Development of New Pentaplex PCR Assay for Differentiating Staphylococci from Other Bacteria with Simultaneous Detection of *Staphylococcus aureus* Genes Encoding Panton-Valentine Leukocidin and Methicillin Resistance

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Authors' contributions

This work was carried out in collaboration between both authors. Author CEO designed the study, performed the wet experiments and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author RJ managed the analyses of the study. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

In some human infections including those of blood, skin and respiratory tract, the causative bacterial agents tend to overlap, especially *Staphylococcus* spp. and *Streptococcus* spp. Such overlaps constitute difficulties in the choice of diagnostic tools, antibacterial chemotherapy and infection control strategies. To resolve this challenge, we developed a pentaplex PCR assay which simultaneously detects sequences for the recognition of bacteria (bacterial 16S rRNA), the genus staphylococcus translation elongation factor Tu (*tuf*), *Staphylococcus aureus* (*spa*), *mecA*-encoded

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staphylococcal methicillin resistance (*mecA*) and the *S. aureus* Panton-Valentine leukocidin (PVL) virulence factor (*pvl*). The new pentaplex PCR assay was validated using standard bacterial strains (N=377) including strains from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA), the National Collection of Type Cultures (NCTC), and the National Collection of Industrial and Marine Bacteria (NCIMB). The new pentaplex PCR assay enables inference of bacterial presence/absence, differentiates between the genus *Staphylococcus* spp. and other bacteria, separates *S. aureus* from other *Staphylococcus* spp., differentiates between methicillin-susceptible and methicillin-resistant staphylococci, and detects the *S. aureus* PVL gene locus. The negative predictive value was 100% while the positive predictive value was 100%. Using a 96-well plate, the time to result was 2.5 hours against ≥24 hours by bacteriological culture. The new pentaplex PCR assay can easily be integrated into routine diagnostic microbiology workflow especially for laboratories with slim budgets which are unable to incorporate next generation sequencing at the moment.

1. INTRODUCTION

Recent studies have illuminated the abundance and diversity of bacteria in/on different parts of the human body [1,2]. As more microbiomic data appear, differential diagnostic tools will be necessary to identify particular genetic factors associated with diseases and their outcomes [3]. The first strains of methicillin resistant *Staphylococcus aureus* (MRSA) were reported in the United Kingdom hospitals about the same time as the introduction of the antibiotic methicillin [4,5]. Following the UK reports, MRSA clones with diverse genetic backgrounds and virulence factors have been reported worldwide [6]. Following the establishment of the centrality of *mecA* gene in the mechanism of staphylococcal methicillin resistance [7], polymerase chain reaction (PCR) detection of *mecA* DNA gained wide acceptance [8].

In 2004, it was observed that some MRSA strains were not detected by *mecA* PCR, rather, five primers specific to different staphylococcal cassette chromosome mec (SCC*mec*) right extremity sequences, including three new sequences and specific to the *S. aureus* chromosomal *orfX*, a gene of unknown function, located to the right of the SCC*mec* integration site, were the most reliable for the identification of some MRSA strains [9]. The *orfX* information informed the development of several commercial MRSA assays including Xpert MRSA assay (Cepheid, California, USA) and BD-GO (Becton-Dickinson, USA). However, neither *orfX* nor the recently reported hybrids and analogues [10,11] reduced the centrality of *mecA* in staphylococcal methicillin resistance.

Whereas *mecA* gene driving methicillin resistance is shared among *S. aureus* and other staphylococci [6,7], Panton-Valentine leukocidin (PVL) encoded by *lukSF-PV* operon and staphylococcal protein A (Spa) encoded by the *spa* gene, both of which are associated with skin and airways diseases, are unique to *S. aureus* [12,13]. The staphylococci and other bacterial species associated with human infections all share the *tuf* gene encoding translation elongation factor Tu as well as the 16S rRNA gene [14].

