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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS ISOLATED FROM HOSPITAL SAMPLES

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ABSTRACT

In the present study, Staphylococcus aureus was isolated from hospital samples and tested it for antibiotic sensitivity test. On antibiotic sensitivity testing Staphylococcus aureus showed resistance to three antibiotics out which two are β-lactum containg antibiotic that is for Methicillin and ampillicin. PCR analysis for mec A gene in Staphylococcus aureus shows that the gene is nearly 310 bp in length. Ammonium sulphate precipitation and sephadex G-100 were used to purifying the PBP2a enzyme. An increase in PBP2a activity showed after purification of crude enzyme by ammonium sulphate precipitation and Sephadex G-100. Molecular weight of the PBP2a from Staphylococcus aureus was determined by SDS-PAGE gel electrophoresis and it was found to be a single protein band with 41 kDa molecular weight.

INTRODUCTION

Staphylococcus aureus is an important opportunistic pathogen responsible for a variety of diseases. Random prescription of antibiotics caused severe antibiotic resistance especially against commonly used drugs (Momtaz and Hafezi, 2014). Methicillin-resistant Staphylococcus aureus (MRSA) has spread to become one of the most important nosocomial and community pathogens worldwide (Gillet *et al.*, 2002). In the case of Methicillin-resistant *Staphylococcus aureus* (MRSA), increased rates of MRSA infections are seen with especially quinolones glycopeptides, and cephalosporins (Tacconelli *et al.*, 2008; Muto *et al.*, 2003). In the case of colonization with *C. difficile the* high risk antibiotics include cephalosporins and in particular quinolones and clindamycin (Vonberg *et al.*, 2009; Kuijper *et al.*, 2007). SCC*mec is* Staphylococcal cassette chromosome *mec* (SCC*mec*). The *mecA* gene crried by this chromosome which it conseder the main reason that made *Staphylococcus* resist to methicillin (Hiramatsu, 1995; Hiramatsu *et al.*, 2001; Kreiswirth *et al.*, 1993; Oliveira *et al.*, 2001; Musser *et al.*, 1992; Givney *et al.*, 1998; Crisostomo *et al.*, 2001; Enright *et al.*, 2002; Lowy *et al.*, 2003; Pantosti *et al.*, 2007). SCC*mec* also contains other genes in addition to *mecA*, involving the cytolysin gene *psm-mec*, which restrains virulence in hospital- acquired MRSA strains (Kaito *et al.*, 2011). Currently; six unique *SCCmec* types with size from 21–67 kb have been discovered. According to the *SCCmec* genes size and the gene transfer (horizontal), a few number of clones may to be accountable for the prevalence MRSA infections (Kuo *et al.*, 2012). mecA is responsible for resistance to methicillin and other β-lactam antibiotics. After obtaining of mecA, the gene should be integrated and localized in the Staph. aureus chromosome. 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 (Berger-Bächi, 1999). PBP2a can continue to catalyze the transpeptidation reaction required for peptidoglycan cross-linking, where the cell wall become able to synthesize in the presence of antibiotic. Today approx. 35% of hospital strains of *Staph. aureus* are resistant to methicillin (or other penicillin antibiotics), and the up growth of vancomycin-resistant *Staph. aureus* (VRSA) has caused additional concern in recent years (Wielders *et al.*, 2002). Resistance occurs when the organism has a mecA gene producing an altered penicillin binding protein, PBP2a and either an oxacillin MIC of 2mg/l or a methicillin MIC of 4mg/l. Infected and colonised patients are the reservoir of MRSA both in hospitals and the community with transmission generally being via contact with health workers (Martin and Hardy, 1991).

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MATERIALS AND METHODS

2.1. Sample collection

The 20 specimens were obtained from different patients, age groups, and years under medical attention from Osmania hospital, Hyderabad. The specimens obtained were wound swabs, nasal swabs and urine samples. These isolates were identified by different methods including the biochemical characterization tests, API-20E, biological identification systems. By using Kirby -Bauer-NCCLS modified disc diffusion method, the antimicrobial susceptibility of S. aureus was determined (Mehndiratta *et al.*, 2001). All the isolates were tested for sensitivity to the following three antibiotics. Each isolate standardized to overnight culture which consists of (10^8 cfu/ml) was used to flood the surface of Mueller Hinton agar (MHA) plates where excess drained off and allowed to dry whereas the lid was in plate. Standard antibiotic discs were put in aseptic place at logical equal distance on the inoculated MHA plates, and then allowed to stand for 1 h. when the duplicate is take place in each plate we transfer it to incubation at 30°C for 24 h therefore the methicillin resistant strains (BSAC 2002) can be identify. Inhibition zones ratio that produced by each antibiotic disc were measured , registered and classified the isolant according to (resistant", intermediate and sensitive) based on information of standard illustrative chart updated by NCCLS standard (NCCLS, 2002).

