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Research Article

MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *Staphylococcus auerus* (MRSA) ISOLATED FROM PUS SAMPLES

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ABSTRACT

Staphylococcus auerus is a gram positive, round shaped bacterium, is a common cause for skin infections. MRSA is any strain of *S. auerus* that was developed by horizontal gene transfer or by natural selection. Methicillin resistant staphylococcus auerus (MRSA) is a noscomial pathogen which shows resistance to methicillin and is one of the main causes for skin infections, respiratory infections and also infections by pus from infected wounds. MRSA shows resistant towards various kinds of β lactam antibiotics. Totally 3 strains of *Staphylococcus auerus* samples were collected from pus infected patients. The resistance of *S.auerus* towards methicillin was detected by disk diffusion test and the gene for resistance was identified by PCR using mecA and ampC primer. There was no zone formation due to its resistance activity against the antibiotics. RFLP was done to investigate the spread of *S. auerus*. Due to the multi-drug resistance pattern of MRSA, nanoparticles were used to determine the resistance activity. Nanoparticles have the potential to be used in place of antibiotics and can control the microbial infections caused by MRSA. The resistance of MRSA against gold, silver and chimosan nanoparticles was examined by MIC (Minimum Inhibitory Concentration) test. The zone of inhibition was formed which concluded that the nanoparticles can be used to control MRSA infections.

Keywords: MRSA, disk diffusion test, PCR, multi-drug resistance, nanoparticles, MIC.

INTRODUCTION

In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world[1]. The widespread use of antibiotics leads to the emergence of multidrug resistant bacteria.*Staphylococcus aureus* is a facultative anaerobic, gram positive coccal bacterium known as "golden staph" which appears as grape like clusters. The increasing resistance of *S.aureus* to a wide range of antibiotics lead to the development of clinically serious problems[2]. *S. aureus* has resistance towards variety of β lactam antibiotics like erythromycin, streptomycin, and tetracycline. It is the common cause of skin infections which can be spread through contact with pus from an infected person's wound. The resistance of *S.aureus* towards Methicillin is referred as Methicillin Resistant *Staphylococcus aureus*(MRSA). MRSA is predominantly a noscomial pathogen, normally does not cause disease unless it enters an opening in the skin which can cause septicaemia, pneumonia and major wound infection[3]. Serious MRSA infections more often occur with people in hospitals and other types of healthcare facilities.MRSA strains can be identified with molecular tests such as PFGE, MLST, SCC mec typing, and spa typing which are done for epidemiological studies; PFGE has been shown to be an accurate and reliable method[4]. Diagnosis includes quantitative PCR procedures for detecting MRSA strains. In humans and animals *S. aureus* infections can be diagnosed by culture and identification of the organism. The laboratory determination of MRSA antibiotic resistance and susceptibility is important. The type of treatment depends on factors such as location, severity and progression of the infection, age and health of the patient.

The main difficulty in treating MRSA infections is compounded by the fact that many strains possess efflux pumps, which export certain tetracyclines, macrolides, and genes which confer resistance to antibiotics[5]. The mecA gene is found in bacterial cells and the common carrier of the gene is MRSA; mec A is a biomarker responsible for resistance to methicillin and β-lactam antibiotics. The mecA gene does not allow the ring like structure ofantibiotics to bind to the enzymes that help from the cell wall of the bacterium and hence the bacteria replicates as normal. The objectives of our study are to identify the gene profile by PCR technique and the gene responsible for antibiotic resistance in *S.aureus.*

MATERIALS AND METHODS

Sources of inoculum and subculturing of microorganism

Methicillin resistant *Staphylococcus aureus* is a bacterium responsible for several infections in humans. MRSA is mainly spread from patients with open wounds and with pus infections in hospitals. The sources of inoculum of the bacterial pathogens, the three strains of *Staphylococcus aureus* S_1 S_2 S_3 were isolated from the pus sample in Abirami hospital Coimbatore, Tamilnadu India .The collected three strains of *Staphylococcus aureus* isolated from the pus sample were cultured in three plates in Muller-Hinton agar and then sub culturing was done by using Luria- Bertani medium. These strains were identified by the subculturing of micro organism[6].