Independently, *S. aureus* strains expressing Spa, PVL, or PBP2a are sources of concern to infection control, laboratory diagnosis and anti-staphylococcal therapy [15]; their convergence highlights a higher threat to public health microbiology [16]. It has been suggested that the application of multiplex PCR for the detection of multiple genetic markers within the same sample will provide a major contribution to the efficiency, logistics and cost-effectiveness of molecular diagnostics [17]. In this regard, some previously developed MRSA and PVL assays have been revisited recently. As Pichon and colleagues envisaged that *S. aureus* harbouring *mecA*$_{(LGA251)}$, a recently identified livestock variant of *mecA*, could be wrongly identified as methicillin-susceptible *S. aureus* (MSSA) in the absence of antimicrobial susceptibility testing (AST), they modified a triplex real-time PCR assay published in 2005 by McDonald and colleagues [18] into a quadruplex assay [19].

There are several reports of simultaneous sequencing and clustering of large numbers of *16S rRNA* genes enabled by next generation sequencing (NGS) [20]. However, multiplex detection of virulence and antibiotic resistance

genes is not common at the moment. NGS machines and analysis of the associated metagenomic sequence data are currently possible only by high-budget laboratories [21]. PCR-based MRSA and PVL assays do not distinguish between staphylococci and other bacterial agents of skin and airways disease. To provide this necessary advancement, we studied a triplex assay for simultaneous detection of *S. aureus* spa*, mecA* and *lukSF-PV* [22] and added two more DNA targets, namely: Bacterial 16S rRNA and staphylococcal *tuf,* thus arriving at a new pentaplex PCR. Sequencing and phenotypic tests were used to validate the new assay according to current practices.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Microbiological Media Used for This Study

All microbiological media and consumables, except otherwise mentioned, were obtained from Oxoid (Oxoid, Basingstoke, UK). Bacterial strains were obtained from ultra-cold (-80°C) storage, sub-cultured in brain heart infusion (BHI) broth and plated out on BHI plates and sheep blood agar (Oxoid, UK) as reported previously [23]. A total of 377 bacterial strains were used for evaluation through four panels (2.2.1 – 2.2.4).

Type culture staphylococcal strains (n=50) listed in Table 1. They were obtained from the Network for Antibiotic Resistance in *Staphylococcus aureus* (NARSA) Strain Repository (www.narsa.net), the National Collection of Type Cultures (NCTC) and the National Centre for Industrial and Marine Bacteria (NCIMB) and used to validate the 5 key markers targeted by the new assay. Necessary genomic information for the reference type cultures were obtained from the homepages of the suppliers.

Randomly selected (n=240) from reference clinical staphylococcal strains (RSS) previously characterized at the Queen's Medical Centre (QMC) Nottingham between August 2003 and December 2004. RSS data from QMC identified them as *S. epidermidis* (n=72) of which 45 were methicillin resistant (MRSE), and *S. aureus* (n=168) including 92 MRSA. They were previously identified phenotypically by coagulase as *S. aureus* and by latex agglutination for MRSA, but were not tested for PVL at the time. The staphylococci (2.1.1 and 2.1.2) were

collected and stored by Dr. Richard Spence who used them for validation of DNA microarrays [23].

Table 1. Staphylococcal type culture strains and their sources

Serial no.	Isolate	Species or group	Source[a]
1	NRS1	*S. aureus*	NARSA[b]
2	NRS102	*S. aureus*	NARSA
3	NRS103	*S. aureus*	NARSA
4	NRS110	*S. aureus*	NARSA
5	NRS111	*S. aureus*	NARSA
6	NRS112	*S. aureus*	NARSA
7	NRS113	*S. aureus*	NARSA
8	NRS114	*S. aureus*	NARSA
9	NRS123	*S. aureus*	NARSA
10	NRS13	*S. aureus*	NARSA
11	NRS147	*S. aureus*	NARSA
12	NRS149	*S. aureus*	NARSA
13	NRS153	*S. aureus*	NARSA
14	NRS157	*S. aureus*	NARSA
15	NRS158	*S. aureus*	NARSA
16	NRS162	*S. aureus*	NARSA
17	NRS164	*S. aureus*	NARSA
18	NRS165	*S. aureus*	NARSA
19	NRS167	*S. aureus*	NARSA
20	NRS170	*S. aureus*	NARSA
21	NRS171	*S. aureus*	NARSA
22	NRS172	*S. aureus*	NARSA
23	NRS176	*S. aureus*	NARSA
24	NRS179	*S. aureus*	NARSA
25	NRS182	*S. aureus*	NARSA
26	NRS185	*S. aureus*	NARSA
27	NRS188	*S. aureus*	NARSA
28	NRS191	*S. aureus*	NARSA
29	NRS192	*S. aureus*	NARSA
30	NRS194	*S. aureus*	NARSA
31	NRS227	*S. aureus*	NARSA
32	NRS229	*S. aureus*	NARSA
33	NRS231	*S. aureus*	NARSA
34	NRS233	*S. aureus*	NARSA
35	NRS244	*S. aureus*	NARSA
36	NRS248	*S. aureus*	NARSA
37	NRS249	*S. aureus*	NARSA
38	NRS255	*S. aureus*	NARSA
39	NRS260	*S. aureus*	NARSA
40	NRS265	*S. aureus*	NARSA
41	NRS70	*S. aureus*	NARSA
42	NRS71	*S. aureus*	NARSA
43	NRS72	*S. aureus*	NARSA
44	NRS8	*S. epidermidis*	NARSA
45	NRS9	*S. haemolyticus*	NARSA
46	NRS69	*S. haemolyticus*	NARSA
47	NCTC12217	*S. lugdunensis*	NCTC[c]
48	NCTC11042	*S. haemolyticus*	NCTC
49	NCIMB9993	*S. epidermidis*	NCIMB[d]
50	NCIMB700787	*S. capitis*	NCIMB