2.2. Genomic DNA extraction from Staphylococcus aureus

DNA was isolated by using Phenol/chloroform method, were the mixture centrifuged to separate cell debris. PCR was carried out by using the cell lysate as template. Check the Resolution of genomic DNA fragments by agarose gel electrophoresis which was performed on 1% agarose gel was melted in 1x TBE buffer till it forms a clear solution.MecA gene amplified of by using PCR. All isolates were tested for the presence of mecA genes as an amplification control by PCR by use the two primers, Forward primer: 5´-TGGCTATCGTGTCACAATCG-3´ Reverse primer: 5´-CTGGAACTTGTTGAGCAGAG-3´ DNA amplification was performed on an Eppendorf Master-Cycler in a final volume of 50µl containing 10x PCR buffer, dNTPs (10mM) 1 µl, Taq polymerase 0.25 µl, Nuclease free water 37.25 µl, Primers (F) 2 µl, Primers (R) 2 µl, DNA 2.5. DNA Samples were denatured for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing temp 58°C for 45 sec, and Polymerization at 72°C for 1 min, denaturation repeated for 29 cycle, the final extension done at 72°C for 10 min. PCR products checked by agarose gel electrophoresis through 2% agarose.

2.3. PRODUCTION OF PBP2a (penicillin binding protein 2a)

Strain was grown in modified tryptone soy broth containing 0.1gm of methicillin at 37 °C for 24 h. After incubation, centrifugation broth at 10000 rpm for 10 min is done and the cells were separated out. Supernatant was used as a crude enzyme.

2.4. Purification of PBP2a (Ammonium Sulphate Precipitation)

Different concentrations of ammonium sulphate were added to the supernatant. After stirring on a magnetic stirrer, it was kept undisturbed at 4°C overnight. Precipitates formed were collected by centrifugation at 10000 rpm for 10 min and re-dissolved in 20 mM phosphate buffer with pH 7.0 and the dialysate of enzyme against the same buffer.

2.5. Dialysis

A typical dialysis procedure for protein samples is as follows: Pre-wet the membrane with 20 mM phosphate buffer then Load sample into dialysis tubing or device and Dialyze for 1-2 hours at room temperature using 20mM phosphate buffer and Change the 20mM Phosphate buffer and dialyze for another 1-2 hours. Finally change buffer and dialyze overnight at 4°C.Then the sample in dialysis bag is used as protein source for further work. Selection of ammonium sulphate precipitated fraction: The fraction which showed the maximum lactamase activity was selected for further purification studies.

2.6. PBP2a activity

PBP2a activity was measured by simple photometric assay developed by Naomi *et al* 1972. Preparing the iodine-tungstate solution was by diluting the stock iodine solution (0.25 M iodine in 1.25 M KI) 1:3 in 1 M sodium tungstate. Assay of PBP2a activity. Determination of penicillinase activity was made by timed iodometric assay ,depend on hydroxamate formation can be determine the colorimetric assay while the photometric assay depend on formation of a colorless iodine - phenolic acid complex and a decrease that occur in optical densityPhotometric assay. The solutions used which consisted of Trismaleate buffer (0.025 M, pH 7.0), substrate (500, umoles/ml in buffer), dissolved enzyme in that buffer as the substrate, and iodine-tungstate solution (0.08 M I_2, 0.4 M KI, and 0.67 M sodium tungstate). Because Penicillinase is known to adsorb easily to the surface of glass, in other to prevent the adsorption. In this assay procedure we did not need to the gelatin where 1% of silicone solution was used for coating the glassware .The reaction was started by transferring 4 ml of the buffer solution containing 25 µ moles of substrate, prewarmed to 30 C, into the silicone-coated test tube containing the enzyme solution. By the addition of 1 ml of the iodine-tungstate solution the reaction was finished. The optical density was measured in a spectrophotometer at 620 nm. Control samples, in which the enzyme or substrate, or both, had been eliminated, were included. One unit of, PBP2ais defined as the amount of enzyme that hydrolyzes 1 mole of benzyl penicillin/hr at 30 C (Dan Ferber, 2002; Pechère, 2001). Sephadex G-100 Gel Filtration Chromatography: Loaded the pellet of protein that produced after dialysis onto a column of Sephadex G-100 (1.5 × 24 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with 20mM phosphate buffer, pH 7. The column was eluted at a flow rate of 30 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. The elution fractions (1mL) were collected and assayed for protein concentration and lactamase assay. The fraction which showed maximum PBP2aactivity was collected and used for SDS-

