A Simple Sample Processing Protocol and Multiplex PCR for Direct Detection of MRSA from Uncultured Clinical Samples—A Pilot Study

Rosy Chikkala[1], Swathi Ch[1], Sireesha Divyakolu[1], Kamaraju Suguna Ratnakar[2], Venkataraman Sritharan[1]*

[1]Molecular Diagnostics and Biomarkers Laboratory, Global Medical Education and Research Foundation, Hyderabad, India
[2]Global Medical Education and Research Foundation, Hyderabad, India
Email: *venkataraman.sritharan@gmail.com

Abstract

Phenotypic tests have limited discrimatory power to identify closely related members of genus *Staphylococcus* and particularly for identification of *S. aureus*. 157 isolates of *S. aureus* obtained from different clinical specimens were included in our study. To present a demonstration of our method's sensitivity and ability to correctly detect *S. aureus* from uncultured clinical specimen, 30 known *S. aureus* positive but leftover uncultured clinical specimens from clinical microbiology laboratory were processed by our protocol and analyzed. All the 30 clinical specimens were confirmed as *S. aureus* among which 26 specimen were identified as MRSA and the remaining 4 as MSSA. These 30 clinical specimens used in the study showed 100% correlation with coagulase test and Cefoxitin disc diffusion method. Though commercial molecular diagnostic kits are available for detecting MRSA from swabs, this is probably the first time that multiplex PCR is being demonstrated directly on a variety of uncultured clinical specimens.

Keywords

1. Introduction

S. aureus is a common pathogen causing minor skin infections to major life threatening diseases such as osteomyelitis, pneumonia and septicaemia [1] [2]. Accurate and rapid identification of *S. aureus* and MRSA directly from clinical samples is essential for the proper management of patients with bacteraemia,

endocarditis, skin infections, abscesses, gastroenteritis, endocarditis, toxic shock syndrome and certain food toxicity [3] [4] [5]. In developing countries, phenotyping tests are mainstay in the diagnosis of staphylococcal infections and among them the coagulase test is usually confirmatory for *S. aureus* [6] [7] [8]. Although these tests efficiently identify *S. aureus*, their performance varies from setting to setting resulting in variable reliability and reproducibility [4] [6]. The genus *Staphylococcus* contains more than 50 species and 30 subspecies which are widespread in nature [9] [10]. Three of them are important human pathogens: 1) *S. aureus*, which causes various pyogenic infections like endocarditis, osteomyelitis, skin and soft tissue infections, toxin-mediated diseases such as food poisoning, toxic shock syndrome and the scalded skin syndrome, 2) *S. epidermidis*, a member of the common skin flora, which causes infections associated with devices, such as catheters and prosthetics and 3) *S. saprophyticus*, which causes urinary tract infections [11]. Single phenotypic tests are inefficient for the identification of *S. aureus*. Indeed, mannitol salt agar (MSA) positive CoNS (*Staphylococcus caprae*, *S. hemolyticus* and *S. saprophyticus*) have been reported in Nigeria and Japan [12] [13]. However, a combination of methods like isolation on MSA and screening by DNase agar improves the outcome [14]. Thus, in certain settings, if used individually to identify *Staphylococcus aureus*, common phenotypic tests may be inconclusive; some isolates will be misidentified. The use of MSA prior to tube coagulase/DNase is highly recommended due to the clumping factor negative and tube coagulase positive *Staphylococci* that are increasingly being recovered from human infections [15]. These isolates also produce a heat stable DNase and can be misidentified as *S. aureus*. However, these strains can be differentiated from *S. aureus* by their failure to produce acid from maltose, lactose and mannitol. Furthermore, rare strains of *S. aureus* can be coagulase negative, some *Staphylococcus* isolates from animals (*S. intermedius*, *S. hyicus*, *S. delphini* and *S. schleiferi* subsp. coagulans) are clumping factor negative but tube coagulase positive [16] [17] differentiation of which requires isolation on MSA also.

