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Research Paper

Detection of mecA Gene and Identification of Potential Methicillin-resistant *Staphylococcus Aureus* in Hospital Wastewater Samples

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important causes of hospital infections worldwide. Methicillin-resistant *S. aureus* (MRSA) tends to be resistant to multiple antibiotics. High-level resistance to antibiotics is caused by the *mecA* gene, which encodes an alternative penicillin-binding protein, PBP 2a. The present study was aimed to detect *mecA* gene in potential methicillin-resistant *Staphylococcus aureus* (MRSA) isolates in clinical wastewater. Three hospital wastewater samples were collected, and the bacteria were isolated in mannitol salt agar (MSA) medium and Baird-parker agar medium. Gram staining and biochemical tests were performed. PCR amplification of extracted DNA from these bacterial isolates was done with 16S rRNA universal primers, specific primers of *S. aureus* and mecA gene primers to screen the clinical bacterial isolates. Sequencing of mecA gene amplicon was also done. The sequences were analyzed using BLAST (NCBI) and EMBOSS Needle tool (EMBL-EBI). Moreover, antibiotic resistance was tested at the levels of 50, 100, 200 and 300 μg/mL ampicillin. PCR amplification with all the primers screened was resulted expected bands for the isolates from Polgahawela and Chilaw wastewater. Two bacterial isolates of Polgahawela hospital effluent were able to grow at 200 μg/mL ampicillin. However, sequence analysis of amplified *mecA* gene product of these two bacterial isolates showed sequence similarity with the penicillin-binding protein (*mecA*) gene of *Staphylococcus aureus* strain and methicillin-resistance gene region of *Staphylococcus sciuri* 28C with 95% and 96% identity, respectively. Pairwise alignment results proved 89.6% sequence similarity between the two sequences. In conclusion, potential methicillin-resistant *staphylococcus aureus* (MRSA) along with *Staphylococcus sciuri* was able to detect only in the clinical effluent collected from Polgahawela base hospital.

Keywords: Antibiotic resistance, methicillin resistance gene, mecA gene

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Introduction

The use of antibiotics together with antibiotic resistance of bacteria in clinical settings is a well-known problem worldwide. The overuse and abuse of antibiotics have not only contributed to the global epidemic of antibiotic resistance but also it caused major environmental problems and the pollutants have largely been overlooked [1]. As a result, the increasing incidence of resistance to a wide range of antibiotic agents by a variety of organisms is a major concern facing modern medicine.
Uncontrolled and excessive use of antibiotics by human and animals result in an increase in antibiotic resistance and cause the spread of resistance genes in environmental samples such as clinical wastewater [2]. Studies have demonstrated that clinical wastewater is highly selective environments and that they contribute to the high rates of resistant bacteria that are being discharged in the natural environment [3].

Methicillin Resistant Staphylococcus aureus (MRSA) is an important human pathogen that is transmitted in both hospitals and the community with the risk of antibiotic resistance. MRSA is a major challenge to the hospitals all over the world [4] due to the emergence and spread of isolates with decreased susceptibilities to several antibiotics classes including methicillin and other members of β-lactam family.

The development of the ability for the antibiotic resistance of *Staphylococcus aureus* is gained by the integration of Staphylococcal cassette chromosome *mec* (SCC *mec*), a mobile genetic element into the chromosome of *Staphylococcus aureus* [5]. SCC *mec* element contains the *mecA* gene which is responsible for the antibiotic resistance.

The methicillin resistance gene *mec*A, encodes for the penicillin-binding protein (PBP2a) with lower affinity to β -lactam antibiotics than the intrinsic set of PBPs of *S. aureus*. PBP2a differs from other penicillin-binding proteins as its active site does not bind methicillin or other β-lactam antibiotics. As a result, attainment of *mec*A confers resistance to

all β-lactams antibiotics including methicillin due to inability of PBP2a to interact with β-lactams moieties [5].

The resistance development in MRSA will cause the infection difficult to treat and make it life threatening. Therefore, the present study focused to screen the *mecA* gene in order to identify *Staphylococcus aureus* including methicillin-resistant strains (MRSA) in hospital wastewater collected in different regions of Sri Lanka.

Materials and Methods

Sample Collection

Clinical wastewater samples were collected from three different hospital locations in Sri Lanka; Polgahawela base hospital (7.34^{0}N, 80.30 ^{0}E), Chilaw hospital (7.57^{0}N, 79.80^{O}E) and National Hospital, Colombo (6.92^{0}N, 79.87^{0}E). Sampling locations were decided based on the sources of antibiotic contaminations as these sites are found to be a few of the most potential contaminated sites in the country. All the wastewater samples were collected from the identified locations as per the EPA guidelines [6]. The collected samples were transported in ice to the laboratory and stored at 4^{0}C until inoculation.