PAGE analysis. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) In the present study, 12% Resolving gel and 4% stacking gel were used to separate the proteins.

The detail protocol for SDS-PAGE is as follows: Buffers: Buffers were filtered through 3MM Whatmann and A was stored at 4°C, B and C were stored at RT, sample buffer was stored in small aliquots in a frozen state.

RESULTS

The bacterial strains were maintained in pure cultures which are isolated from hospital samples. The bacterial strains were streaked on Mannitol salt agar to the identify *Staphylococcus species.* Isolate on mannitol salt agar medium. Since the high salt concentration of Mannitol salt agar, staphylococci was selective. The Acid that formed from mannitol fermentation use in the pH indicator which phenol color (red) change from red (alkaline) to yellow (acid).

3.1. Morphological Characteristics

Grams staining, capsule and spore staining, motility and colony morphology of the isolates were studied for identification. The results of these observations are listed in Table 1.

Table 1. Identification Morphological Characteristics

Property	Name of test	Isolate
Morphology of the cells in nutrient broth	1.Gram stain	Gram-positive cocci
	2.Motility	Non Motile
	3.Spores	Non Sporulating
	4.growth	Turbid, with oozing

3.2. Biochemical Tests. Catalase activity

When hydrogen peroxide was added to the slants slow appearance of gas bubbles was observed. Novobiocin Sensitivity Test: The isolate is sensitive to antibiotic novobiocin. Fermentation of carbohydrates: After incubation, it was observed that only few carbohydrates were utilized by isolate. Table 2 shows the results of twelve carbohydrate fermentation test. Based on above carbohydrate and biochemical characterization isolate was identified as *Staphylococcus aureus* according to bergey's manual of bacteriological classification.

Table 2. Fermentation of carbohydrates approach

S.No	Carbohydrate	Results
01	Maltose	+
02	Rabinose	_
03	Xylose	_
04	Sucrose	+
05	Fructose	_
06	Cellobiose	_
07	Melibiose	_
08	Saccharose	_
09	Raffinase	_
10	Trehalose	+
11	Glucose	_
12	Lactose	+

3.3. Antibiotic sensitivity

In this test, nine antibiotics are used to the resistant nature of staphylococcus (table.3). The diameter of the zone of inhibition produced by each antibiotic disc was measured and registered. The isolates were classified as "resistant", "intermediate" and "sensitive" based on the standard interpretative chart updated according to the current NCCLS standard. Sensitive-when the diameter of the zone of inhibition is above 10 mm. Intermediate- when the diameter of the zone of inhibition is below 10 mm resistant- when there is no zone of inhibition. In the present study, staphylococcus is resistant to three antibiotics, intermediate to two antibiotics and sensitive to four antibiotics.

Table 3. Drug resistance of MRSA

Name of antibiotic (100µg/ml)	Antibiotic sensitivity test result
Methicillin	Resistant
Ampicillin	Resistant
Erythromycin	Resistant

3.4. Genomic DNA isolation

Genomic DNA was isolated from Staphylococcus aureus and the purity of the DNA was measured (table.4). Genomic DNA appeared as a single lane in agarose gel.

Table 4. Concentration of genomic DNA isolated from Staphylococcus aureus

Sample	N.A Conc. (ng/µl)	A260	A280	260/280
S. aureus (DNA)	46.5	0.93	0.499	1.86

3.5. Purification of PBP2a

An increase in PBP2a activity took place after purification of crude enzyme by ammonium sulphate precipitation and sephadex G-100. Crude PBP2a was partially purified by ammonium sulphate precipitation (80%) followed by dialysis. The partially purified sample was further purified by sephadex G-100 column chromatography and the fraction with highest PBP2a activity was pooled out. Maximum PBP2a activity was observed between fractions 33 to 35. The fractions of maximum PBP2a activity were mixed together and SDS-PAGE (12%) was run to determine the molecular weight (Sharma *et al.* ?).