aGenomic information available at suppliers' website, bNARSA = Network for Antibiotic resistance in Staphylococcus aureus, cNCTC = National Collection of Type Cultures, d NCIMB = National Collection of Industrial and Marine Bacteria

Additional CoNS (n=40) donated by researchers at the Nottingham University's Centre for Biomolecular Sciences (CBS) who previously identified them as *S. auricularis* (3 strains)*, S capitis* (2 strains)*, S. caprae* (2 strains)*, S. chromogenes* (1 strain)*, S. cohnii* (2 strains)*, S. epidermidis* (2 strains), *S. hemolyticus* (4 strains)*, S. hominis* (4 strains), *S. intermedius* (2 strains)*, S. lugdunensis* (1 strain), *S. saprophyticus* (3 strains), *S. sciuri* (4 strains), *and S. warneri* (1 strain)*.*

Non-staphylococcal bacterial strains (n=47) previously characterized by CBS microbiology research groups including some strains of *Escherichia coli, Pseudomonas* spp., *Klebsiella* spp., *Aeromonas* spp., *Salmonella* spp., *Citrobacter* spp., *Proteus* spp., and Group A streptococci (GAS).

2.2 Bio Safety

Safety is emphasised in our laboratory. Given that most NARSA isolates are potentially hazardous, work on them were carried out in enhanced level 2 (BSL2+) suite designated for such pathogens. Local bacterial strains were handled completely in the general (BSL 2) laboratory space where they have been circulating prior to this study.

2.3 Preparation of Bacterial Inoculum for PCR and Phenotypic Tests

For each bacterial strain used in this study, a 0.5 McFarland standardised inoculum was prepared and used for PCR and for phenotypic tests.

2.4 Extraction of Bacterial DNA for PCR

Extraction of DNA from NARSA isolates grown on BHI plates was performed by heating and centrifugation as reported previously [24]. Briefly, 0.5 mL live bacterial suspension was killed (95°C, 10 minutes). Following a quick centrifugation (13000 RPM, 20 seconds), the DNA-rich supernatant was transferred into a fresh 0.5 mL Eppendorf tube for PCR in the general (BSL 2) laboratory.

DNA extraction was waived for local strains. Isolates were applied directly into the PCR from BHI broths, blood agar plates, BHI plates and from 0.5 McFarland broths.

2.5 Design of Oligonucleotide Primers

The sequences of the oligonucleotide primers used in the new pentaplex PCR assay are presented in Table 2. The primers targeting *spa, pvl* and *mecA* were published originally for a triplex real-time PCR assay [22]. The primers targeting 16S rRNA and *tuf* were designed for this study. Numerous nucleotide sequences for each marker were sourced from the National Centre for Biotechnology Information (NCBI) and entered into clusta lW suite at the European Bioinformatics Institute (www.ebi.ac.uk/tools/clustalW) for multiple alignments which allowed identification of highly conserved motifs (HCMs) for hybridization of primers targeting bacterial 16S rRNA and staphylococcal *tuf* genes. Primers were located manually according to Mount [25]. All primers were purchased from Sigma Genosys (Sigma, UK).