Methicillin resistance in *Staphylococci* is conferred by *mecA* gene that produces altered PBP2a. Detection of *mecA* gene remains the gold standard for identification of methicillin resistance; however it does not confirm the species *S. aureus* [18]. There is no consensus on the genomic target that could be used to confirm the *S. aureus*. A number of auxiliary factors which influence methicillin resistance by regulating cell wall metabolism have been used by different laboratories to identify *S. aureus*. Notable among them are the *fem*A or *fem*B and *fem*X (factor essential for methicillin resistance) genes [19] [20]. However, failure to confirm the species of *S. aureus* as reported by others earlier [19] [21] [22] and our own report downplays the reliability of *fem*A or *fem*B as genomic target in species identification [23]. The exact reason for the false negative results with *fem* genotyping is not yet known.

The speed with which MRSA is detected has a significant role to play in any successful strategy to impede the pathogen from dissemination. Since MRSA detection by culture requires 2 - 3 days, quick detection techniques using PCR

methods have made headway. The execution of these rapid tests minimizes the time of detection of MRSA from 48 - 72 [24] to 2 - 5 h [25] [26]. Clinical evaluation data have shown that MRSA can thus be detected with very high sensitivity. Currently there are commercial kits which can only detect MRSA *(S. aureus)* from nasal swabs and positive blood cultures. Presently available molecular tests which are PCR based for MRSA detection include the HyplexStaphyloResist PCR (BAG, Lich, Germany), the GenoType MRSA direct assay (Hain Lifescience, Germany), LightCycler *Staphylococcus* and MRSA detection kit (LC assay; Roche Diagnostics, Germany), the IDI-MRSA assay (GenOhm, San Diego, BD Diagnostics), and the recently introduced GeneXpert MRSA assay (Cepheid, Sunnyvale). Rossney *et al.* [26] evaluated Xpert MRSA assay, which is run on the GeneXpert real-time PCR platform (Cepheid) for clinical samples like swabs from nose, throat, and groin/perineum sites. 90% Sensitivity and 97% specificity was reported for clinical specimens from all sites, but for throat specimens they reported poor sensitivity of 75%. Boyce *et al.* compared BD GeneOhm (MRSA) real-time PCR assay formerly called IDI-MRSA with CHROM agar MRSA assay. BD GeneOhm PCR assay had sensitivity of 100% and specificity of 98.5% with a turn-around time of 14.5 h [25]. Levi *et al.* evaluated LC *Staphylococcus* assay on pooled patient screening swabs which showed 90.8% specificity and 95.7% sensitivity [27]. All these kits which could detect MRSA rapidly and were easy to use had major limitations like being very expensive, could be performed only in swabs of nasal, groin and blood samples and results need to be compared with culture. In addition, the Xpert MRSA assay requires more interpretation than currently suggested by the manufacturer therefore more expertise is required. Table 1 shows the kits used in MRSA identification and their limitations in detail. It may be noted that many of these kits are not validated on clinical samples other than swabs. In this study we used *nuc* gene as genetic marker for PCR amplification to identify clinical isolates of *S. aureus* in comparison to some of the conventional phenotyping methods. We also designed a simple sample processing protocol and multiplex PCR using *nuc* as species marker instead of *fem* sequence. We have demonstrated in this study the potential of the sample processing protocol and multiplex PCR on 30 uncultured left over but characterised clinical samples as a pilot study.

2. Materials & Methods

2.1. Culture Isolation and Characterization

157 isolates of *S. aureus* obtained from different clinical specimens were included in our study. *S. aureus* colonies were identified by standard microbiological tests which included isolation on mannitol salt agar, coagulase test and DNase test. All *S. aureus* isolates were screened with Cefoxitin (30 μg) and Oxacillin (1 μg) disc on Mueller Hinton Agar to identify MRSA and MSSA. Thermonuclease activity was measured by streaking isolates on Methyl Green DNase Agar (Himedia Pvt Ltd) plates, and measure the zone of clearance after 24 h at 37˚C.

Table 1. Comparison of commercial MRSA detection kits.