Isolation of Bacteria from Clinical Wastewater Samples

The collected samples were initially inoculated on modified Mannitol Salt Agar (MSA) containing (gL-1): K_2HPO4 2.75 g, KH_2PO4 2.25 g, $(NH_4)_2SO4$ 1 g, $MgCl_2.6H_2O$ 0.2 g, NaCl 0.1 g, $FeCl_3.6H_2O$ 0.02 g, $CaCl_2$ 0.01 g at pH 7 by spread plate method in the specific medium for *Staphylococcus aureus* and incubated at 37 °C for overnight [7].

Biochemical Characterization of Bacterial Isolates

The colonies isolated on Mannitol Salt Agar (MSA) were further screened by using biochemical tests; catalase test and KOH tests and gram staining. Stock cultures of *Staphylococcus aureus* and *E.coli* isolates were used as positive and negative controls respectively for the biochemical tests.

KOH Test

Each bacterial isolate was mixed with two drops of 3% KOH solution on top of the object glass and stirred circularly for 5 - 10 seconds with one needle and observed the formation of mucus. If the mucus is formed on top of the glass, the object indicates the isolate is a gram-negative bacterium, if it is not slimy it indicates gram positive bacteria.

Catalase Test

Catalase test method was carried out with 3% H_2O_2. Each bacterial isolate was placed it on the object's glass and mixed with 100 µl of 3% H_2O_2. The formation of bubbles was observed. If there are bubbles which indicates positive catalase bacteria if there are no bubbles, which includes negative catalase bacteria.

Identification of Staphylococcus Aureus

Moreover, colonies were inoculated on Baird-parker agar for further confirmation as it is a differential selective medium. This isolation was done at the Microbiology Unit of Ceylon Agro Industries (CAI) Prima group.

Extraction of Bacterial Genomic DNA from the Isolates

The screened bacterial isolates were grown in LB media to extract the total genomic DNA. From each sample, 2.5 mL was centrifuged at 4 500 rpm at 4 ˚C for 5 min. The pellet was suspended in TE buffer (50 mM Tris/HCl and 5 mM EDTA of pH 8.0) and 10 µL of crystalline lysozyme (1 mg/mL) was added. The suspensions were incubated for 60 min at 37 ^{0}C. Then, 6 µL of proteinase K (10 mg/mL) was added followed by 30 µL of SDS (20%), and the samples were mixed thoroughly and incubated at 37 ˚C until the suspension become relatively clear and viscous. Then, 100 µL of 5 M NaCl was added, and the samples were incubated at 65 ˚C for 2 min, followed by adding 80 µL of preheated CTAB/NaCl solution and incubating at 65 ˚C for 10 min. The suspension was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 5 min at 15 000 rpm. The supernatant was transferred

into a new microfuge tube and 0.7 volume of isopropanol was added to the supernatant along the wall of the tube. It was centrifuged for 15 min at 13 000 rpm at 4 ˚C. The pellet was washed with 500 µL of 70% ethanol and re-centrifuged at 13 000 rpm for 10 min at 4 ˚C. Ethanol was completely removed by air drying. The DNA was re-suspended in 20 µL of Tris-HCl (pH 8.0) and 10 µL of each extracted genomic DNA sample was subjected to electrophoresis on a 0.8% agarose gel containing 0.5 µg/mL ethidium bromide. The gel was run at 60 V for 45 min and visualized under UV light.

Table 1. Primer sequences used for screening of *Staphylococcus Aureus*

Primer	***Direction***	***Sequences (5′-3′)***	***Amplicon Sizes (bp)***
16S rRNA	F	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAG AGT TTG ATC CTG GCT CAG	1484
	R	TAC GGG TAC CTT GTT ACG ACT T	
***S. aureus* Specific primer**	F	TCT TCA GCG CAT CAC CAA TGC C	229
	R	TTC GTA CCA GCC AGA GGT GGA	
mecA	F	TCCAGATTACAACTTCACCAGG	162
	R	CCACTTCATATCTTGTAACG	

Amplification with 16s rRNA Universal Primers

The DNA extracted from the selected isolates was amplified with 16s rRNA universal primers of, forward primer with GC clamp and reverse primer (Table 1). The PCR mixture consisted of 40 ng of bacterial genomic DNA, 2 µL of dNTPs (200 µM), 0.8 µL of primers (0.4 µM), 0.5 units of *Taq* polymerase, 2 µL of $MgCl_2$ (25 mM) and 4 µL of 5X PCR buffer with a final volume of 20 µL. The PCR program consisted of an initial denaturation for 5 min at 94 ˚C followed by 30 cycles of 1 min at 94 ˚C, 2 min at 59 ˚C and 2 min at 72 ˚C with a final extension of 10 min at 72 ˚C was used for the PCR amplification.

