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Identification of Multi-Resistant *Staphylococcus aureus* in Clinical Specimens

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Abstract: In the present study, highly resistant *S. aureus* were isolated. Identification of multiple genes (*mec*A, *erm*A, *van*A) for 5 multi-resistant *S. aureus* strains have been attempted. These isolates contain 533 bp *mec*A fragment, 139 bp *erm*A fragment and 1030 bp *van*A fragment by multiplex PCR method. Also antimicrobial susceptibility testing by disk diffusion method was evaluated for 360 *S. aureus* isolates against 20 antimicrobial agents. Among the 360 isolates, 117 isolates were resistant to >14 antimicrobial agents. The prudent use of antibiotics and rapid and continuous screening for resistant microorganisms should be more focused to prevent the emergence and spread of multi resistant MRSA strains.

Key words: *S. aureus*, MRSA, erythromycin, vancomycin, VRSA, *mec*A, *erm*A, *van*A, India

INTRODUCTION

Staphylococcus aureus is one of the most important human pathogens causing skin and tissue infections, deep abscess formation, pneumonia, endocarditis, osteomyelitis, toxic shock syndrome and bacteremia (Tenover and Gaynes, 2002). The first antibiotic used in the control of *S. aureus*, penicillin G developed resistance within 2 years of its introduction and has increased gradually over the past 50 years. Methicillin-resistant strains of Staphylococci were identified immediately upon the introduction of methicillin into clinical practice. Methicillin-resistant *S. aureus* (MRSA) was initially identified for the first time in 1961 (Jevons, 1961; Barber, 1961).

MRSA is frequently resistant to most of the commonly used antimicrobial agents including the aminoglycosides, macrolides, chloramphenicol, tetracycline and fluoroquinolones (Mandell *et al*., 1995). In addition, MRSA strains should be considered to be resistant to all cephalosporins, cephems and other β-lactams (such as ampicillin-sulbactam, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, piperacillin-tazobactam and the carbapenemes) regardless of the *in vitro* test results obtained with those agents (National Committee for Clinical Laboratory Standards, 2000).

Until recently, the antibiotic vancomycin has been the last weapon against strains of *S. aureus* that shows resistant to all other antibiotics (especially MRSA). After extensive research it was found that *S. aureus* acquired resistance to vancomycin also (Hiramatsu *et al*., 1997) Worldwide, many strains of *S. aureus* are already resistant to all antibiotics and thus the organism has progressed one step closer to becoming an unstoppable killer. The emergence and rapid spread of this organism has created important new challenges for infection prevention and control services in hospitals and other health care facilities.

In Indian hospitals, MRSA is one of the common causes of hospital-acquired infections and different hospitals have reported anywhere from 30-80% methicillin resistance based on a ntibiotic sensitivity tests (Anupurba *et al*., 2003). Interestingly, there appears to be significant variable in the epidemiology and prevalence of MRSA in different parts of the world and even in different regions of a country (Voss *et al*., 1994). Hence, constant monitoring of these strains is essential in order to control their spread in the hospital environment and transmission to the community.

The objective of the present study is to identify the life threatening, highly antibiotic resistant *S. aureus* strains and further to identify multiple genes responsible for resistance to these antimicrobial agents from the clinical specimens from Hyderabad, South India.

MATERIALS AND METHODS

Staphylococcal strains: A total of 360 *Staphylococcus aureus* isolates were investigated from a period between March 2008 to February 2009. These strains were collected from various clinical specimens including pus, urine, wound swabs, blood, sputum, CSFand pleural fluid from Osmania Hospital and Durgabai Deshmukh hospital and research center, Hyderabad, Andhrapradesh, India.

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Media and culture conditions: All clinical samples were first inoculated into blood agar and brain heart infusion agar plates (Hi-media, India) and incubated at 37°C for 24 h. Coagulase, catalase tests were carried out on the *Staphylococci* isolates as per standard procedures. Mannitol fermentation was observed by inoculating the isolates onto mannitol salt agar (Hi-media, India) and plates were incubated at 37°C for 24-48 h (Baird, 1996).

