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Emergence of Multi-Drug Resistant Clinical Strains of *Staphylococcus aureus*

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ABSTRACT

Indiscriminate and wide spread use of antibiotics has lead to the development of multi-drug-resistant strains of pathogenic *Staphylococcus aureus.* Information regarding increase (in percentage) of existing resistance as well as emergence of new resistance to different antibiotics used for staphylococcal infections are insufficient. This study explores a comparative analysis of growing resistance to different antibiotics mainly ampicillin, methicillin, erythromycin, gentamicin, clindamycin and vancomycin against *S. aureus* isolated from Kolkata hospitals during two phases. During first phase (126) and second phase (67) non-repeat clinical strains of *S. aureus* obtained from different hospitals of Kolkata were identified by standard biochemical methods. However, PCR amplification of *nuc* gene and rDNA was also performed for identification of *S. aureus*. Antibiotic susceptibility pattern was determined by Disc Agar Diffusion tests and *mecA* was identified by PCR. Comparative analysis of antibiotic resistance pattern of the strains isolated during two phases showed significant difference (p=0.05) with 75% increase of resistance to erythromycin followed by 30% increase to ampicillin, chloramphenicol and streptomycin with the appearance of vancomycin resistance. Gentamicin and methicillin resistance have increased by 22% and 7% respectively. On the other hand, *mecA* was obtained by PCR from vancomycin resistant *S. aureus* strain, which was also resistant to methicillin, erythromycin and clindamycin. This study reveals tremendous increase of resistance to erythromycin and a remarkable increase to other antibiotics with emergence of multi-drug-resistant clinical strains of *S. aureus*. This trend in increasing resistance to the commonly used antibiotics against *S. aureus* cannot be controlled until and unless antibiotics are used more prudently.

Key words: Erythromycin resistance, *mecA*, MRSA, multi-drug resistance, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus, a major cause of potentially life-threatening infections acquired in health care and community settings. *S. aureus* causes a wide variety of suppurative diseases in man, including superficial and deep abscesses, and wound infections [1]. In addition, Staphylococcus can cause skin, heart valve, blood and bone infection, which can lead to septic shock and death. More than 90 % of *S. aureus* elaborate penicillinases or beta-lactamases and 20 – 30 % of *S. aureus* are methicillin resistant (MRSA) [2, 3]. The prevalence of MRSA in India is also on the rise and there are reports of detecting MRSA in community acquired infections though the prevalence is much lesser [4, 5]. MRSA strains also demonstrate a high degree of resistance to other antibiotics especially beta-lactams, and non-ß-lactam antibiotics, such as macrolides, lincosamides, quinolones, tetracyclines and aminoglycosides. In particular, the majority of MRSA strains are not susceptible to macrolides and aminoglycosides, because the genes *ermA* and *aadD* encoding resistance to these drugs are usually conserved within *mec* DNA, and located upstream and downstream, respectively, of the *mecA* gene [6]. Erythromycin resistance in *Staphylococcus aureus* is part of the macrolide-lincosamide-streptogramin B resistance phenotype. This phenotype was first described by Chabbert shortly after the introduction of erythromycin in clinical practice [7].

This is an endeavor to get a clear picture of the current trends in antibiotic resistance among the clinical strains of *S. aureus* isolated from Kolkata hospitals and to make a comparative analysis of antibiotic resistance pattern among the strains isolated in two phases.

MATERIALS AND METHODS

Bacterial strains:

First phase collection: Earlier (a few years back), one hundred twenty six (126) strains of *S. aureus* were collected from the Calcutta Medical College & Hospital (CMC). Second phase collection: Sixty seven (67) non-repeat clinical isolates of *S. aureus* have been collected recently from various Kolkata hospitals, which are the Calcutta Medical College & Hospital (CMC), School of Tropical Medicine (STM), Institute of Child Health (ICH), R. G. Kar Medical College & Hospital (RGK), Nilratan Sirkar Medical College and Hospital (NRS) and Seth Sukhlal Karnani Medical College & Hospital (SSKM). All these strains were collected to study the antibiotic resistance profile of *S. aureus*. For collection of strains, no specific criterion was adapted. All cultures were grown in nutrient agar (NA) medium and purified by single colony isolation technique in NA containing 10 % sodium chloride.