2.6 Performance and Optimization of New Pentaplex PCR

PCR amplification was performed incorporating 5.0 µL of the template in a 40 µL PCR using Eppendorf mastercycler (Eppendorf, Hamburg, Germany) following the cycling conditions described by Nakagawa et al. [22] with modifications. Briefly, an initial single cycle for 5 minutes at 94°C was followed by 40 cycles consisting of 15 seconds at 94°C (denaturation) and 5 seconds at 60°C (amplification) with one final run of 30 seconds at 72°C (final polymerase extension). The reaction was then cooled to 4°C for post-amplification processes. PCR optimization followed previously described methods [26,27]. The optimized factors driving the new pentaplex PCR assay are listed in Table 3.

2.7 Gel Resolution and Gel Purification of PCR Products

PCR products were resolved using conventional Tris-EDTA buffer submarine electrophoresis (200 V, 1 h) in 2% agarose gel containing Ethidium bromide (0.5 g/L) and visualised in UV trans-illuminator (UVP, UK). PCR products were purified from agarose gels using GenElute™ (Sigma, UK) according to manufacturer's instructions.

Table 2. Sequences of oligonucleotide primers used in the new pentaplex PCR assay

Target DNA	Amplicon size (bp)	Primer identity	Primer sequence 5'→ 3'	Reference/source[a]
16S	174	16S-1	CTAGTAATCGCGGATCAGCAT	This study
		16S-2	GATACGGCTACCTTGTTACGACTT	study[c]
mecA	155	mecA-1	TGGTATGTGGAAGTTAGATTGGGAT	[22]
		mecA-2	CTAATCTCATATGTGTTCCTGTATTGGC	
tuf	143	tuf-1	TACCAGCATTAGTAGTATTCTTAAACAAAGTTG	This study
		tuf-2	TGCTGAACCAGCGATTACAG	s
pvl	118	pvl-1	TTACACAGTTAAATATGAAGTGAACTGGA	[22]
		pvl-2	AGCAAAAGCAATGCAATTGATG	
spa	101	spa-1	CAGCAAACCATGCAGATGCTA	[22]
		spa-2	CGCTAATGATAATCCACCAAATACA	

aThe *mecA*, *pvl*, and *spa* primers were designed by Nakagawa et al. [22] while the two sets of differential primers targeting bacterial 16S rRNA and staphylococcal *tuf* were designed in this study

Table 3. Optimal constituents of the new pentaplex PCR

Factor	Optimal value/concentration
Primers	0.75 µM
dNTPs	0.500 mM
Taq DNA polymerase	5.0 U (NEB)
Thermopol buffer (pH 8.8 at 25°C)[a]	5 µL amounting to: 20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100.
Time to complete a 50-cycle PCR run	77 minutes

[a] Our laboratory maintains controlled temperature, approximately 25°C

2.8 Sequencing of PCR Products and Sequence Analysis

Sequencing reactions were generated by BigDye[TM] protocol (Applied Biosystems, USA) and analysed on Prism 310 Genetic analyser (Abbott Laboratories, USA). Identity of PCR products were analysed by BLAST searches on the NCBI databases and used to confirm the amplified sequences as *spa, tuf,* 16S rRNA*, pvl,* or *mecA.* The obtained sequence data were combined together to identify the strains as MRSA, PVL-positive MRSA (PPMRSA), MSSA, PVL-positive MSSA (PPMSSA), methicillin susceptible CoNS (MSCoNS), methicillin resistant CoNS (MRCoNS), or other bacteria.

2.9 Validation of the New Pentaplex PCR Assay Using Conventional Phenotypic Tests

Without disclosing their genotypic and phenotypic properties, bacterial strains were taken from the freezer and tested on the new pentaplex PCR assay as RSS001, RSS002, RSS007, etc,. The finding of Gram positive cocci in clusters was used to characterise *Staphylococcus* spp. Tube coagulase test was used to differentiate *S. aureus* from CoNS. *Staphylococcus* spp. were examined for staphylococcal methicillin resistance by oxacillin salt agar screen (OSAS) according to Clinical and Laboratory Standards Institute (CLSI) recommendation [28] with modification of the oxacillin content based on a more recent work used in the UK to support the real-time PCR detection of *mecA* and *mecA(LGA251)* in *S. aureus* [19]. Briefly, duplicate Mueller-Hinton agar plates supplemented with 4% NaCl and 0.5 mg/L oxacillin were incubated in ambient air at 30°C or 35°C for 24 hours. Plates were examined carefully with transmitted light for evidence of small colonies (>1 colony) or a light film of growth indicating oxacillin resistance. Non-staphylococcal bacteria were not tested for methicillin resistance. Obtained results were compared against phenotypic tests used to support the new assay as well as the QMC phenotypic information held by Dr. Spence.