Antimicrobial susceptibility test: Susceptibility was measured by disc agar diffusion method using the following discs; Amikacin (Ak) 10 μg, Ampicillin (A) 10 μg, Amoxicillin (Am) 25 μg, Cephalexin (Cp) 30 μg, Cephotaxime (Cx) 30 μg, Ceftazidime (Ca) 30 μg, Chloramphenicol (C) 30 μg, Clindamycin (Cd) 10 μg, Ciprofloxacin (Cf) 5 μg, Co-trimoxazole (Co) 25 μg, Erythromycin (E) 15 μg, Gentamycin (G) 50 μg, Nalidixic Acid (Na) 30 μg, Norfloxacin (Nx) 10 μg, Oxacillin (Ox) 1 μg, Penicillin G (P) 10 U, Rifampin (R) 15 μg, Streptomycin (S) 25 μg, Tetracycline (T) 30 μg, Vancomycin (Va) 30 μg.

Mueller-Hinton agar plates were overlaid with the inoculum (turbidity equivalent to that of a 0.5 McFarland standard) of the *S. aureus* clinical strains. Zone diameters were measured at 24 and 48 h as recommended by the National Committee for Clinical Laboratory Standards (2000).

Primers: The primer sets used in this study is shown in Table 1 have been described by Martineau *et al.* (1998, 2000), Perez-Roth *et al.* (2001), Louie *et al.* (2000) and Tiwari and Sen (2006).

Multiplex polymerase chain reaction: The multiplex PCR was performed according to Perez-Roth *et al.* (2001) in a total volume of 25 μL containing 80 mM MgCl2, PCR buffer, 3.5 mM DNTP mix (Fermentas), 10 picomole μL-1 of each of the primers shown in the Table 1 and 1 unit of Taq polymerase with 1 μL of bacterial suspension. Amplifications were carried out by using a thermal cycler with the following thermal cycling profile: an initial denaturation step at 94°C for 5 min was followed by 10 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 45 sec) and 25 cycles of amplification (denaturation at 94°C for 45 sec, annealing at 50°C for 45 sec and extension at 72°C for 1 min) ending with a final extension step at 72°C for 10 min. After amplification, 10 μL of the reaction mixture was loaded onto a 2% agarose gel and electrophoresed to estimate the sizes of the amplification products with a 100-bp molecular size standard ladder.

Table 1: Primers used for multiplex PCR for the identification of *S. aureus* and detectionof its antibiotic resistant genes

Gene	Primer sequences (5'-3')	Amplicon size (bp)
*mec*A	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533
*erm*A	TATCTTATCGTTGAGAAGGGATT CTACACTTGGCTTAGGATGAAA	139
*van*A	CATGAATAGAATAAAAGTTGCAATA CCCCTTTAACGCTAATACGATCAA	1030

RESULTS AND DISCUSSION

According to the results of cultural and biochemical properties, 360 isolates used in the present investigation were identified as *S. aureus*. All the 360 strains were positive for coagulase, mannitol fermentation and catalase test. The antibiotic-resistance profile was determined by disc-diffusion method and oxacillin agar screen plate were used to identify MRSA. The present study included 20 antimicrobial agents for 360 *S. aureus* isolates, the results shows 117 isolates (32.5%) were resistant to >14 antimicrobial agents. The resistance pattern to different antimicrobial agents is as shown in the Fig. 1.

Higher resistance rates were noted for penicillin G (90%), oxacillin (79.1%) followed by ceftazidime (68.3%), ampicillin (63.3%), cephotaxime (53.6%), cotrimoxazole (50%), norfloxacin (49.7%), cephalexin (48.6%), nalidixic acid (48.6%), rifampin (45%). Low rate of resistance were seen for streptomycin (21.1%), ciprofloxacin (20.2%), clindamycin (15.5%) and chloramphenicol (11.6%). But majority of the *S. aureus* strains were susceptible to vancomycin but only 7 strains (1.9%) were resistant to vancomycin. All VRSA were resistance to methicillin and only 5 were resistant to erythromycin.

