein 2a (PBP 2a).[1] MRSA incidence varies between countries; in Malaysia, the MRSA infection rate was 10.0/1000 hospital admissions and the incidence density of MRSA infection was 1.8/1000 patient days.[2] The major reservoirs of MRSA are colonized or infected patients and, occasionally, hospital personnel.[3] These data support the view that early detection and prevention of colonization of intensive care unit (ICU) patients with MRSA could reduce the frequency of MRSA infection and assist in the design of effective prevention strategies against MRSA infection.[4] Rapid detection and isolation of patients colonized with MRSA and rapid implementation of contact precautions should result in a reduction of nosocomial MRSA transmission.[5] Guidelines for the identification of MRSA recommend culture.[6] Chromogenic media yield reliable negative results at 20 hours,[7] but isolation, sensitivity testing and the confirmation of MRSA takes 48—72 hours. Diagnosis of MRSA within a few hours by the use of molecular approaches could lead to the early implementation of appropriate antibiotics and reduced hospital bed stays. As molecular methods become commonplace for MRSA, screening of all patients admitted to ICU wards becomes a viable option.

Molecular methods, particularly polymerase chain reaction (PCR), have become more important in the diagnosis of MRSA and have greatly improved the speed, sensitivity, and specificity of diagnostic tests, which in turn facilitates early detection of MRSA patients and helps in infection control and prevention.[8] PCR methods for detection of the *mec*A gene, the Panton—Valentine leukocidin (PVL)-encoding gene *luk*S and the species-specific gene *fem*A have required the use of separate assays.[8,9] Multiplexed PCR techniques to identify the methicillin-resistance gene *mec*A, the *S. aureus*-specific gene *fem*A, and the PVL virulence gene simultaneously have been developed by our research group and others.[10—13] A PCR test was designed on the basis of the distribution of virulence determinants *seh*, *etd*, and *arc*A within the *S. aureus* population.[14] but these genetic determinants are most likely to be specific for community-acquired MRSA clones. However, another PCR test was developed to discriminate *S. aureus* from coagulase-negative staphylococci and to determine methicillin resistance from blood cultures[15]; this assay is used for blood culture only and requires further incubation of clinical samples on blood culture broths. Conventional PCR assays require multiple pipetting steps, skilled personnel for master mix preparation, and cold-chain transportation and storage. Handling of PCR reagents requires dealing with microvolumes, where processing variation can detrimentally affect a test result. Furthermore, PCR error is compounded by the multiple pipetting steps of small volumes of reagents, which may lead to errors. These factors adversely influence the use of PCR as a screening and diagnostic tool of choice for MRSA, even though PCR provides higher levels of sensitivity and specificity. In this study, a thermostabilized PCR assay that is cold chain free and requires only the addition of DNA samples and PCR-grade (DNase-free) water was developed for the detection of MRSA. A PCR assay with specific primers was designed for detection of the MRSA gene *mec*A[10]; the 16S rRNA gene of the *Staphylococcus* genus, the *S. aureus*-specific gene *fem*A, and the virulence gene *luk*S uses an internal amplification control (IC) for monitoring PCR inhibitors. The whole PCR mix containing the specific primers was thermostabilized into a dried pellet, using a technique similar to that described earlier.[16,17] The ready-to-use dried reagent mix was analyzed to determine the limit of detection (LoD) and evaluated with clinical specimens.

Materials and methods

Bacterial strains

The reference strains used in this study (Table 1) were obtained from: the Belgian Coordinated Collections of Microorganisms, Ghent, Belgium; the Institute for Medical Research, Malaysia; and the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia.