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Confirmation of species identification:

Identification of the clinical isolates of *S. aureus* was performed by traditional biochemical tests, including catalase, coagulase, mannitol fermentation tests, and Gram-staining [8,9]. PCR amplification of *nuc* gene [10] was performed. PCR amplification of 16S rDNA [11] was also performed.

Antibiotic susceptibility testing:

Antibiotic resistance profile was determined by Disc Agar Diffusion (DAD) technique [12,13], using eighteen antibiotic discs. Among these antibiotics, some discs were prepared in this laboratory and a few were obtained commercially from Himedia (Mumbai, India). The name of antimicrobials, its manufacturer and the concentration in µg of antimicrobials per disc, were as follows: amoxycillin (AMX, Rexcel, India; 30µg), ampicillin (AMP, Biochem Pharmaceutical Industries, India; 10µg), cefepime (FEP, Unichem Laboratories, India; 30µg), cefotaxime (CTX, Alkem Laboratories Ltd, India; 30µg), cefuroxime (CXM, Glaxo Smith Kline, India; 30µg), cephalexin (LEX, Ranbaxy, India; 30µg), chloramphenicol (CHL, Sigma, USA; 30µg), ciprofloxacin (CIP, Pharma(Ran), India; 5µg), clindamycin (CLI, Indipharma, India; 2µg), erythromycin (ERY, Alembic, India; 15µg), gentamicin (GEN, Nicholas, India; 10µg), methicillin (MET, Himedia, India; 5µg), oxacillin (OXA, Himedia, India; 1µg), rifampicin (RIF, Lupin, India; 5µg), roxithromycin (ROX, Alembic, India; 15µg), streptomycin (STR, Synbiotics Ltd. India; 10µg), trimethoprim-sulfamethoxazole (1:5) (SXT, Piramal Health Care, India; 5µg) and vancomycin (VAN, Lilly Pharma, Germany; and Himedia, India; 30µg). *S. aureus* ATCC25923, an all sensitive reference strain was used as a quality control strain for DAD test.

The test bacteria, taken from an over-night culture (inoculated with a single colony) were freshly grown in MHB for 4 hours at 37^0 C. A bacterial lawn was prepared on MHA plate by spreading with this 4hours culture. Filter paper discs of 6 mm size soaked in antibiotic solutions (conc. of the antibiotics per disc are given above) were put in the inoculated MHA plate and incubated overnight at 37^0 C. The zone of bacterial growth inhibition surrounding the antibiotic disc was measured and compared with a standard for each drug. This result gives a profile of drug susceptibility vis-à-vis antibiotic resistance.

Determination of Minimum Inhibitory Concentration:

Minimum Inhibitory Concentration (MIC) of vancomycin was determined by broth microdilution method using Mueller-Hinton broth (MHB, dehydrated, Himedia, Mumbai) as recommended by National Committee for Clinical Laboratory Standards, renamed as CLSI [14]. The cut–off MIC for vancomycin resistance used by us was 32µg/ml. Most isolates of *S. aureus* are susceptible to vancomycin. The concentration of vancomycin required to inhibit these strains (called the minimal inhibitory concentration or MIC) is typically between 0.5 and 2 micrograms/mL (µg/mL). In contrast, *S. aureus* isolates for which vancomycin MICs are 4-8 µg/mL are classified as vancomycin-intermediate, and isolates for which vancomycin MICs are ≥16 µg/mL are classified as vancomycin-resistant. The revised definitions for classifying isolates of *S. aureus* are based on the interpretive criteria published in January 2006 by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [14].

Preparation of genomic DNA:

Genomic DNA was prepared following the standard protocols.

Polymerase Chain Reaction (PCR):

The PCR amplification was performed with the thermal cycler ABI 9700 (ABI, Foster city, USA), in a volume of 50 µl. For amplification of *nuc* gene and *mecA*, the following components were used: 1.5 mM $MgCl_2$, 200 µM each of dATP, dTTP, dGTP, dCTP, 2 µM of each primer, 0.1 µg of template DNA, and 1.25 U of *Taq* polymerase (Invitrogen, Brazil).

i) PCR amplification of *nuc* gene :

Partial *nuc* gene was amplified using *nuc* Forward (5'-GCGATTGATGGTGATACGGTT-3') and *nuc* Reverse (5'-AGCCAAGCCTTGACGAACTAAAGC-3') PCR primers, which were selected on the basis of the published nucleotide sequence of the 966 bp *nuc* gene derived from *S. aureus* Foggi strain [10]. The cycling parameters consisted of 30 cycles of denaturation at 94^0C for 30 sec, primer annealing at 50^0C for 1 min and extension at 72^0C for 1 min 30 sec.

ii) PCR amplification of *mecA* :

For amplification of *mecA*, oligonucleotide primers *mecA*-Forward (5'-TGGCTATCGTGTCACAATCG-3') and *mecA*-Reverse (5'-CTGGAACTTGTTGAGCAGAG-3'), were used [15]. The reaction condition was 30 cycles of denaturation at 94^0C for 40 sec, primer annealing at 52^0C for 45 sec and extension at 72^0C for 30 sec.

