Isolation and Characterization of Methicillin Resistant *Staphylococcus aureus* from Hospital Effluents

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ABSTRACT

Antimicrobial resistance is recognized as a major emerging public health problem by the World Health Organization (WHO). Exerting pressure on microorganisms, misuse of antibiotics are the main epidemiological factors responsible for the emergence of antimicrobial resistance in health care settings. Sri Lanka has higher risk of hospital acquired antimicrobial resistance. Methicillin Resistant *Staphylococcus aureus* (MRSA) has more than eighty percentage of incidence than the other antimicrobial resistant microbes. The aim of the present study is to develop drug targets for the MRSA infection. As a preliminary step of this study, the potential MRSA was isolated and identified. Hospital effluents collected from three different locations were placed on a selective manitol salt agar media to isolate the *Staphylococcus aureus*. Randomly selected four bacterial isolates, two from Polgahawela base hospital and two from Chillaw hospital were tested by biochemical tests and molecular methods. The DNA was extracted from those isolates and amplified with 16s rRNA primer, *Staphylococcus* specific primers, and *mecA* specific primers respectively. The *mecA* amplified fragments of two different isolates (SAP 1 and SAP 2) from Polgahawela hospital effluents were sequenced and analyzed to confirm the antibiotic resistant bacteria. Yellow colonies on the selective media obtained from Polgahawela base hospital effluents and Chillaw hospital effluents indicate the presence of *Staphylococcus aureus*. The selected isolates showed positive results for all the biochemical tests in the present study. The amplified products of 16s rRNA primers and *Staphylococcus* specific primers also confirmed the presence of *Staphylococcus aureus* in hospital effluents. Moreover, amplification with *mecA* indicated that the selected four isolates are potential MRSA. However, sequencing results of Polgahawela isolates confirmed the presence of *mecA* gene. Therefore, the expression of *mecA* gene should be detected to confirm their methicillin resistant to identify the presence of MRSA in these effluents.

KEYWORDS: Antibiotic resistance, *mecA*, Methicillin resistant *Staphylococcus aureus*

INTRODUCTION

Antimicrobial resistance is an important concern for the public health authorities at global level. Easy availability and higher consumption of medicines have led to disproportionately higher incidence of inappropriate use of antibiotics and greater levels of antibiotic resistance in developing countries compare to developed countries.

Sri Lanka is a developing country which has achieved a good health index compare to most other Asian countries during the past few decades. Currently, the country faces the problem of an increasing trend in antimicrobial resistance in microorganisms in both health care and community settings probably due to multiple reasons. Some of these organisms are extended spectrum β lactamase (ESBL) producing coliforms, multidrug-resistant *Pseudomonas* and *Acinetobacter* spp, methicillin resistant *Staphylococcus aureus* (MRSA), penicillin resistant *Streptococcus pneumoniae* and quinolone resistant *Salmonella typhi* and *Salmonella paratyphi* (Patabendige *et al.*, 2011). In addition, a case study indicates the very higher proportion of MRSA in hospital associated *Staphylococcus aureus* (86.5%) and community associated infection (38.8%) which were recorded in Sri Lanka (Song *et al.*, 2011).

Methicillin resistant *Staphylococcus aureus* (MRSA) is a specific strain of *Staphylococcus aureus* that has developed antibiotic resistant to all β-lactam antibiotics (Gunawardena *et al.*, 2012). The β-lactam ring is a part of the core structure of several antibiotic families, the principal ones being the penicillins, cephalosporins, carbapenems, and monobactams, which are, therefore, also called β-lactam antibiotics. *Staphylococcus aureus* is a gram positive, non-spore forming, non-motile and facultative anaerobic bacteria. *Staphylococcus aureus* is known to cause skin and soft tissue infections such as abscesses (boils), furuncles and cellulitis. Although most infections are not serious, *Staphylococcus aureus* can cause serious infections such as blood stream infections, pneumonia or bone and joint infections leading for mortality and morbidity (Anon, 2010). Methicillin resistant *Staphylococcus aureus* is a major concern as an important nosocomial pathogen (Mahalingam *et al.*, 2014) with high risk of antibiotic resistance. Moreover, in recent years in Sri Lanka, MRSA has shown the distinctive

transformation of infection tendency from traditional hospital acquired to community associated infection (Gunawardena *et al.*, 2012).

Under new selective pressure *Staphylococcus aureus* has evolved and developed resistance to many antibiotics. The evolution of *Staphylococcus aureus* is associated with serious community acquired and nosocomial infections (Day *et al.*, 2001).

The development of antibiotic resistant ability of *Staphylococcus aureus* is gained by the integration of Staphylococcal cassette chromosome *mec* (SCC *mec*) element into the *Staphylococcus aureus.* Staphylococcal cassette chromosome *mec* element contains the *mecA* gene which is responsible for the antibiotic resistance. The *mecA* encodes penicillin-binding protein 2a (PBP2a), which 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 (Jayatilleke and Bandara, 2012).