Amplification with the Specific Primers of Staphylococcus Aureus

The extracted genomic DNA was amplified with specific primers of *Staphylococcus aureus* (Table 1) to confirm the presence *of Staphylococcus aureus* [8]. The PCR mixture was prepared with 40 ng of bacterial genomic DNA, 2 μL of dNTPs (2 mM), 1μL of primers (0.4 μM), 0.5 units of *Taq* polymerase, 2 μL of $MgCl_2$ (25 mM) and 4 μL of 5X PCR buffer with a final volume of 20 μL. The PCR program consisted of an initial denaturation for 2 min at 95 ˚C followed by 30 cycles of 45 sec at 95 ˚C, 1 min at 55 ˚C, 1 min at 72 ˚C, with a final extension 72 ˚C for 5 min was used for the PCR amplification.

PCR Amplification with mecA Specific Primers

The extracted DNA of selected isolates was subjected to PCR for amplification of the *mecA* gene which is responsible for the antibiotic resistance using primers of *mecA* gene [9]. The PCR mixture consisted of 40 ng of bacterial genomic DNA, 2 μL of dNTPs (200 μM), 0.8 μL of primers (0.4 μM), 0.5 units of *Taq* polymerase, 2 μL of $MgCl_2$ (25 mM) and 4 μL of 5X PCR buffer with a final volume of 20 μL. The PCR program consisted of an initial denaturation for 4 min at 94 ˚C followed by 32 cycles of 30 sec at 94 ˚C, 30 sec at 55.5 ˚C and 50 sec at 72 ˚C with a final extension of 10 min at 72 ˚C was used. Amplified PCR products were subjected to electrophoresis using a 1% agarose gel.

Antibiotic Susceptibility Testing

The selected isolates of *S. aureus* confirmed by the preliminary investigations were used for the antibiotic screening test. Overnight culture of few isolates was inoculated on mannitol salt agar media supplemented with four different concentrations (50 μg/ml, 100 μg/ml, 200 μg/ml, 300 μg/ml) of ampicillin and without any antibiotics as a control. This experiment was repeated three times. Then the plates were incubated at 37^oC.

Analysis of mecA Sequences

Two *mecA* amplified products of the isolates selected on ampicillin media were sent for bidirectional sequencing (both reverse and forward

directions) to Genetech Molecular Diagnostic Laboratory, Colombo 8. Sequences were analyzed by Basic Local Alignment Search Tool (BLAST) and EMBOSS Needle tool (EMBL-EBI) to confirm the presence of *mec*A gene.

Results and Discussion

Isolated Staphylococcus in Selective Medium

As an initial approach, the research focused on isolating *Staphylococcus aureus* from clinical wastewater. Yellow colonies surrounded with yellowish zone in the selective media indicate the presence of *Staphylococcus group* in Polgahawela base hospital effluents and Chillaw hospital effluents. But no yellow colonies obtained in the Colombo general hospital effluents. *Staphylococcus* is a salt tolerant bacterium which has the ability to ferment mannitol. MSA media contains the phenol red indicator which turns to yellow when mannitol is fermented. From this screening, two yellow colonies were selected from each hospital isolates (SAP 1 and SAP 2 from Polgahawela base hospital effluents, SAC 1 and SAC 2 from Chillaw hospital effluents) for further testing.

Biochemically Identified Staphylococcus Aureus

All isolates showed violet coloured coccal shaped cells under the light microscope except the negative control (*E.coli*) for the gram staining. Violet colour indicates the presence of gram-positive bacteria. The gram staining results indicated that the all the selected isolates were gram positive cocci (Table 2). Gram-positive and negative cells vary according to their cell wall thickness and stain differently as violet and red for gram-positive and gram negative respectively. The KOH test further confirmed that the isolates are gram-positive. The isolates did not show string formation for the KOH test as they were gram-positive except the negative control (Table 2).

The catalase test proved that all the selected isolates were catalase positive. Bubbles were visible in all bacterial isolates. Bubbles can be

observed when hydrogen peroxide is oxidized into water and oxygen. Catalase breaks H_2O_2 and protects bacteria as it becomes toxic to the organism. Lack of bubbles indicate the absence of catalase.