2.10 Statistical Analysis

Negative predictive value (NPV) and positive predictive value (PPV) of the new pentaplex PCR assay were analysed according to the Clinical and Laboratory Standards Institute (CLSI) Guidelines for molecular diagnostics [29].

3. RESULTS AND DISCUSSION

Use of bioinformatics tools enabled the selection of primer pairs for the amplification of a 174 bp region from ubiquitous bacterial 16S rRNA gene and a 143 bp region from the translation elongation factor Tu (*tuf*) gene of *Staphylococcus* spp. Those two primer sets were added into the previously described triplex assay to scale up to the new pentaplex assay thus allowing all the 5 targets to be amplified simultaneously (Fig. 1).

Testing of 377 bacterial isolates on the new assay showed the new assay detected the expected genes from all the bacterial strains studied. The new assay was able to differentiate between *Staphylococcus* spp. and non-staphylococcal bacteria. A wide spectrum of *S. aureus* types, including methicillin-susceptible and methicillin-resistant strains of diverse SCC*mec* backgrounds previously tested on Dr. Richard Spence's microarray were identified.

As evident in Fig. 2, the pentaplex PCR was able to distinguish staphylococci from non-staphylococcal bacteria such as *E. coli, Proteus spp*., and Group A Streptococcus (GAS). The detection of the bacterial 16S rRNA as the only marker in the PCR (Fig. 2, Lane 1) clearly indicated bacterial presence of non-staphylococcal background. The pattern of Fig. 2 Lane 1 was found with GAS, *E. coli*, *Proteus* spp. and other non-staphylococcal bacteria. This is an advantage of the nes assay which is not common to most PCR assays reported in literature. Numerous PCR assays designed to detect *S. aureus* PVL and MRSA lack the quality of showing the presence of eubacteria of non-staphylococcal background. This is especially important in pure cultures. Very few such PCRs carry a DNA band for a sequence unique to *S. aureus* such as the staphylococcal 16S rRNA, a role well played by the staphylococcal *tuf* in the new pentaplex PCR assay. The detection of the broad-range 16S rRNA would be very useful in the differential diagnosis of skin and soft-tissue infections in which the clinical presentations of staphylococcal and streptococcal diseases are often similar and tend to overlap. As the *tuf* marker was not detected from non-staphylococcal bacteria (100% NPV), its detection strongly evidenced the presence of a member of the genus *Staphylococcus*, thus conferring a double assurance of staphylococcal diagnosis. This is essential both for the institution of empirical therapy and for the purposes of infection control.

Using the new pentaplex PCR assay, gene detection for *spa, tuf,* 16S rRNA*, pvl,* and *mecA* which were combined to inform identification of MRSA, PVL-positive MRSA (PPMRSA), MSSA, PVL-positive MSSA (PPMSSA), methicillin susceptible CoNS (MSCoNS), methicillin resistant CoNS (MRCoNS), and other bacteria corroborated the phenotypic tests used as well as the previous QMC data. All (100%) of the 377 bacteria studied generated the bacterial 16S rRNA marker thus yielding a 100% PPV. All other markers were generated with very high level specificity. No spurious amplification (100% NPV) and no expected marker was left undetected (100% PPV). All other markers were generated with very high level specificity. No spurious amplification (100% NPV) and no expected marker was left undetected (100% PPV).

BLAST data was inadequate in discriminating between PCR fragments of 16S rRNA from bacterial strains of staphylococcal and non-staphylococcal background. Using the new assay, the staphylococcal *tuf* showed greater discriminatory power than 16S rRNA. This attribute of the new assay will be helpful in separating clinical isolates of staphylococci (positive for *tuf*) from other bacteria (negative for *tuf*). This finding is supported by previous reports [30] and helps to explain the cause of failure of tiny 16S rRNA PCR fragments for taxonomical work as such tiny PCR fragments of 16SrRNA are not discriminatory enough [31].