The resistant development in MRSA will cause the infection difficult to treat and make it life threatening. Overall objective of the present study is to develop drug targets for the MRSA. Therefore, the initial approach of this study focused to identify the MRSA isolates and confirm the antibiotic resistance by the detection of *mecA* gene.

MATERIALS AND METHODS

Experimental Location

The study was carried out at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka from December 2015 to June 2016.

Collection of Hospital Effluents

The samples were collected from three different hospitals: Colombo general hospital, Polgahawela base hospital and Chillaw base hospital and immediately used for the inoculations.

Isolation of Staphylococcus aureus

The collected samples were inoculated on Manitol Salt Agar (MSA) which is a specific medium for *Staphylococcus aureus* by spread plate method and incubated at 37 °C.

Biochemical Tests for the Isolates

The isolated colonies were further tested 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.

Moreover, Baird parker test was done at the Microbiology Unit of Ceylon Agro Industries (CAI) Prima group for further confirmation.

Extraction of Bacterial Genomic DNA from the Isolates

The identified bacterial isolates were grown in LB media to extract the total genomic DNA. From each samples, 2.5 mL was centrifuged at 4 500 rpm at 4 °C for 5 min. The pellet was suspended in of 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 °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.

PCR Amplification of the Extracted DNA

The DNA extracted from different isolates was subjected to PCR for the molecular identification. The DNA was amplified initially with universal 16s rRNA primers for bacterial identification and then with specific primers of *Staphylococcus aureus* to confirm the presence of this organism, finally with *mecA* specific primers to detect antibiotic resistance of *Staphylococcus aureus*.

Amplification with 16s rRNA Universal Primers

The DNA was amplified using a set of universal primers of 16s rRNA, forward primer with GC clamp (5'- CGC CCG CCG CGC GCG

GCG GGC GGG GCG GGG GCA CGG GGG GAG AGT TTG ATC CTG GCT CAG–3') and reverse primer (5'–TAC GGG TAC CTT GTT ACG ACT T–3').

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* to confirm the presence of *Staphylococcus aureus* using Forward primer (5 '- TCT TCA GCG CAT CAC CAA TGC C-3') and reverse primer (5'- TTC GTA CCA GCC AGA GGT GGA -3') (Pradhan *et al.*, 2011).

The PCR mixture was prepared with 40 ng of bacterial genomic DNA, 2 µL of dNTPs (2 mM), 1 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 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.

Amplification of mecA Primers

The DNA of selected isolates were subjected to PCR, amplifying the *mecA* gene which is responsible for the antibiotic resistance using forward primer of *mecA* gene (5'-TCCAGATTACAACTTCACCAGG–3') and reverse primer of *mecA* gene (5'–CCACTTCATATCTTGTAACG–3') (Amini *et al.*, 2012).

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.

From the *mecA* amplification, amplified products of SAP 1 (*Staphylococcus aureus* isolate number 1 from Polgahawela hospital) and SAP 2 (*Staphylococcus aureus* isolate number 2 from Polgahawela hospital) were sequenced by submitting the PCR amplified products to Genetech Molecular Diagnostics and School of Gene Technology, Colombo 08 for further analysis.

RESULTS AND DISCUSSION

Identification of Staphylococcus aureus

As an initial approach, the research focused on isolating *Staphylococcus aureus* from hospital effluents. Yellow colonies appeared with yellowish zone around them on selective media indicate the presence of *Staphylococcus aureus* in Polgahawela base hospital effluents and Chillaw hospital effluents. But no yellow colonies obtained in the Colombo general hospital effluents. *Staphylococcus aureus* is a salt tolerant bacterium which has the ability to ferment mannitol. Manitol Salt Agar 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.

Biochemical Tests of the Identification of the Isolates

The results of biochemical tests of all selected manitol positive isolates confirmed the biochemical characteristics of *Staphylococcus aureus*.

Gram Staining and KOH Test

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 1). 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.

Catalase Test

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 indicates the absence of catalase.

Table 1. Biochemical test results

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

Baird Parker Agar Test

The selected isolates tested by Baird Parker agar showed black, convex, and shiny colony with a clear zone in Baird Parker agar (BPA) confirming the presence of *S. aureus*. Baird Parker Agar is a selective medium for the isolation and identification of coagulase-positive *Staphylococci*. Baird Parker agar act as selective agents for *Staphylococci* (Anon. 1996). Egg yolk is the substrate to detect lecithinase production and lipase activity. *Staphylococci* produce dark gray to black colonies due to tellurite reduction; *Staphylococci* that produce lecithinase break down the egg yolk and cause clear zones around respective colonies. An opaque zone of precipitation may form due to lipase activity which is specific for *Staphylococcus aureus* (Anon. 1996).