Statistical Analysis:

The statistical analysis of antibiotic resistance between the two periods was performed by following the conventional two- tail't'- test.

RESULTS AND DISCUSSION

126 pathogenic strains of *Staphylococcus aureus* were obtained from various patients from the Calcutta Medical College and Hospital in 1st phase and 67 strains from the Calcutta Medical College and Hospital as well as from other Medical Colleges and Hospitals of Kolkata in 2nd phase. The clinical records of the strains of *S. aureus* collected from the Calcutta Medical College and Hospital in 1st phase showed that strains collected from male patients were 62.7% and from female patients were 37.3%. Strains collected from indoor patients were 76% and from outdoor patients 24%. The sources were pus, blood, sputum, urine, throat swab, wound swab, vaginal swab, cerebrospinal fluid (CSF) etc. Maximum numbers of *S. aureus* strains were found from the pus.

The strains were gram-positive, catalase and coagulase positive, and mannitol fermenting.
Antibiotic resistance profile (of the strains collected in 1st phase) obtained by Disc Agar Diffusion (DAD) tests showed that maximum strains of *S. aureus* were resistant to beta-lactam group of antibiotics, i.e. 86% strains of *S. aureus* were penicillin resistant followed by 66% to ampicillin. MRSA were found to be 23%. Next peak of resistance was observed of tetracycline (54%), followed by chloramphenicol (41%). Aminoglycosides resistances were found to be as follows streptomycin, 38%; kanamycin, 34%; gentamicin, 3%. Macrolide (erythromycin) resistance was 17%.

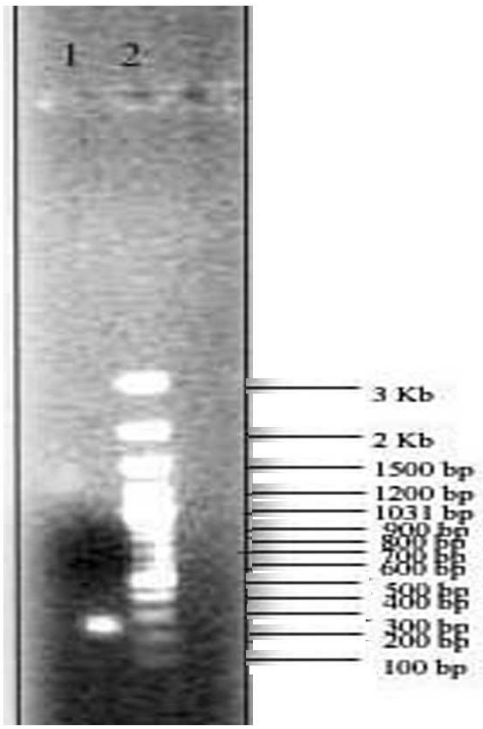

Figure 1: Agarose gel electrophoresis of PCR amplified *nuc* gene of a clinical strain of *S. aureus*. Lane: 1, amplified product of *nuc* gene (270 bp). Lane: 2, 100 bp DNA ladder as molecular size marker.

Sixty seven clinical isolates of *S. aureus* collected from various Kolkata hospitals in 2nd phase were also identified by gram-staining and other traditional biochemical tests. They were also gram-positive, catalase and coagulase positive, and grown on mannitol-salt-agar (MSA) medium. From these strains (2nd phase) one strain of *S. aureus*, which was found to be vancomycin resistant, was identified by PCR amplification of *nuc* gene encoding thermonuclease, which is highly specific for *S. aureus* [Figure 1]. The PCR amplification of rDNA for this strain was also performed [data not shown].