Table 2. Biochemical tests for *S. Aureus*

Strain	**Gram test**	**KOH test**	**Catalase test**
SAP 1	Positive	Negative	Positive
SAP 2	Positive	Negative	Positive
SAC 1	Positive	Negative	Positive
SAC 2.	Positive	Negative	Positive
S. aureus	Positive	Negative	Positive
E.coli	Negative	Positive	Positive

Identification of Staphylococcus Aureus

The selected isolates tested by Baird Parker agar showed black, convex, and shiny colonies with a clear zone in Baird Parker agar (BPA) confirming the presence of *S. aureus*. Baird-parker agar (BPA) medium was chosen for the isolation of *Staphylococcus* spices in this study because the medium is the recommended selective medium used specially for the identification of *Staphylococcus aureus***.**

The results of the growth of colonies of aureus and non-aureus *Staphylococcus* from the samples of Polgahawela clinical wastewater studied are shown in Figure 1. Colonies of *S. aureus* have a type of the selective medium of BPA [10]. The BPA medium is a differential selective medium for the isolation and detection of *Staphylococcus sp* and *S. aureus* bacteria groups.

Figure 1: *Staphylococcus* species isolated in Baird-parker agar medium

PCR Amplification with Selected Primers

PCR of 16s rRNA amplification *with 16s rRNA Primers* resulted in the expected band of 1484 bp region. This clearly confirm the presence of 16s rRNA gene in all the screened isolates confirming the bacterial genome with the required quantity and purity for further PCR amplification. Further, PCR amplification with *Staphylococcus aureus* specific primers resulted in the expected band of 230 bp region and it clearly confirms the presence of *Staphylococcus aureus*.

PCR Amplification of the Extracted DNA with mecA Gene Specific Primers

The PCR of *mecA* amplification resulted in the expected band of 162 bp region (Figure 2). It shows that the amplified isolates SAP 1, SAP 2, SAC 1 & SAC 2 have the *mecA* gene. MRSA is resistant to methicillin by acquiring a gene producing a modified penicillin binding protein (PBP2a) which is encoded by the *mecA* gene locates in a mobile genetic element [11]. Resistance for the methicillin was conferred by *mecA* gene [12]. Amplification with *mecA* gene (Figure 2) confirmed strongly that the screened isolates are potential MRSA.

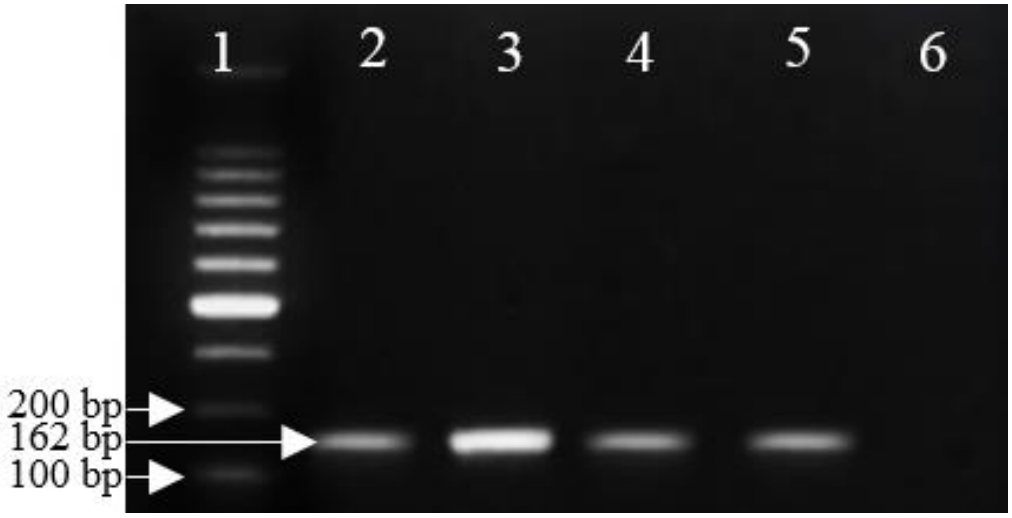

Figure 2. Agarose gel electrophoresis of PCR amplified of *mecA* gene primer. *Lane 1: 100 bp ladder, Lane 2: SAP 1, Lane 3: SAP 2, Lane 4: SAC 1, Lane 5: SAC 2 and Lane 6: negative control.*

Analysis of mecA Sequences

Sequence results of the *mecA* amplification indicated SAP2 isolate has the best match to the *Staphylococcus aureus* strain 28C penicillin-binding protein (*mecA*) gene with 95% identity while SAP1 isolate expressed 96% of identity to *Staphylococcus sciuri* which is a distantly related species of *Staphylococcus aureus* (Table 3). Output of EMBOSS Needle tool (EMBL-EBI) proved 89.6% sequence similarity between the two sequences (Table 4).