Kit Name	Company	Specimen	Target Genes	Time	Comments	References
IDI-MRSA™ Assay	Infection Diagnostic (I.D.I.) Inc	Nasal Swabs & Isolated Colonies	orfX sequence and a sequence of SCC*mec* near the integration site	48 h	1) Only for nasal swabs and culture isolates. 2) US FDA approved.	[36]
Real MRSA and Real MRCONS multiplex real time PCR assay kit	M and D, Wonju, Republic of Korea	Culture isolates and Blood culture	16S rRNA, *nuc* and *mec*A	4 h	1) Wang et al reported false negative results due to the presence of PCR inhibitors or polymicrobial infections in direct blood samples 2) Expensive	[37]
BacLite Rapid MRSA Test	3M Company	Nasal swabs	Adenylate Kinase activity	5 h	1) This company claimed that swabs are confirmed negative in 5 h and positives the next day 2) It uses Bioluminescence combined, a sensitivity 94.6% and specificity 99%. 2) Evaluated only in nasal swabs and inguinal swabs. 3) Johnson *et al.* reported false positives due to the presence of MRCON and a sensitivity of 90.4% and specificity of 95%.	[38]
HyplexStaphyloResist PCR Enzyme Linked Immunosorbent assay	BAG, Lich, Germany	Nasal, Throat, Perineum and wound Swabs	*mec*A, Coagulase gene and *nuc*	6 h	1) Coagulase gene polymorphism has been reported. 2) False positives were reported 3) Only for swabs.	[39]
REBA Sepsis –ID Test PCR Reverse Blot Hybridization Assay	Optipharm	Blood Culture Bottles	*mec*A, vanA and vanB	4 h	1) This kit distinguishes Gram-positive bacteria, Gram-negative bacteria and fungi using pan-probes and antibiotic resistance genes. As well as identifies MRSA and VRE. As claimed by Optiparm. 2) Used as Diagnostic tool as well as for research for detection of several pathogens 3) Park SD reportedthat no specific probes for extended-spectrum β-lactamases and carbapenemases for the detection of antibiotic-resistant GNB. 4) No IVD certification 5) Detects only in blood culture bottles 6) Wang HY reported of false negatives.	[40] [41]
Xpert MRSA assay	Cepheid, Sunnyvale, CA, USA	Lower-respiratory-tract specimens, Nasal swabs and blood cultures	MRSA-specific DNA sequence within the SCC*mec*	2 h	1) Cepheid claimed that this kit can't be used for all types of specimens. 2) Oh A-C *et al.* reported false negatives and false positives 3) US FDA approved	[42]
Hain GenoQuick (GQM) methicillin resistant *Staphylococcus aureus* (MRSA) assay	Hain Life science	Nasal and groin swabs	MRSA-specific chromosomal sequences	2.5 h	1) Only Nasal and groin swabs can be processed. 2) IVD approved.	[43]
Light Cycler MRSA advanced test	Roche	Nasal Swabs	orfX sequence and a sequence of SCC*mec* near the integration site	3 h	1) *mecA* gene is present in many other Coagulase negative Staphylococcal species, thereby causing false identification. 2) W C Yam *et al.* evaluated this kit and reported 83.3% sensitivity and 99% specificity. 3) Detects only in nasal swabs which is a major drawback. 4) IVD and US FDA Approved	[44] [45]