The new pentaplex PCR assay completes 40 cycles in 37 minutes. The total time from sample preparation to gel documentation, also called the turn-around time (TAT), was 4 hours for a 96-well plate. This is a very good outcome compared with ≥24 hours required to identify staphylococcal methicillin resistance when microbiological agar is in use. This is especially important in blood cultures where the finding of GPC in clusters from positive blood cultures has become a public health concern.

In addition to the PCR primers listed in Table 2, other PCR factors optimized for the new pentaplex PCR assay are summarized in Table 3. Early in the history of PCR, optimization of PCR assays used to be so cumbersome that the list of factors identified as having influence on PCR was never to be complete [32].
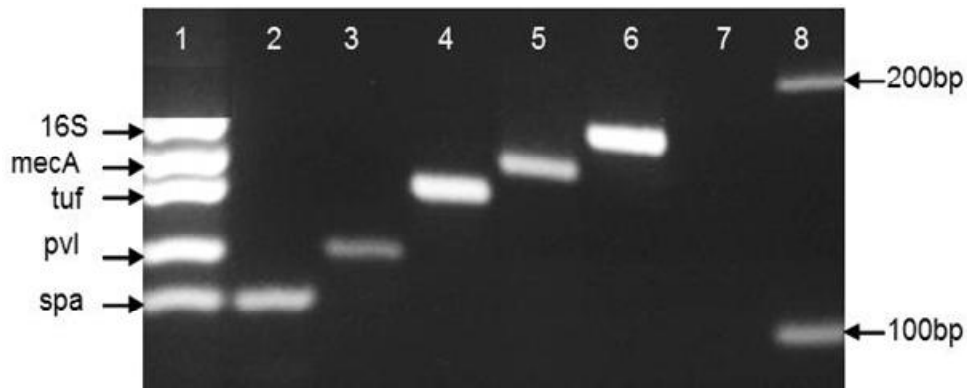
Fig. 1. The new pentaplex PCR assay and consituent monoplexes resolved in 2.5% agarose
Lane 1 is the new pantaplex PCR showing from top to bottom respectively, the bands for: Bacterial 16SrRNA (174 bp),mecA (155 bp),staphylococcal tuf (143 bp), pvl(118 bp),and spa (101 bp).Singal target PCRs are: spa(Lane 2), pvl (Lane 3), staphylococcal tuf (Lane 4) mecA (Lane 5), bacterial 16S rRna (Lane 6).Controls are: Negative PCR control with PCR grade water as template (Lane 7), 100bp DNA marker(Lane 8)
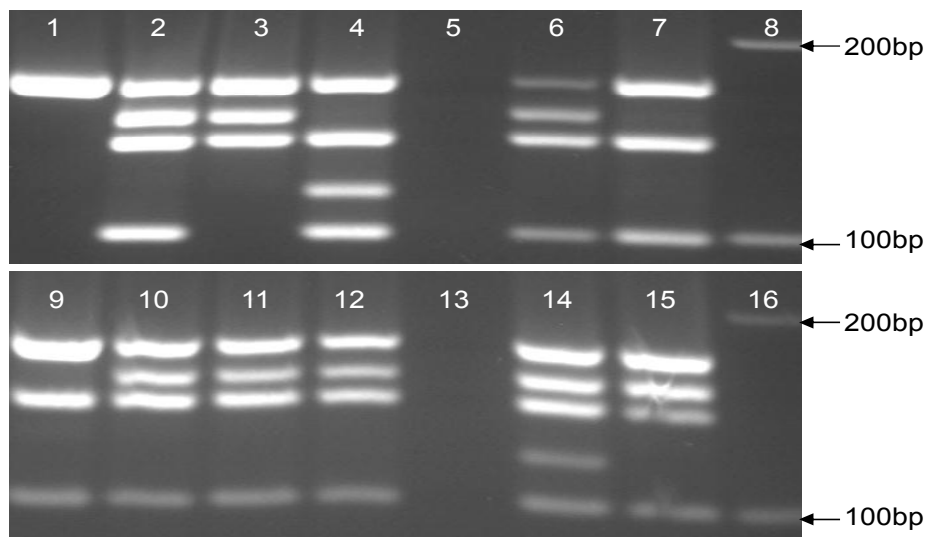