A rapid and reliable molecular approach (multiplex PCR) for the detection of multidrug resistance MRSA was used to confirm the presence of resistance genes in *S. aureus*. Multiplex PCR was done for the amplification of *mec*A for methicillin resistance, *erm*A for erythromycin resistance and *van*A gene for vancomycin resistance. Amplification of these three genes was done for 5 *S. aureus* strains which is resistant to methicillin, erythromycin, vancomycin and were also resistant to >17 antimicrobial agents. These isolates contain 533 bp *mec*A fragment, 139 bp *erm*A fragment and 1030 bp *van*A fragment by multiplex PCR method.

Nosocomial infections caused by multi resistant Staphylococci are a growing problem for many healthcare institutions (Kloos and Bannerman, 1995). The emergence of resistant pathogens, particularly gram positive pathogens is an important factor in the morbidity and mortality of the hospitalized patients. According to a recent report by the World Health Organization, Drug resistant infections in rich and developing nations alike are threatening to make once treatable diseases incurable. This chilling announcement fits most accurately *Staphylococcus aureus*, the number one cause of

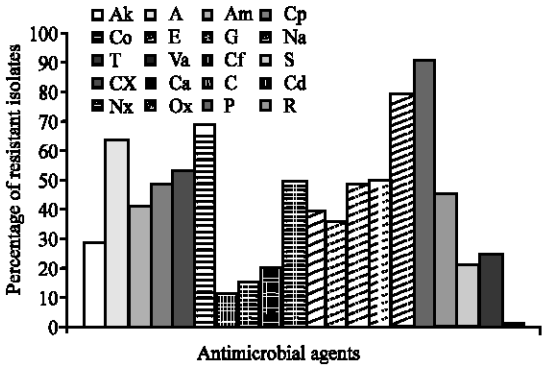

Fig. 1: Resistance pattern of *S. aureus* isolates to antimicrobial agents

potentially life-threatening hospitalized patients. Among 360 isolates, 285 (79.1%) were resistant to oxacillin and were positive for the *mec*A gene. The other antimicrobial susceptibility tests revealed that the isolates had the characteristics of general multidrug resistance. Majority of the MRSA isolates were resistant to penicillin, oxacillin, ampicillin, amoxycillin, cephalexin, cephotaxime and ceftazidime and were less susceptible to vancomycin, chloramphenicol, ciprofloxacin and tetracycline (Fig. 1). In the face of this growing resistance among these organisms, the selection of the correct antimicrobial and nonpharmacological interventions, based on correct identification and susceptibility test data has become increasingly challenge.

During the last decade, several studies have demonstrated the extremely high capacity of PCR for specifically detecting bacteria and genes of interest (Salisbury *et al*., 1996). That ability has revealed PCR as a powerful tool in clinical microbiology studies (Cockerill, 1999). Genotyping identification of *S. aureus* and its antibiotic resistant genes has been used based on the detection of different specific target sequences like *mec*A (oxacillin resistance), *erm*A (erythromycin resistance), *van*A (vancomycin resistance). Although, previous studies have reported the application of PCR for the accurate detection of the various antibiotic resistant genes and the possibility of simultaneous identification of *S. aureus* no reports were evidenced for the identification of *S. aureus* and the detection of methicillin, erythromycin and vancomycin resistance in a single tube.

CONCLUSION

In this study, researches have identified increased resistance of *S. aureus* strains from Hyderabad, South India and further identification of multiple genes (resistant to antimicrobial agents) from multi resistant *S. aureus* has been carried out by multiplex PCR technique. Although, the study has been limited to Hyderabad, the emergence of multi resistant *S. aureus* strains might also be prevalent in other parts of India. Hence, the prudent use of antibiotics and rapid and continuous screening for resistant microorganisms should be more focused to prevent the emergence and spread of multi resistant *S. aureus* strains.

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