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Amplification of Methicillin Resistance Gene (*mec-A*) from *Staphylococcus aureus* Isolates from India

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ABSTRACT

Staphylococcus aureus strains were isolated from 261 clinical samples located at various regions of Tamil Nadu, India. All isolates were grown properly using standard methodology and were used for the isolation of chromosomal DNA. The chromosomal DNA was used for the confirmation of a methicillin resistant gene named *mec-A*. The strains possessing the *mec-A* gene were highly resistant to the antibiotic, methicillin. The *mec-A* gene (size = 310 bp) was successfully detected by Real Time-PCR.

Keywords: AGE, methicillin resistance, RT-PCR
Abbreviations: AGE, agarose gel electrophoresis; **MRSA**, methicillin-resistant *Staphylococcus aureus*; **PCR**, polymerase chain reaction

INTRODUCTION

Staphylococcus aureus is a Gram-positive bacterium which occurs naturally in the skin of warm-blooded animals, including human beings (Boyce and Medeiros 1987). It can produce enterotoxin in humans and animals. *S. aureus* is also known to be the common cause of nosocomial community-acquired infections and surgical wound infections (Blanc *et al*. 2001). *Staphylococcus* can also cause serious infections such as surgical wound infections and pneumonia. In the past, most serious *S. aureus* infections were treated with antibiotics but subsequently after the organism were found to have developed resistance to various drugs including penicillin and its related antibiotics (Smith *et al.* 2001). 70-80% of *S. aureus* isolates was found to be resistant to penicillin (Atkinson and Lordain 1984). Methicillin and other semi-synthetic penicillins were successful in treating penicillin-resistant *S. aureus* infections until the 1980's, when Methicillin-resistant *S. aureus* (MRSA) became endemic in many hospitals (Panlio *et al.* 1992).

In 1963, the USA introduced methicillin in clinical practices against naturally occurring strains of *S. aureus* resistant to penicillin and other antibiotics. They had not encountered resistance to methicillin even after a course of extensive tests. However, Jevons *et al.* (1963) stated that there were three strains among a total of 5,440 isolates tested, which showed increased resistance to methicillin, the MRSA (Sutherland and Robinson 1964. In 2000, vancomycin antibiotic treatment failed in the United States, prompting Japan and France to retrospectively analyse 42 cases of septicemia caused by epidemic MRSA 15 (EMRSA-15). The most prevalent epidemic strain of MRSA in the UK was between 1994 and 1998. Mortality was lowest (4%) in patients with rifampicin-isolates treated with vancomycin and rifambin. It increased to 38% in patients but in whom the organism became resistant to rifambin during therapy, and it reached 78% in patients who had rifambin-resistant isolates or in whom rifambin was contraindicated (Burnie *et al.* 2000). In 1997, a vancomycin-resistant *S. aureus* (VRSA) was isolated (Kuroda *et al.* 2001).

Tenover *et al*. (1995) interpreted the DNA banding patterns generated by PFGE and transformed them into random genetic events that can alter the patterns. Most commonly random changes in genetic elements, including point mutations, insertions and deletions of DNA, altered PFGE banding patterns and only few bands were detected. Struelens *et al*. (1995) reported the accurate detection of methicillin-resistance among *S. aureus* isolates using 7% NaCl containing Muller–Hinton Agar medium. Detection of the *mec-A* gene by PCR and/or DNA probe hybridization was reliable and rapid method. A multi-resistant MRSA clone with a high level of methicillin resistance was discovered. The *mec-A* gene was therefore considered a useful molecular marker for methicillin-resistance in all *Staphylococci.* PCR appeared to be a rapid, sensitive and specific assay for detection compared with southern blot hybridization, macro restriction and finger printing techniques (Vannuffel *et al*. 1995). Geha *et al*. (1994) analyzed a multiplex PCR array for detection of Staphylococcal *mec-A* gene (the structural gene for PBP2a) which indicated methicillin-resistance because this gene is responsible for the production of penicillin binding protein 2a, which binds to penicillin and related antibiotics and inactivates them.