Continued

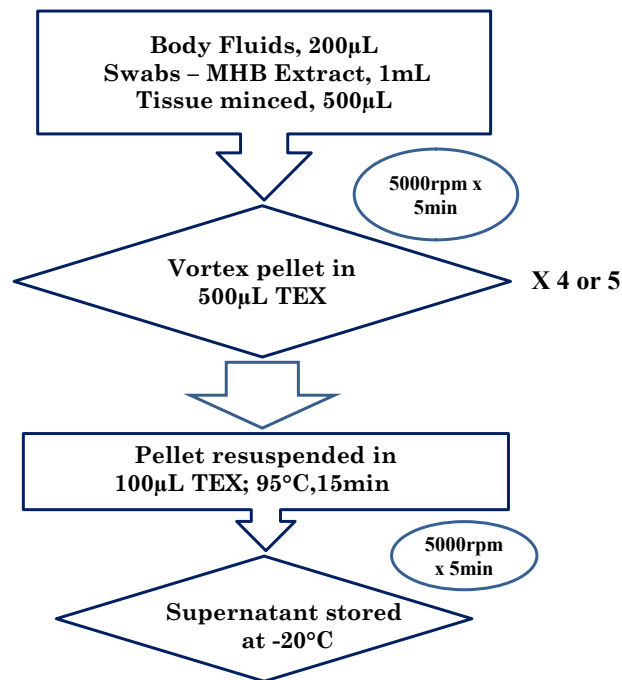|						
QX100 droplet digital PCR system	Bio-Rad	Nasal Swabs and isolated colonies	*mec*A and SA0140 protein gene	48 h	1) Expensive 2) Specific equipment for DNA extraction and sample processing is required. 3) Not IVD approved	[46]
BDMaxStaphSR assay kit	Becton-Dickinson	Nasal Swabs	*mec*A /*mec*C, *nuc* and orfX sequence	4 h	1) Only Nasal swabs can be processed. 2) False negatives were reported. 3) US FDA approved 4) Used for diagnostic purpose	[47] [48]
MRSA/SA ELITe MGB®	EliTech Molecular Diagnostics	Nasal Swabs and blood cultures	*mec*A/*mec*C, Species specific marker and 16s rRNA	5 h	1) Only nasal swabs can be processed. 2) IVD and US FDA approved	[35]
BD GeneOhm MRSA Assay	BD Diagnostics	Nasal and non-nasal swabs only	orfX sequences	2 h	1) US FDA approved for direct detection in nasal swabs. 2) Katja Lucke *et al.* reported 84.3% sensitivity and 99.2% specificity. 4) False negative results due to sequence variations 5) IVD approved.	[49] [50]
Duplex Light Cycler PCR Assay	Roche	Bacterial colonies	*mec*A and SA442 species specific marker	26 h	Only for culture confirmation	[51]
TEX Method		Uncultured Clinical Samples	*mec*A, 16S rRNA, and *nuc*	5 h	1) Rules-in or rules-out S. *aureus* and non-S. *aureus* species 2) Universal, simple and affordable sample processing protocol for PCR DNA target preparation. 3) Not commercially available yet.	[29]

2.2. Genotyping of Clinical Isolates of *S. aureus*

DNA was isolated [28] from a few isolates of *S. aureus* for initial optimization of PCR, precipitated with isopropanol and finally dissolved in 10 mM Tris-EDTA buffer (pH8.0). For subsequent screening of all the isolates, cell free DNA lysate was prepared by TEX (Tris buffer pH 8.0-EDTA-Triton X-100) method [29] Primers and the thermal cycling conditions are detailed out in Table 2. *Staphylococcus* genus was confirmed with 16S rRNA [30] and MRSA was confirmed by the detection of *mec*A [31] while *nuc* was evaluated as genomic target for species identification [32] [33] against microbiology methods.

2.3. Processing Uncultured Specimens

A total of 30 clinical specimens confirmed to contain *S. aureus* including swabs, endotracheal secretions and pus samples collected from Microbiology laboratory. These were the left over samples and collected after 24 h of storage at 4°C. All of them were processed by the TEX method and the DNA lysates were stored at −20°C until use. The flowchart of processing the samples is as shown in the scheme Figure 1.

Table 2. Primers used for study.