Table 3. Identification of Staphylococcal isolates by using mecA gene sequencing

Strain	**28C penicillin-binding protein (mecA) gene**	**Identified Bacteria (%Similarity)**
SAP1	Staphylococcus sciuri (96%)	Staphylococcus sciuri
SAP2	Staphylococcus aureus (95%)	Staphylococcus aureus

Table 4. Pairwise alignment of reverse primer sequences

```
n160525-_I  GtcAgtA--TTATCTTaTTGCcaCCTTTTCCaTTAATTTTATAACTTGTTTTGcCGTCTA
n160525-_M  GatAagAatTTATCTTtTTGCatCCTTTTCCgTTAATTTTATAACTTGTTTTGtCGTCTA

n160525-_I  AtGTCTTATTgTTTAAGCCAATCATgGCTGTTAATATTTTTTGAGTCGAACCTGGTGAAG
n160525-_M  AaGTCTTATTaTTTAAGCCAATCATaGCTGTTAATATTTTTTGAGTCGAACCTGGTGAAG

n160525-_I  TTGTAATCTGGAAgtTtTaTtTTTTTTTgtcCCcCtCtTTTTtTtTTtcTTTTTtTtt
n160525-_M  TTGTAATCTGGAAaaTcTcTaTTTTTTTT---CCtCcCcTTTTTaTcTTatTTTTTcTac

n160525-_I  TCCTccTACTTTTTTTTTTTTtTtCCCCtcTCTtTttCtTtTCCCcTcTTTTTTCtTtcC
n160525-_M  TCCTttTACTTTTTTTTTTTTcTcCCCCctTCTcTccCcTcTCCCtTtTTTTTTCcTctC

n160525-_I  C----TtTCtCTCTTttTCTCTTTtCttttTttTTCtTTCTCcTTTCctTTcTTtTtTTT
n160525-_M  CctctTcTCcCTCTTccTCTCTTTcCccccTccTTCcTTCTCtTTTCtcTTtTTcTcTTT

n160525-_I  TCcTcCctTTtTTcTTTccTTCTcCttCCTTTtCccTTCTCtTTCTTCTTcTcTttTtCt
n160525-_M  TCtTtCtcTTcTTtTTTttTTCTtC--CCTTTcCttTTCTCcTTCTTCTTtTtTccTaCc

n160525-_I  cTCcTTCCCtttccttttttttttTTTCtCTcCCtCCcttCCCtctCCtCCTTtCCcCC
n160525-_M  tTCtTTCCC----------cTTTCcCTtCCCcCCtccCCCctcCCcCCTTcCCtCC

n160525-_I  CCCttCtCtCtCCCtCCCCcCtCttCCCCaTCCtTCCCCTCCCCCtCCCaCCCCCtcCCt
n160525-_M  CCCccCcCcCcCCCcCCCCtCcCccCCCCcTCCcTCCCCTCCCCCcCCCcCCCCCctCCc

n160525-_I  CCCCtCCCCCtCCCCCtCcCtCtCtCCCCtCCCCCTCctccc
n160525-_M  CCCCcCCCCCcCCCCCcCtCcCcCcCCCCcCCCCCTC-----
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Similar residues are colored as the most conserved one (according to BLOSUM62). Average BLOSUM62 score: *Max: 3.0* *Mid: 1.5* *Low: 0.5*

Antibiotic-Resistant Bacterial Isolates

In the primary screening to detect the antibiotic resistance with one of the β-lactam antibiotics, the different concentrations of ampicillin were used. Only two isolates (SAP1 & SAP2) out of four were able to grow at the level of 200 µg/ml ampicillin indicating that the isolates may have developed resistance to the screened antibiotics.

Conclusions

Potential *Methicillin Resistant Staphylococcus aureus* (MRSA) was detected in the Polgahawela clinical waste along with *Staphylococcus sciuri*. The presence of MRSA strains in hospital associated environment in Polgahawela highlighted the need of identifying appropriate measures to control infections. Further studies on antibiotic resistance level for different β-lactam antibiotics and gene expression studies of *mecA* will confirm the resistance capacity of isolated bacteria.

Conflicts of Interest

The authors declare no conflict of interest.

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