Antibiotic resistance profile of the strains (obtained in 2nd phase) found by Disc Agar Diffusion (DAD) tests showed maximum resistance to ampicillin (95%), followed by erythromycin (92%), roxithromycin (86%), chloramphenicol (71%), streptomycin (67%), gentamicin (25%), methicillin, oxacillin (30%), Strains were also resistant to cotrimoxazole (80%), amoxycillin (78%), ciprofloxacin (77%), and clindamycin (57%). Cephalosporin group of antibiotics e.g., cefepime (4th generation cephalosporin), cefotaxime (3rd generation), cefuroxime (2nd generation), and cephalexin (1st generation), resistances were also found to a great extent. Resistances to vancomycin and rifampicin were also observed. The majority of methicillin resistant *S. aureus* (MRSA) strains were also resistant to erythromycin, streptomycin, kanamycin and clindamycin. The methicillin resistant strains MC48 and STM2 were also found to be erythromycin and clindamycin resistant. STM2 was also resistant to the glycopeptide antibiotic vancomycin. 49% of clinical strains of *S. aureus* were found to be vancomycin resistant apparently by disc agar diffusion test. The size of zone of inhibition of bacterial growth for vancomycin sensitive strain is 15 mm or more (for *S. aureus* ATCC25923 it is 17-21 mm); 14 mm or less for VRSA. The sizes of zone of inhibition of 32 strains among 67 strains of *S. aureus* were found to be 12-14 mm. But for *S. aureus* STM2 it was only 6 mm. So for further confirmation, MIC determination was performed. The MIC value of the strain STM2 was found to be 64 µg/ml but, for the remaining 32 (49%) strain, it was only 1-2 µg/ml.

Finally a comparative analysis of antibiotic resistance pattern of the clinical isolates of *S. aureus* obtained in 1st phase and 2nd phase was done [Figure 2], which showed 30% increase of resistance to ampicillin, chloramphenicol, and streptomycin. Maximum increase of resistance (75%) was observed to erythromycin. Gentamicin resistance has increased by 22%. Seven percent increase of resistance to methicillin was also noticed. The statistical analysis of antibiotic resistance between the two phases was performed

by following the conventional two-tail't'- test and it was found that the difference of antibiotic resistance between these two periods is significant at P level of 0.05.

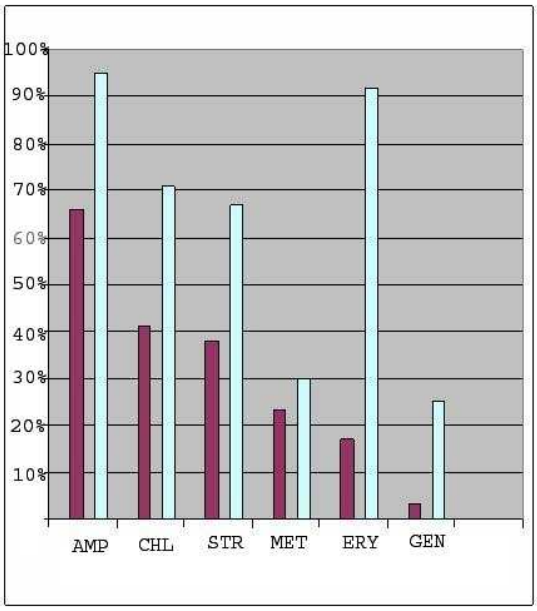

Figure 2: A comparison of antibiotic resistance pattern of clinical strains of *S. aureus* obtained during 1st phase (Red Bar) and 2nd phase (Blue Bar). Antibiotics are, AMP: ampicillin; CHL: chloramphenicol; STR: streptomycin; MET: methicillin; ERY: erythromycin; GEN: gentamicin.

A dramatic increase in the number of health care and community associated infections due to macrolide resistant *S. aureus*, clindamycin resistant *S. aureus* and MRSA highlight the success of this species *S. aureus* as a pathogen and its ability to adapt under pressure from antimicrobial agents. More than 30% of *S. aureus* have been reported to be MRSA. Vancomycin (the drug of last resort for enterococcal and staphylococcal infections) resistant S. *aureus* (VRSA) has also been reported from India [16]. Erythromycin resistance and clindamycin (used for soft tissue and bone infection) resistance are increasing very sharply. Apart from this it has been reported that methicillin resistant *S. aureus* is also resistant to all other beta-lactam and non-beta-lactam antibiotics [6]. But there is no report of the pattern / trends in increasing resistance to different antibiotics against clinical strains of *S. aureus* in the last few decades and the emergence of new antibiotic resistance from Kolkata as well as India, which motivated us to make a thorough investigation about the current trends in antibiotic resistance among the clinical strains of *S. aureus* from Kolkata (India).