Gene	Sequence 5'-3'	PCR conditions		Size	Reference
16S rRNA F	GTGCCAGCAGCCGCGGTAA	94˚C × 5 min			
		94˚C × 30 s			
16S rRNA R	AGACCCGGGAACGTATTCAC	55˚C × 30 s	35 cycles	886 bp	[30]
		72˚C × 50 s			
		72˚C ×10 min			
nuc F	GCGATTGATGGTGATACGGT	94˚C × 5 mins			
		94˚C × 30 s			
nuc R	AGCCAAGCCTTGACGAACTAAAGC	55˚C × 30 s	35 cycles	270 bp	[32]
		72˚C × 50 s			
		72˚C ×10 mins			
mecA F	TCCAGATTACAACTTCACCAGG	94˚C × 5 min			
		94˚C × 30 s			
mecA R	CCACTTCATATCTTGTAACG	55˚C × 30 s	35 cycles	162 bp	[31]
		72˚C × 50 s			
		72˚C ×10 min			

Figure 1. Flow chart for processing of uncultured clinical samples.

Three sets of primers were used, one for *nuc* (species specific gene), one for 16S rRNA (genus specific gene) and another one for *mecA* (methicillin resistance gene). The reaction conditions for the multiplex PCR are described in Table 1. The PCR reaction mixture in a volume of 20 μL contained the following; 1.5 mM $MgCl_2$, 30 pmol of each primer of *mecA* and *nuc*, 10 pmol of primers of 16S rRNA, 200 μmol of dNTP along with 1 U of Taq (KAPA Taq DNA Polymerase, KAPA Biosystems Inc.) and 10 μL of cell free lysate as DNA template. *S. aureus* ATCC 6538P was used as positive control MRSA (PC). Other gram posi-

tive and gram negative ATCC cultures were used to check the specificity of our multiplex PCR (NC). Gram positive ATCC cultures included ATCC 27626 *Staphylococcus epidermidis*, ATCC 6305 *Streptococcus pnuemoniae*, ATCC 29212 *Enterococcus faecalis*, and ATCC 700221 *Enterococcus faecium*. Gram negative ATCC cultures comprised of ATCC 19606 *Acinetobacter baumanii*, ATCC 10418 *E. coli*, ATCC 70060 *Klebsiella pneumoniae*, ATCC 13048 *Enterobacter aerogenes*.

3. Results

All isolates were screened and confirmed by coagulase test. 157 coagulase positive isolates were included as *S. aureus* in this study. We screened these isolates for methicillin resistance (MRSA) by Cefoxitin and Oxacillin Disc diffusion test, 105 isolates were MRSA and 52 were MSSA. However, in genotyping, 106 isolates were identified as MRSA (*mecA*-PCR positive) and the remaining 51 isolates were classified as MSSA (*mecA*-PCR negative). One isolate which was indeterminate (22 mm) by Cefoxitin disc method was found to harbour *mecA*. The Oxacillin disc diffusion test did not compare well with *mecA* PCR test; 67% sensitivity, 94% specificity, 96% positive predictive value (PPV) and 58% negative predictive value (NPV). Cefoxitin disc diffusion test compared well with *mecA* PCR with sensitivity of 99.06%, specificity 100%, Positive Predictive Value of 100%; and a Negative Predictive Value of 98%. The results are presented in Table 3(a) and Table 3(b).

3.1. Genotyping of Cultured *S. aureus* (n = 157)

We used only coagulase positive isolates and found thermonuclease gene reliable for *S. aureus* detection from our previous study [23] hence we used thermonuclease *nuc* gene for *S. aureus* detection from uncultured clinical samples. The sensitivity of *nuc* PCR were 95% (149/157) respectively (Figure 2). We used Medcalc software (MedCalc Statistical Software version 15.6.1, MedCalc Software, Ostend, Belgium; https://www.medcalc.org; 2015) for statistical analysis.

3.2. Genotyping of *S. aureus* (n = 30) from Uncultured Clinical Specimen

We optimised a triplex PCR for detection of *mecA*, *nuc* and 16S rRNA with 31 uncultured left over specimen from microbiology laboratory. This multiplex PCR produced distinct amplicons of expected size for *mecA* (162 bp), *nuc* (270 bp) and 16s rRNA (886 bp) when analysed on agarose gel (Figure 3). Multiplex PCR result of other bacterial isolates is presented in Figure 4 and Figure 5. Results of our multiplex PCR were compared with microbiology laboratory results. All the uncultured specimens were correctly identified as *S. aureus*, 26 of them tested positive for *mecA* correlating with phenotype (Cefoxitin disc diffusion) as MRSA. This is probably the first time that multiplex PCR is being demonstrated directly on a variety of uncultured clinical specimens.