Clinical samples

The study was approved by the Research and Ethics Committee of the School of Medical Sciences, Hospital Universiti Sains Malaysia (HUSM), in accordance with the Helsinki Declaration. Nasal swabs (235) were collected from inpatients in all of the wards in the HUSM from March to August 2008. Informed consent was obtained from all participants before specimen collection. Samples were

Table 1 Bacterial species and strains used in this study and results of PCR assay

No. Reference strains	16S rRNA[a]	*mec*A[b]	*luk*S	*fem*A	Internal control
***Staphylococcus* spp. strains (*n* = 7)**					
1. *S. aureus* (ATCC 33591)	+	+	−	+	+
2. *S. aureus* (ATCC 43300)	+	+	−	+	+
3. *S. aureus* (ATCC 25923)[d]	+	−	+	+	+
4. *S. aureus* (ATCC 49775)	+	−	+	+	+
5. *S. aureus* (ATCC 51153)[e]	+	−	−	+	+
6. *S. epidermidis* (ATCC 14990)	+	−	−	−	+
7. CoNS methicillin-resistant[e]	+	+	−	−	+
Gram-positive bacteria (*n* = 8)					
1. *Streptococcus* spp. Group A (ATCC 19615)[e]	−	−	−	−	+
2. *Streptococcus* spp. Group B (ATCC 12401)[e]	−	−	−	−	+
3. *Streptococcus* spp. Group G[e]	−	−	−	−	+
4. *Bacillus subtilis* (ATCC 6633)[e]	−	−	−	−	+
5. *Listeria monocytogenes* (ATCC 7644)[e]	−	−	−	−	+
6. *Enterococcus faecium* LMG 16192[c]	−	−	−	−	+
7. *Enterococcus faecalis* (ATCC 29212)[e]	−	−	−	−	+
8. *Corynebacterium* spp.[e]	−	−	−	−	+
Gram-negative enteric pathogens (*n* = 15)					
1. *Escherichia coli* (EHEC)[e]	−	−	−	−	+
2. *E. coli* (EPEC)[e]	−	−	−	−	+
3. *E. coli* (ETEC)[e]	−	−	−	−	+
4. *Klebsiella pneumoniae* (ATCC 10031)[e]	−	−	−	−	+
5. *Shigella sonnei* (ATCC 25931)[e]	−	−	−	−	+
6. *Shigella flexneri* (ATCC 12022)[e]	−	−	−	−	+
7. *Shigella boydii* (ATCC 9207)[e]	−	−	−	−	+
8. *Proteus mirabilis* (ATCC 29245)[e]	−	−	−	−	+
9. *Salmonella typhi*[e]	−	−	−	−	+
10. *Pseudomonas aeruginosa* (ATCC 27853)[e]	−	−	−	−	+
11. *Yersinia enterocolitica* (ATCC 23715)[e]	−	−	−	−	+
12. *Vibrio cholerae* (O1 classical)[e]	−	−	−	−	+
13. *Citrobacter freundii* (ATCC 8090)[e]	−	−	−	−	+
14. *Gardnerella* spp.[e]	−	−	−	−	+
15. *Candida albicans* (ATCC 10231)[e]	−	−	−	−	+

[a] *Staphylococcus* genus.
[b] Methicillin-resistant genotype.
[c] Reference strains from Belgian Co-ordinated Collections of Micro-organisms (BCCM), Ghent, Belgium.
[d] Obtained from Institute for Medical Research, Malaysia.
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Table 2 Sequences of primers used for the PCR assay

Primers	Primer sequence (5′—3′)	Gene target	Gen Bank accession number	Product size
16S rRNA-F	GCA AGC GTT ATC CGG AAT T	**16S rRNA**	D83356	597 bp
16S rRNA-R	CTT AAT GAT GGC AAC TAA GC			
*mec*A-F	ACG AGT AGA TGC TCA ATA TAA	***mec*A**	NC_003923M	293 bp
*mec*A-R	CTT AGT TCT TTA GCG ATT GC			
lukS -F	CAG GAG GTA ATG GTT CAT TT	***luk*S**	AB186917	151 bp
lukS -R	ATG TCC AGA CAT TTT ACC TAA			
*fem*A-F	CGA TCC ATA TTT ACC ATA TCA	***fem*A**	CP000255	450 bp
*fem*A-R	ATC ACG CTC TTC GTT TAG TT			
IC-F	AGC GTC CAT TGT GAG A	***hem*M**	AF227752	759 bp
IC-R	ATT CTC AGA TAT GTG TGG			

analyzed simultaneously by molecular and conventional culture-based techniques.