The results obtained through the entire biochemical test and gram staining results clearly confirm the presence of *Staphylococcus aureus*.

Extraction of Genomic DNA of the Isolates

CTAB extraction protocol (Moore *et al.*, 2004) was used for the extraction of genomic DNA of bacteria. Extracted DNA was in sufficient quantity (2 μg/μL - 5 μg/μL) and quality for PCR. (Figure 1)

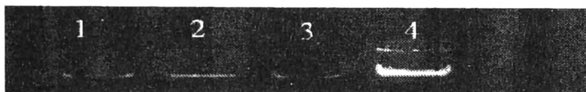

Figure 1. Agarose gel electrophoresis of extracted genomic DNA. *Lane 1: SAP 1, Lane 2: SAP 2, Lane 3: SAC 1, Lane 4: SAC 2*

PCR Amplification of the Extracted DNA

PCR amplification done with 16s rRNA primers, *Staphylococcus* specific primers, *mecA* specific primers confirmed the presence of expected bands as indicated below.

PCR Amplification with 16s rRNA Primers

PCR of 16s rRNA amplification resulted the expected band of 1484 bp region. This clearly confirm the presence of 16s rRNA gene in all the isolates. These results confirm that the extracted DNA is bacterial genome with the optimum quantity and purity. (Figure 2)

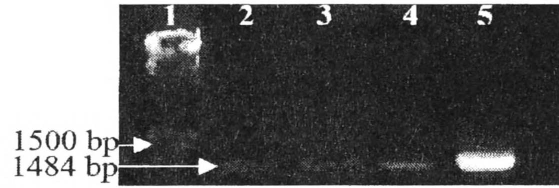

Figure 2. Agarose gel electrophoresis of PCR amplified 16s rRNA gene. *Lane 1: Lamda Hind III, Lane 2: SAP 1, Lane 3: SAP 2, Lane 4: SAC 1 and Lane 5: SAC 2*

PCR Amplification with Staphylococcus aureus Specific Primers

PCR of *Staphylococcus aureus* specific primers amplification resulted the expected band of 230 bp region. This clearly confirms that all four isolates are *Staphylococcus aureus*. (Figure 3)

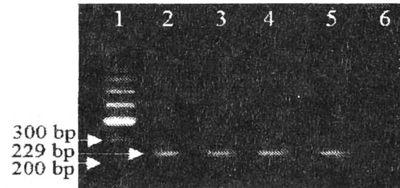

Figure 3. Agarose gel electrophoresis of PCR amplified produts of *Staphylococcus aureus* specific primers. *Lane 1: 100 bp ladder, Lane 2: SAP 1, Lane 3: SAP 2, Lane 4: SAC 1, Lane 5: SAC 2 and Lane 6: negative control*

PCR Amplification with mecA Specific Primers

PCR of *mecA* amplification resulted the expected band of 162 bp region. It shows that the amplified isolates 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 (Khaldi *et al.*, 2015). Resistance for the methicillin was conferred by *mecA* gene (Schwartz *et al.*, 2002). Amplification with *mecA* gene confirmed strongly that the isolated colonies are potential MRSA. (Figure 4)

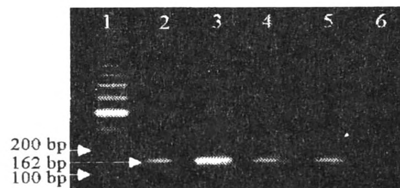

Figure 4. Agarose gel electrophoresis of PCR amplified of *mecA* gene primer. *Lane 1: 100 bp ladder, Lane 2: SAP 1, Lane 3: SAP 2, Lane 4: SAC 1, Lane 5: SAC 2 and Lane 6: negative control*

Sequence results of the *mecA* amplification indicate SAP 2 isolate has the best

match to the *Staphylococcus aureus* strain 28C penicillin-binding protein (*mecA*) gene with 95% identity which clearly confirm the presence of MRSA in the effluents. However, SAP 1 isolate also has 96% of identity to *Staphylococcus sciuri* which is a distantly related species of *Staphylococcus aureus.*

CONCLUSIONS

All the biochemical tests and molecular tests used for the study, clearly confirm the presence of *Staphylococcus aureus* in Polgahawela base hospital effluents and Chillaw hospital effluents. *The mecA* amplification also confirms the presence of *mecA* gene which confers the antibiotic resistance. In addition, the sequence of *mecA* gene of SAP 2 isolate further confirms that the isolate *Staphylococcus aureus* is a potential MRSA. However, further studies are needed to detect the expression level of *mecA* gene which will confirm the capability of antibiotic resistance of MRSA in the effluents.

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