3.6. Molecular weight determination in SDS-PAGE

Molecular weight of the PBP2afrom *Staphylococcus aureus* was determined by SDS-PAGE gel electrophoresis. Single protein band was observed when stained with Coomassie blue and it clearly indicated the purity of the protein. The Molecular weight of the purified PBP2awas calculated to be about 41 kDa.

DISCUSSION

Recent research has revealed that colonization of MRSA poses a substantial threat for the hospital environment, resulting in nosocomial infections. The word colonization here does not

mean the normal sites of colonization (nostrils and in the skin of the axilla/groin), but rather unhygienic surfaces, cotton swabs in the hospital near areas. According to NNIS data for the year 2011, 59.5% of *S. aureus* strains causing infections in hospitals were MRSA. This is not only true for Europe and United States but for India as well. Initially, occasional reports on MRSA were available, but now it has become one of the established nosocomial pathogens (Nelson *et al.,* 2007). Although no surveillance system exists, the figures obtained from some large medical care facilities including tertiary care hospitals is alarming, with percentages as high as 51.6% to 54.8% (Mehndiratta *et al.,* 2001; Anapurba and Sen, 2003). This is much higher than the range of 20 to 32.8% shown by earlier reports [27, 28].Our sample size was very limited so we cannot represent the entire case; however ,our data showed significant result at least based on this hospital, we can say that MRSA are rising in the environment of our hospital. This study was conducted using isolates from swab samples, keeping in mind the spread of MRSA in the hospital environment. The analysis of the antibiotype of *S. aureus* isolated in this study showed resistance against different groups of antibiotics including β-lactams of second- and third-generation. Of the 20 isolates, 12 were drug resistant, 4 isolate were had resistance against many antibiotics (5 antibiotics), while the remaining 4 were resistant to one or more antibiotics (Table 3). Plasmid profiling showed that these isolates had plasmids of similar molecular size. Although high methicillin resistance in isolate of *S. aureus* was found in four sample of our study, its emerging profile (18% exhibiting Methicillin resistance) is a sign of danger for both community acquired and hospital-associated infections. Methicillin resistance is either due to expression of *mecA* gene or the synthesis of methicillinase or due to both. (Dominguez *et al.,* 2002). The isolate was subjected to PCR amplification of *mecA* gene. PCR results revealed that isolate was carrying *mecA* gene. The present study demonstrates the production of methicillinase (PBP 2a) in MRSA strain isolated from hospital samples. we also suggest that *S. aureus*, that suspected to be carriers of *mecA*, phenotypic and genotypic analysis must be study to confirm their MR status. According to this study carried out, the least effective antibiotics against MRSA are Methicillin, Ampicillin and cephalosporin, whereas the most effective are Chloramphenicol, Gentamicin, and tetracycline. It is highly recommended that hospitals should deal very carefully with the cotton swabs and they should be sterilized before they are thrown out.

Conclusion

In the present study, *Staphylococcus aureus* was isolated from hospital samples and tested it for antibiotic sensitivity test. On antibiotic sensitivity testing *Staphylococcus aureus* showed resistance to three antibiotics out which two are β-lactum containg antibiotic that is for Methicillin, ampillicin and glycopeptides antibiotic vancomycin. Plasmid curing was done to confirm the presence of genes related to genomic or plasmid. The results showed that the genes are present in plasmid as the cured *Staphylococcus aureus* has not shown resistance to same antibiotics. PCR analysis of methicillin resistant gene in *Staphylococcus aureus* shows that the gene is nearly 1200 bp in length. Similar gene with nearly 1200 bp length is seen in case of Enterococci species. (Berger-Bächi, 1999). The PBP2a enzyme was purified by ammonium sulphate precipitation and sephadex G-100. An increase in (PBP2a) activity occurred after crude enzyme purification by ammonium sulphate precipitation and sephadex G-100. Molecular weight of the PBP2a from *Staphylococcus aureus* was determined by SDS-PAGE gel electrophoresis and it was found to be 41 kDa. Single protein band was observed which clearly indicated the purity of the protein.

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