Thermostabilization of PCR reagents

Staphylococcus genus 16S rRNA, *mec*A, *fem*A, and *luk*S of *S. aureus* and PCR internal control specific primers were used based on previous study (Table 2).[10]

Each PCR master reagent mix contained 2 pmol of each primer for the 16S rRNA gene, 10 pmol of each primer for the *mec*A gene, 10 pmol of each primer for the *fem*A gene, 6 pmol of each primer for the *luk*S gene, 8 pmol of each primer for the IC in PCR buffer (Promega, Madison U.S.A), 3.125 mM $MgCl_2$ (Promega, Madison U.S.A), 400 mM each dNTP (Fermentas, Vilnius, Lithuania), 1.875 U of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) and 4.6 pg of IC DNA template.

For the positive control, 40 ng of genomic DNA of both MRSA (ATCC 43300) and methicillin-sensitive *S. aureus* (ATCC 25923) reference strains were added to the mixture before lyophilization, whereas the negative control received only PCR-grade (DNase-free) water (GIBCO) in place of the DNA. Trehalose as an enzyme stabilizer was added to the PCR reagent mix and the final mixture was dried for 2 hours at a reduced pressure of 4 Pa in a vacuum concentrator (Thermo Scientific Heto) connected to a freeze-dryer (LyoLab 3000 Thermo Scientific Heto).[16,17] After that, the PCR tubes were stored in a sealed aluminum pouch.

PCR protocol and clinical evaluation

PCR was performed by addition of 18 μL of PCR-grade (DNase-free) water and 2 μL of DNA template to a tube containing thermostabilized PCR reagents. The mixture was vortex-mixed briefly to dissolve the dried pellet. Each of the positive and negative control tubes received 20 μL of PCR-grade (DNase-free) water. The positive control was included to ensure the integrity of the PCR process and the negative control was included to check for the presence of contaminating DNA. The PCR was done with a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with one cycle of initial denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds at 60°C, extension at 72°C for 30 seconds, followed by an extra cycle of annealing at 60°C for 30 seconds and a final extension at 72°C for 5 minutes. The amplification products were resolved by 2.0% (w/v) agarose gel electrophoresis, stained with ethidium bromide (Sigma), visualized under UV light and photographed (ChemiImager 5500, Alpha Innotech).

A nasal swab moistened with sterile saline was inserted about 2.54 cm into the nares and rolled five times in each. Collected specimens were inoculated into a single tube of selective broth (3 mL) consisting of mannitol salt broth (HiMedia, Mumbai, India) supplemented with Cefoxitin (Sigma) at 6 μg/mL (MSC-6). Cultures were incubated at 37°C with shaking (~200 rpm) for about 4 hours.

Afterwards, 1 mL of the culture was used for conventional identification and bacterial lysates for PCR were prepared by centrifugation of a 2 mL culture at 10,000*g* for 3 minutes; the supernatant was removed and the pellet resuspended in 50 μL of DNase-free distilled water, kept in a boiling waterbath for 10 minutes then centrifuged at 10,000*g* for 3 minutes. The resultant supernatant was used as a template for the PCR assay.

The diagnostic evaluation of the thermostabilized PCR was done with 235 nasal swabs. The results were compared to the conventional microbiological, biochemical, and antimicrobial susceptibility E-test, which is considered to be the gold standard.[18] The sensitivity, specificity, positive predictive value and negative predictive value were calculated on the basis of the CLSI Guidelines.[19]

Determination of stability and analytical sensitivity of the thermostabilized PCR mix

The stability of the thermostabilized PCR assay was estimated using accelerated aging techniques with elevated temperatures, known also as the Q_{10} method. This technique is based on brief storage of a medical device at elevated temperatures and correlating temperature with time.[20] The thermostabilized PCR tubes were kept at 37°C for up to 60 days and tested at intervals. The longest duration that the thermostabilized PCR maintained its activity was calculated according to this formula provided by Clark[20]:

Age of the thermostabilized PCR tubes = 2 months at 37°C
Ambient temperature = 24°C
$Q_{10} = 1.8$
Acceleration factor (based on 13°C temperature difference): $(1.8)^{1.3} = 2.14$
Length of time at elevated temperature = 2 months
Estimation of shelf life:
Accelerated age = age × acceleration factor
2 months × 2.14 = 4.28 months
Shelf life = accelerated age + actual age
4.28 + 2 = 6.28 months

A series of six samples was prepared by diluting a high concentration standard. Each sample was tested 10 times. LoD of the thermostabilized PCR at the bacterial level was determined using 2 μL of template from lysates of serially diluted bacterial suspensions ranging from 10^8 colony-forming units (CFU)/mL to 10^3 CFU/mL, whereas LoD at the genomic DNA level was determined using various amounts of extracted genomic DNA ranging from 100 ng to 10 pg per PCR.

Statistical methods

The relationship between the visible positive bands on the agarose gel and the corresponding log concentrations of each bacterial suspension CFU was examined using probit regression analysis (IBM SPSS for Windows 19.0; SPSS, Inc., Chicago, IL, USA).

Results and discussion

The thermostabilized PCR was standardized with known MRSA (ATCC 33591) and methicillin-sensitive *S. aureus*

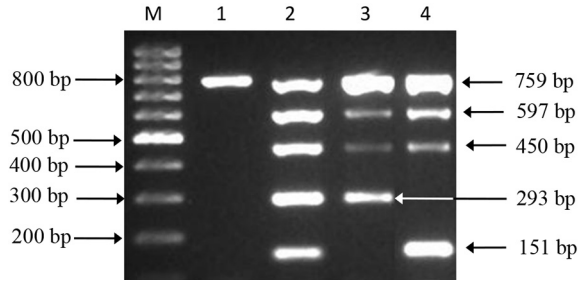

Figure 1. Representative agarose gel electrophoresis of thermostabilized polymerase chain reaction assay using known strains. Lanes: M, 100 bp DNA ladder; 1, valid negative control; 2, positive control; 3, methicillin-resistant *Staphylococcus aureus* (ATCC 33591); 4, methicillin-sensitive *S. aureus* (ATCC 25923).

(ATCC 25923). The PCR test is positive for MRSA when all four amplicons—293 bp, 450 bp, 597 bp, and 759 bp—are detected, which indicates the presence of the target *mec*A, *fem*A and 16S rRNA genes of MRSA and the IC for PCR, respectively. Methicillin-sensitive *S. aureus* is detected when only amplicons of 450 bp (*fem*A gene), 597 bp (16S rRNA gene), and 759 bp (IC) are present, without the presence of a 293 bp amplicon of the *mec*A gene. The presence of the 151 bp *luk*S gene among MRSA and methicillin-sensitive *S. aureus* indicates the strain is a PVL toxin producer.

The PCR is considered a valid negative when only the PCR IC amplicon is observed (Fig. 1). If all five amplicons are absent, the PCR is defined as inhibited and the test should be repeated.

The thermostabilized PCR assay was evaluated with 235 nasal swabs and the diagnostic performance was determined.

Among the 235 nasal swabs, 166 were CoNS and the other 69 were *S. aureus*, of which 15 were resistant to methicillin. MSC-6 broth was used as a selective enrichment to differentiate between different isolates, all 54 methicillin-sensitive *S. aureus* and 166 methicillin-sensitive CoNS revealed no turbidity or change in color indicator of the MSC-6 broth. However, all MRSA showed turbidity, ferment mannitol, and turn the MSC-6 broth into a yellow color after 4 hours' incubation.