MATERIALS AND METHODS

Sample collection

Various clinical laboratories in northern (Salem and Vellore) and southern districts (Madurai and Trichy) of Tamil Nadu, India were opted for sample collection. Pus samples were collected from respective districts. Patients belonging to various age groups (viz., < 20 years-old, 21-40 years-old, 41-60 years-old and > 60 years-old) were selected for sample collection. The patient's data was collected from Gokulam Hospital, Gobi Hospital, Bose Clinical Laboratory, Bio Micro Laboratory, GMKV Hospital, MK Diagnostics and KAPV Hospital in India. Clinical samples were collected and transported to the laboratory after bedside inoculation using standard microbiological methods.

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Original Research Paper

Pus

Pus was removed according to the WHO (1999). Swab intact pustules with 70% ethyl alcohol were allowed to dry. Either piercing with a sterile needle or absorbing the contents with a sterile swab or aspirating the contents was done. A sterile swab to sample areas of superficial pyoderma, preferably sampling from the margin of the lesion was used. Submission of biopsies for bacterial culture in a sterile universal was done.

Identification of *S. aureus*

All clinical samples were carefully analyzed for the identification of *S. aureus* strains using standardized methods. Gram staining, hemolytic activity, the coagulase test, the gelatinase test, growth on mannitol salt agar and the antibiotic assay test for the detection of MRSA were conducted according to Benson (2002). In the latter, isolated *S. aureus* were cultured in the nutrient broth. Various antibiotic discs obtained from Hi-Media Laboratory, India were used for the antibiotic assay. In the present study the following antibiotic discs [methicillin (5 µg/disc), penicillin G (10 U/disc), tetracycline (30 µg/disc), gentamycin (10 µg/disc), bacitracin (10 µg/disc), ampicillin (10 µg/disc), novobiocin (30 µg/disc), doxycycline (30 µg/disc), and amikacin (30 µg/disc)] were placed on the *S. aureus* swabbed MHA with 7% NaCl, and incubated for 12 hrs at 35°C for screening MRSA.

Confirmation of MRSA using Real Time PCR amplification of *mec-A* gene

Bacterial strains such as MRSA isolates, MSSA isolates and MTCC 87 were used in this study.

1. Chromosomal DNA was prepared by using Murray and Thompson (1980) methodology.
2. Oligonucleotides were obtained from Medox India, Ltd.
3. 5′-GTT GTA GTT GTC GGA TTT GG-3′ (upstream primer) was obtained from Medox India, Ltd.
4. 5′-CTT CCA CAT ACC ATC TTC TTT AAC-3′ (downstream primer) was obtained from Medox India, Ltd.
5. Finzyme PCR kit (Finland).
6. Name of the Instrument: Eppendorf Master Cycler.

The DNA extracts (600 ng) were amplified by RT-PCR in a final volume of 50 µl containing 0.25 mM of each dNTP, 40 pmol of each primer, 0.5 IU *Taq* DNA polymerase and buffer provided by the manufacturer. The denaturation was performed for 1 min at 95°C; the annealing for 1 min at 58°C, and the primer extension for 2 min at 72°C with a total of 30 cycles. A sample of 10 µl from each reaction was analyzed by gel electrophoresis in a 0.8% agarose gel with ethidium bromide, and results were observed.