Fig. 2. Agarose gel (4%) resolution of pentaplex PCR products on various bacterial isolates
Group A Streptococcus (GAS) showing only the DNA band for bacterial 16SrRNA (Lane 1), PVL-negative MRSA S. aureus strain Mu50 showing, from top to bottom, the four DNA bands respectively marking the bacterial 16S rRNA, mecA, staphylococcal tuf, and spa (Lane 2), Meticillin Resistant Staphylococcus epidermidis strain NRS8 showing the markers for bacterial 16S rRNA, mecA and staphylococcal tuf (Lane 3), PVL-positive MSSA S. aureus strain NRS158 showing the DNA bands for bacterial 16S rRNA, staphylococcal tuf, pvl, and spa, from top to bottom, respectively (Lane 4), No Template DNA (Lane 5), PVL-negative MRSA S. aureus Sanger 252 showing, from top to bottom, the four DNA bands respectively depicting the bacterial 16S rRNA, mecA, staphylococcal tuf, and spa (Lane 6), PVL-negative MSSA S. aureus NCTC8325 strain RN6390B showing the bands for bacterial 16S rRNA, staphylococcal tuf, and spa (Lane 7), 100bp DNA Marker (Lane 8), PVL-negative MSSA S. aureus Sanger 476 harbouring SCC476 showing the DNA bands for bacterial 16S rRNA, staphylococcal tuf, and spa (Lane 9), PVL-negative MRSA harbouring SCCmec type 1 showing the bands for bacterial 16S rRNA, mecA, staphylococcal tuf, and spa (Lane 10), PVL-negative MRSA harbouring SCC mec type 2 showing the bands for bacterial 16S rRNA, mecA, staphylococcal tuf, and spa (Lane 11), PVL-negative MRSA harbouring SCCmec type 3 showing the bands for bacterial 16S rRNA, mecA, staphylococcal tuf, and spa (Lane 12), No Template DNA (Lane 13), PVL-positive MRSA S. aureus MW2 harbouring SCCmec type 4 showing respectively, from top to bottom, all five markers respectively for bacterial 16S rRNA, mecA, staphylococcal tuf, pvl, and spa (Lane 14), PVL-negative MRSA S. aureus N315 showing the bands for bacterial 16S rRNA, mecA, staphylococcal tuf, and spa (Lane 15), 100bp DNA Marker (Lane 16)

Though every effort was made to ensure the new assay continues to be useful, we are aware of the new MRSA genomes harbouring the *mecC* or *mecA(LGA251)* homologues of *mecA*. In applying the phenotypic tests to support the new pentaplex PCR assay, we did not encounter strains negative by *mecA*-PCR and phenotypically oxacillin-resistant, or positive by *mecA*-PCR and phenotypically oxacillin-susceptible at 0.5 mg/L breakpoint, which is an attribute of the *mecA(LGA251)* strains [19,33]. Since many PCR-based MRSA assays have failed and it is currently perceived that MRSA PCR with only one set of primers for detection of methicillin resistance does not work because of the *mecA(LGA251)* strains [19,33], we are keeping our eyes on the new pentaplex PCR assay as well as evolving genomes in case any new strain renders the new assay invaluable.

4. CONCLUSION

Taken together, speed and specificity attributes suggest that the new pentaplex PCR assay is readily adaptable for use in routine diagnostic microbiology laboratories as it will enable the implementation of timely and properly guided therapy and infection control strategies. The only limitation of the new pentaplex PCR assay is the time needed to resolve the PCR amplification products by gel electrophoresis. To exclude the gel resolution time, laboratories with fat budgets and real-time PCR skills can always increase on the speed of the new pentaplex PCR assay by converting the same into real-time PCR since the detectable fragments are all tiny bits which can be possible with real-time PCR. However, the availability of real-time PCR machines capable of simultaneously emitting florescence signal through five channels makes the new assay a good one for laboratories to integrate into their workflow. While we look forward to more real-time PCR machines with ≥5 emission channels and NGS and metagenomic instrumentation capable of sequencing longer templates, the new pentaplex PCR will find use in diagnostic microbiology especially in the developing world where microbiology is known for characteristic slim budgets.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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