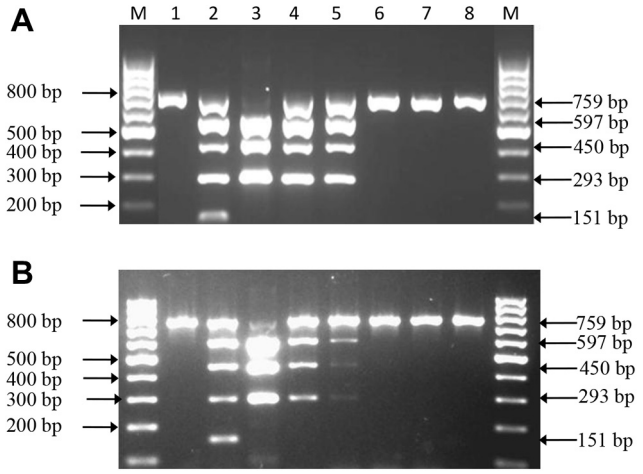

Figure 3. Comparison of (A) the analytical sensitivity of the conventional wet polymerase chain reaction (PCR) and (B) thermostabilized PCR mix. Various amount of methicillin-resistant *Staphylococcus aureus* cells were used, ranging from 10^8 to 10^3 CFU. Lanes: M, 100 bp DNA ladder; 1, negative control; 2, PCR positive control; 3, 10^8 CFU; 4, 10^7 CFU; 5, 10^6 CFU; 6, 10^5 CFU; 7, 10^4 CFU; and 8, 10^3 CFU.

The PCR results for all specimens were comparable with conventional results. A representative agarose gel showing the results of the thermostabilized PCR using nasal swab specimens is shown in Fig. 2. The thermostabilized PCR positively identified all MRSA strains, where genes *mec*A, *fem*A, 16S rRNA, and the IC were all amplified, and all methicillin-sensitive *S. aureus* strains, where only genes *fem*A, 16S rRNA, and the IC were amplified; whereas other staphylococcal species have amplified only the 16S rRNA gene and the IC. Moreover, all other nonstaphylococcal species tested showed negative results where only IC amplicons were detected. The overall analysis of the thermostabilized PCR showed 100% sensitivity, specificity, positive predictive value, and negative predictive value. The high positive predictive value (100%) suggested great reliability and accuracy of the test, and the 100% negative predictive value showed perfect discrimination of methicillin-sensitive *S. aureus* strains. The thermostabilized PCR was evaluated for stability using the Q_{10} method.[20] and was found to be stable for 6.28 months at an ambient temperature of 24°C when mathematically

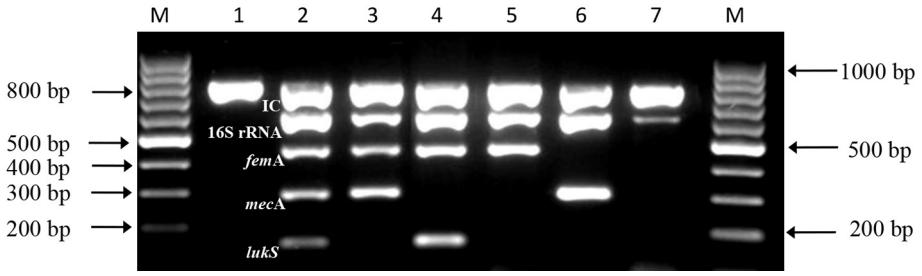

Figure 2. Representative agarose gel electrophoresis of thermostabilized polymerase chain reaction assay profile for nasal swab specimens. Lanes: M, 100 bp DNA ladder; 1, negative control; 2, staphylococcal positive control; 3, methicillin-resistant *Staphylococcus aureus*; 4, methicillin-sensitive *S. aureus* and PVL producer; 5, methicillin-sensitive *S. aureus*; 6, methicillin-resistance of non-*S. aureus*; 7, methicillin-sensitive non-*S. aureus*.