4. Discussion

Thermostable nuclease gene *nuc* was reported to have 100% sensitivity and specificity for the identification of *S. aureus* isolate [32] [33]. A few studies have employed *fem*A and *nuc* along with *mecA* as molecular targets for identification of *S. aureus* and characterization of MRSA [34]. Some commercial kits are available for directly detecting MRSA directly from nasal swabs and blood cultures [34] (Table 1). Several kits target *orf*X and sequence near to SCC*mec* region in the genome while one kit (BDMaxStaphSR kit, Becton Dickinson) also targets *nuc* in addition to *mecA* and *orf*X [35]. We reported poor sensitivity when *fem*A was used as species identification genetic marker in PCR [23]. Therefore, we evaluated *nuc* as species specific marker along with *mecA* and 16S rRNA simultaneously to identify MRSA from methicillin resistance non-*S. aureus* species. A non-*S. aureus* methicillin resistant species is indicated when our multiplex PCR result is negative for *nuc* but positive for *mecA* and 16S rRNA targets. Figure 4. Lane 1. exemplifies this where *S. epidermidis* does not amplify *nuc* sequence but only shows amplicons from 16S rRNA and *mecA*. The

Table 3. Antibiotic Susceptibility Testing. (a) Oxacillin vs *mecA*-PCR (n = 157); (b) Cefoxitin vs *mecA*-PCR (n = 157).

(a)

Oxacillin	*mecA* PCR (+)	*mecA* PCR (−)	Total
(+)	70	3	73
(−)	35	49	84
Total	105	52	157

(b)

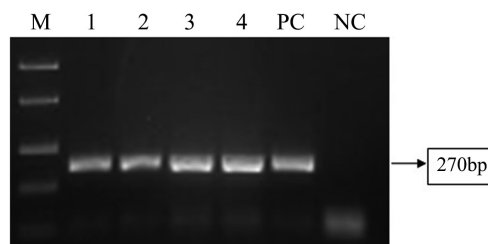| Cefoxitin	*mecA* PCR (+)	*mecA* PCR (−)	Total
(+)	105	0	105
(−)	1	51	52
Total	106	51	157