Strains collected from indoor patients were 76 % and from outdoor patients were 24%. Hence the maximum strains of *S. aureus* collected from the Calcutta Medical College and Hospital were indoor strains. The resistance pattern of clinical isolates of *S. aureus* of 1st phase showed that penicillin resistance had reached very close to 90% as reported by other authors also [2, 3]. Ampicillin resistance was also found to be very high i.e. 66%. But at that period erythromycin and other aminoglycosides resistance was very low.

But the antibiotic resistance profile of the strains of 2nd phase showed maximum resistance to ampicillin (95%), followed by erythromycin (92%). The β-lactam group of antibiotics became almost useless as because ampicillin resistance has reached to nearly 100%, followed by amoxycillin, and cephalosporins. Resistance to macrolides has increased significantly very high in the 2nd phase i.e. erythromycin, 92% and roxithromycin, 86%; most probably due to indiscriminate and wide use of erythromycin and roxithromycin for any skin infection as well as other infectious diseases. Many times we get infections due to multiple micro-organisms; that is why clinicians use broad-spectrum antibiotics. As a result the co-infecting other pathogens and non-pathogens become resistant to all commonly used antibiotics. Here, in this study, we can see that ciprofloxacin, chloramphenicol, cotrimoxazole resistances have reached to 77%, 71%, and 80% respectively which are not generally used for *S. aureus* infection. So the multi-drug-resistance phenomena are increasing more and becoming more complicated due to acquiring multi-drug-resistance genes from the gene-pool existing in nature due to indiscriminate and wide use of antibiotics. The MRSA strains utilize PBP2A (*mecA*) for the formation of cell-wall. But the other penicillin resistant *S. aureus* strains form cell-wall using other PBPs. This result also showed high ampicillin resistance (95 %) and amoxicillin resistance (78 %). This large difference of sensitivity is due to the variation of minimal inhibitory concentration (MIC) of antibiotics. The MIC of an antibiotic is not same for all strains; that is why, the sensitivity varies from strain to strain. It is one of the reasons of strain difference.

We have obtained PCR amplified product of *mecA* gene from VRSA STM2 (data not shown), which was also methicillin resistant, erythromycin resistant and clindamycin resistant. So the *mecA* gene in VRSA STM2 may also contain erythromycin resistance gene *ermA*, which strongly supports the statement of Chambers, the majority of MRSA strains are not susceptible to macrolides and aminoglycosides, because the genes *ermA* and *aadD* encoding resistance to these drugs are usually conserved within *mec* DNA [6].
Among the strains collected in 1st phase and 2nd phase we have obtained many multi-drug-resistant (MDR) *S. aureus* strains; because they are methicillin resistant, erythromycin resistant, clindamycin resistant as well as aminoglycosides resistant. Those are MC3711, MC139, MC154, MC188, MC327, MC387, MC703, MC790,

MC905, MC123, MC2887, MC207, MC48, MC50, STM2, NRSp146, NRSpL, NRSp157. We have obtained plasmid DNAs of different sizes from MC123, MC154, MC790 and MC2887 also, which may harbor *mecA* and *erm* genes in these organisms having Chabbert phenotype [7].

Finally, a comparative analysis of antibiotic resistance pattern of clinical isolates of *S. aureus* obtained in 1st phase and 2nd phase was done [Figure 2], which showed 30% increase of resistance to ampicillin, chloramphenicol, and streptomycin. Maximum increase of resistance (75%) was observed to erythromycin which is alarming and a great concern to the globe. Gentamicin resistance has also increased by 22%.

CONCLUSION

Clinicians' prior choice is erythromycin or roxithromycin for any staphylococcal / any gram-positive bacterial infections. But 92% of *S. aureus* have become erythromycin resistant today. 30 % *S. aureus* are methicillin resistant; and if they contain *mecA* then there is a possibility to harbor *erm* gene classes and *aadD* genes in these strains, which might be a great risk factor for any severe gram-positive bacterial infection. So there is a great problem about the next choice of antimicrobial agent for treatment of staphylococcal and other gram-positive bacterial infections. Hence the time has come for thinking about the prudent use of antibiotics and carrying out antibiotic stewardship with more research all over the globe.

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