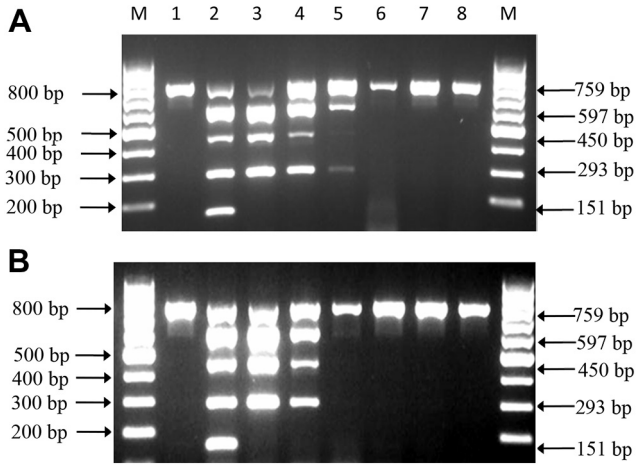

Figure 4. Comparison of (A) the analytical sensitivity of the conventional wet polymerase chain reaction (PCR) and (B) the thermostabilized PCR mix. Various amount of methicillin-resistant *Staphylococcus aureus* genomic DNA template were used, ranging from 100 ng to 1 pg. Lanes: M, 100 bp DNA ladder; 1, PCR negative control; 2, PCR positive control; 3, 100 ng; 4, 10 ng; 5, 1 ng; 6, 100 pg; 7, 10 pg; and 8, 1 pg.

correlated with its stability at 37°C (data not shown). Thus this thermostabilized PCR mixture could be transported and stored without a cold chain, a useful attribute for distributing this MRSA screening test to many regions without fear of degradation of reagents or decreased reliability of the test. This strategy has been used similarly for mycobacteria and *Salmonella typhimurium*[16,17]; however, the thermostabilization procedure was modified and optimized to suit the PCR in this study because the PCR parameters and compositions were different from those in earlier studies. Probit regression analysis gave a probit value of 6.066, which converts to a LD95 value (concentration detectable 95% of the PCR samples) of 10^6 (log10 LD95 = 6), indicating that the LoD is about 10^6 CFU and those samples containing that concentration would be detected 95% of the time. The recorded LoD at the bacterial cell level and the genomic DNA level were comparable with the conventional wet form (Figs. 3 and 4). However, for the thermostabilized PCR assay, there was a one log lower sensitivity than that for the wet PCR assay for DNA levels.

The simplicity and ease-of-use of the thermostabilized PCR would outweigh the decreased sensitivity compared to that of the more expensive real-time PCR. The LoD of the latter was reported at 610 CFU/mL, which is equivalent to a 58 CFU/swab.[21]

The thermostabilized PCR includes a new conserved *Staphylococcus* genus-specific primer, which allowed us to detect most species and strains of staphylococci, and incorporation of both *mec*A and *fem*A gene-specific primers in the thermostabilized PCR assay that recognizes only MRSA[10] has shown potential as a reliable test to identify all MRSA strains. Furthermore, the thermostabilized PCR developed here detects the *luk*S virulence gene. The *luk*S gene partly encodes PVL toxin, which is the main virulence factor responsible for the manifestation of severe pneumonia and skin, soft tissue, bone, and joint infections, and is closely associated with community-acquired MRSA strains.[22–24] Detection of PVL toxin has been described[13,14] and has proved useful for identifying strains of MRSA often associated with epidemics. Thus, combined detection of the 16S rRNA, *mec*A, *fem*A, and *luk*S genes would enable efficient screening and detection of a PVL-producer MRSA nasal carrier in community and among patients newly admitted to ICUs. Rapid detection of MRSA-colonized patients has the potential to improve patient care and positively affect hospital infection control practices. Conventional methods, such as culture and biochemical tests for detecting MRSA nasal carriers, are time-consuming; as a result, molecular diagnosis is increasingly important for rapid detection. However, to allow PCR tests to be more accessible to remote or resource-limited areas in which MRSA nasal carriers are more prevalent, we have developed a cold chain-free, ready-to-use PCR kit for screening and detecting MRSA nasal carriers.

In conclusion, unlike traditional PCR, the thermostabilized PCR developed requires only two pipetting steps, which helps reduce cross-contamination caused by multiple pipetting steps. The thermostabilized PCR for MRSA incorporates an IC to detect inhibitors, which could cause false-negative results. Therefore, the thermostabilized PCR is suitable for detection of nasal colonization with MRSA with same-day results, allowing more efficient and effective use of infection control resources to control MRSA in healthcare facilities.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by a short-term grant (304/PPSP/6131535) from Universiti Sains Malaysia, Malaysia. We thank the following for providing the bacterial strains used in this study: the Belgian Coordinated Collections of Microorganisms, Ghent, Belgium; the Institute for Medical Research, Malaysia and the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia.

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