Figure 2. Results of *nuc* PCR. Legend: Lane M—Low range Molecular Size Ladder 100 bp, Lane 1 - 4—*nuc* PCR positive *S. aureus* isolates, Lane PC—positive control, Lane NC—negative control.
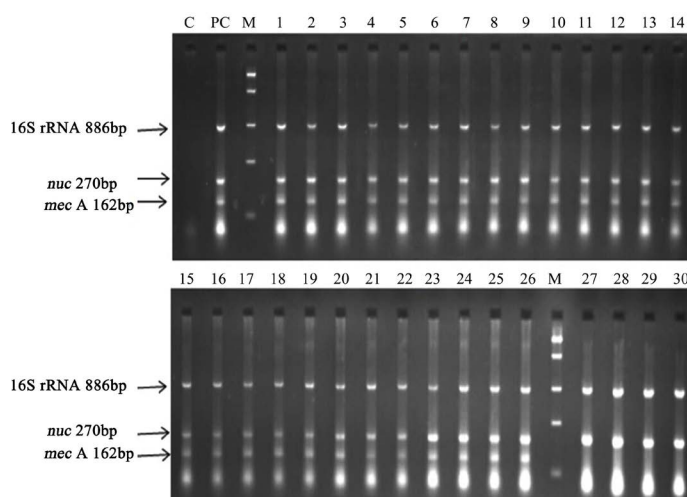
Figure 3. Multiplex PCR Analysis of Uncultured Clinical Samples. Legend: Lane C—Negative Control, Lane PC—Positive Control, Lane M—Molecular Size Marker (100 bp), Lane 1—Wound Swab, Lane 2—Nasal Swab, Lane 3 & 4—Wound Swab, Lane 5—Pus Swab, Lane 6—ET Secretion, Lane 7—Nasal Swab, Lane 8—Vaginal Swab, Lane 9—Pus Swab, Lane 10—Abcesses, Lane 11—Fluid, Lane 12—Pus Swab, Lane 13—MRSA Screen, Lane 14—Wound Swab, Lane 15—ET Secretion, Lane 16—Vaginal Swab, Lane 17—Pus Swab, Lane 18—Wound Swab, Lane 19—MRSA Screen, Lane 20—Pus Swab, Lane 21—Skin Peel, Lane 22—Pus Swab, Lane 23—Abscesses, Lane 24—Nasal Swab, Lane 25—Wound Swab, Lane 26—Skin Peel Methicillin Resistant *S. aureus* Clinical Specimens, Lane M—Molecular Marker (100 bp), Lane 27 & 28—Pus Swab, Lane 29—Nasal Swab, Lane 30—Wound Swab Methicillin Sensitive *S. aureus* Clinical Specimens.
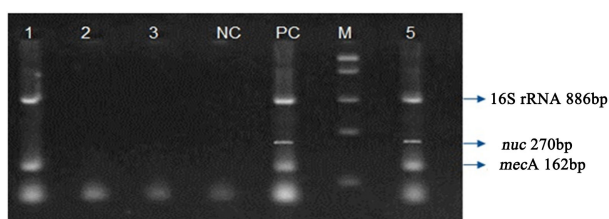

Figure 4. Multiplex PCR Analysis of Gram negative Type Strains and Uncultured Clinical Samples. Lane 1: ATCC 27626 *Staphylococcus epidermidis*, Lane 2: *Streptococcus pnuemoniae*-ATCC 6305, Lane 3: *Enterococcus faecalis*-ATCC 29212, Lane NC: Negative Control, lane PC: Positive Control ATCC 6538 MRSA, Lane M: Molecular marker (100 bp), Lane 5: Groin swab.
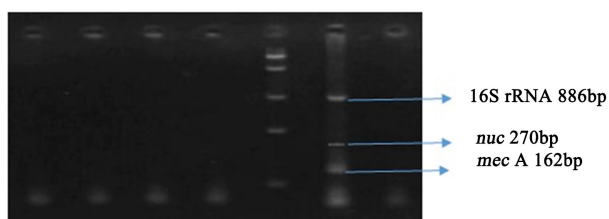

Figure 5. Testing the specificity of the Multiplex PCR. Agarose Gel analysis of analysis of non-S. aureus bacterial type strains. Lane 1: ATCC 19606-*Acinetobacter baumanii*, Lane 2: 10418-*E. coli*, Lane 3: 70060-*K. pneumoniae*, Lane 4: ATCC 13048-*Enterobacter aerogenes*, Lane M: Molecular marker, Lane PC: Positive Control ATCC 6538 MRSA, Lane NC: Negative Control.

specificity of our multiplex PCR is further demonstrated when the DNA lysates of *Streptococcus pnuemoniae*-ATCC 6305, Lane 2 and *Enterococcus faecalis*-ATCC 29212, Lane 3 of **Figure 4** did not show any amplicons. Out of our 30 uncultured clinical samples 2 were endotracheal secretions, which are usually known to harbor mixed microbial populations. Our protocol worked with these endotracheal secretions also and identified correctly the pathogen as *S. aureus*. We first wanted to demonstrate that our protocol works well with uncultured clinical specimen with results comparable to conventional microbiology. Our intention was to show that adopting such a protocol would enable same day reporting (6 - 8 h) of MRSA status of a given sample to the clinician thus facilitating a quick therapeutic decision making. Our protocol requires an extensive evaluation and validation to determine the diagnostic sensitivity, specificity and predictive values which are in progress.

Acknowledgements

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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