RESULTS AND DISCUSSION

Totally 261 pus samples were collected from patients at Madurai, Trichy, Salem and Vellore districts of Tamil Nadu, India. They transferred to NA medium. The colonies were pinheaded, white, smooth, circular and convex in appearance and coagulase positive. All the isolated were Gram positive, and produced β-haemolysis on blood agar medium and turned pink to yellow colour on MSA medium. Colonies were transferred to gelatin medium using stab culturing method and incubated overnight. The gelatin failed to solidify at 4°C the following day, indicating the gelatin liquefying nature of the isolates. As per Kirby-Bauer disc diffusion antibiotic assay, the isolates showing resistance towards methicillin were selected for further study. To confirm the MRSA isolated in the present study RT-PCR analysis was conducted and the presence of the *mec-A* gene was tested. Interestingly, 180 isolates of MRSA were also RT-PCR positive. The specific DNA band was observed at 310 bp in all the strains isolated in the present study (**Fig. 1**).

Barber and Waterworth in 1962, were unable to confirm the difference in the destruction of the isoxazolylpencillins by Stewart's strain, compared with methicillin-sensitive *Staphylococci.* Most researchers have found that cultures of resistant *Staphylococci* are composed of a mixture of cells,

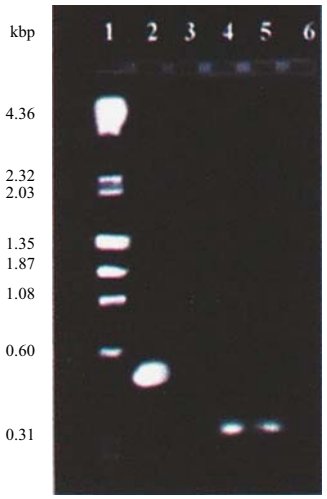

Fig. 1 *mec-A* gene amplification of MRSA isolates. 1 – Marker, 2 – MTCC 87, 3, 6 - MSSA, 4, 5 – MRSA strains.

the majority showing sensitivity to methicillin and a much smaller percentage representing highly resistant variants. But the present study clearly differentiates between MRSA and MSSA culture isolates. Most scientists introduce 5% NaCl into the growth medium. However, an osmotic pressure study was conducted to identify MRSA and MSSA, and in which all isolates showed methicillin sensitivity in the absence of 5% NaCl (Alfizah *et al*. 2002). In the present study also, MRSA isolates showed a similar character. Strains of *Staphylococci* with multiple drug resistance are widespread. They appear and spread rapidly after the introduction of new antimicrobial agents. Similar observations found in the following experimental studies. The genome types represented stable differences in the investigated *S. aureus* strains. The DNA fragment patterns remained unchanged even after numerous DNA preparations. Such findings were also reported by Vannuffel *et al*. (1995). Vangriethuysen *et al.* (1999) amplified a 298-bp fragment of the *mec-A* gene with the primers 5′-GTT GTA GTT GTC GGA TTT GG-3′) and 5′-CTT CCA CAT ACC ATC TTC TTT AAC-3′ specific for the *mec-A* gene. Their study slightly varied from the current research because, they got 298 bp fragmented *mec-A* gene in MRSA isolates. A second set of primers was included in each reaction mixture to amplify a polymorphic region of the coagulase gene that varied between 350 and 600 bp. The coagulase primers specific for the coagulase gene were 5′-CTG GTA TCC GTG AAT A-3′ (upstream) and 5′-TTG TAT TGA CTG TAT GTC TTT GGA-3′ (downstream). The latter primers provided an internal control to check the presence of *S. aureus* DNA and for the absence of PCR inhibitors. MRSA isolates yielded two PCR products, the *CoA* amplicon and the 298-bp *mec-A* amplicon. Cui *et al.* (2003) amplified DNAs encompassing the entire SCC *mec-A* sequence by long-range PCR with several primer sets. The region from the left extremity to the CCR genes (L-C region) was covered by primer sets α5 and cLs1 (CA05) or CL 26(8/6-3p). Primers, α6 and mcR8 were used to detect the 1-R region (Ito *et al.* 2001; Levy *et al*. 2001) spanning a region from Tn 554 to the right extremity of SSC *mec*, which was amplified by long-range PCR with two sets of primers, TnpA 1016 and mN13 and cR1.

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