Screening of methicilllin resistant *Staphylococcus aureus*

Screenings of MRSA were determined by inoculating the bacterial culture. Screening can be used to identify the presence

or absence of a bacterial culture that usually takes a day for a result. The three plates were inoculated with the Muller-Hinton agar and then made to solidify. Then the three strains of *Staphylococcus aureus* S1, S2, S3 was swabbed inside the three plate separately. Then the methicillin combs were placed slantingly and incubated in shaker for 24 hours to identify the zone formation. The 10ml of Luria Bertani medium was prepared and transferred to the three test tubes. The grown three strains of *Staphylococcus* bacterium are swabbed using the cotton stick and put into the three test tubes separately and the screening is done[7,8].

Minimum inhibitiory concentration

The minimum inhibition concentration (MIC) is a lowest inhibitory concentration of an antibiotic that prevents the visible growth of bacterium i.e., at which it has bacteriostatic or bactericidal activity. Minimum inhibition concentration of bacteria is a test carried out by using Hi-comb MRSA screens. This test is simple, reliable method for determining the antimicrobial susceptibility of the bacteria *Staphylococcus aureus* S_1 S_2 S_3.Minimum inhibition concentration test can be carried out by inoculating a plate with the Muller-Hinton agar and after that *Staphylococcus aureus* is swabbed inside the plate. And Hi-comb MRSA strips are placed slanting, in the petriplate and incubated for 24 hours[9].

DNA isolation

DNA isolation is a process of purification of DNA from the sample using the combination of physical and chemical methods. The procedure follows the isolation of DNA without any minimal of break. The genomic DNA was isolated by phenol chloroform method. Finally from the collected pellet the presence of genomic DNA was analysed by agarose gel electrophoresis unit by using TAE buffer[10].

Plasmid isolation

Plasmid is a double stranded extra chromosomal DNA of bacteria. The size of plasmid ranges from 1-1000 kilo base pairs. The resistance of *S.aureus* is mainly offered by its plasmid. Alkaline lysis method was used to isolate plasmid DNA by breaking the cells open and fron the pellet the presence of plasmid DNA is analysed by agarose gel electrophoresis unit by using 1*TAE buffer[11].

PCR amplification at mecA gene and ampC gene

The isolated DNA was subjected to Polymerase Chain Reaction (PCR). High level resistance to methicillin is caused by MecA gene. Amplification of DNA was performed using template DNA 2µl, MecA primer 2µl, ampC primer 2µl, PCR master mix 10µl, Taq polymerase 2µl, PCR buffer 4µl, Molecular biology grade water 5µl. PCR master mix is used to amplify the DNA and the PCR buffer is used to control the reaction[12].

Table 1 Primers used for gene amplification

Primer	Primer sequence
ampC (R)	5'AAT GGG TTT TCT ACG GTC TG 3'
ampC (F)	5'GGG CAG CAA ATG TGG AGC AA 3'
MecA (R)	5'AGG TGC TCA TCA TGG GAA AG 3'
MecA (F)	5' CTT TAT CGG CCC TCA CTC AA 3'

The sample was loaded in the PCR thermal cycler which consists of 25 cycles for amplification. The conditions for cycles follow: initial denaturation-95^0 C (30s); denaturation-95^0 C (2min); annealing- 56 ^{0} C (15s) ; extension-72 ^{0}C (15s); final extension-72 ^{0}C (2min). The amplified DNA sample was visualized by 1% agarose in agarose gel electrophoresis unit using 1* TAE buffer[13].

Restriction fragment length polymerisation

Restriction Fragment Length Polymorphism(RFLP) exploits variations in homologous sequence that can be detected by the presence of fragment of different length of polymerisation. The DNA is broken in pieces the digestion of DNA sample by the specific restriction endonuclease enzyme such as E-CoRI, SaU3AI, BamH I. Restriction fragment length polymerisation RFLP was carried out by suspending template DNA 2µl, Restriction enzyme 1µl, restriction buffer 2µl, distilled water 2µl and PCR was performed and was incubated for 3hours. The restriction enzyme was used to, break the DNA sample into pieces and the restriction fragments are separated according to the length by the restriction enzyme. The broken DNA sample by restriction enzyme was visualized by agarose in agarose gel electrophoresis unit using 1*TAE buffer[14].

Table 2 Enzymes used for RFLP

Enzyme	Recognition sequence
EcoRI	GAATTC CTTAAG
Sau3A I	GATC CTAG
BamH I	GGATCC CCTAGG

Remedial measures

Wounds infected with MRSA should be kept clean and covered with clean, dry bandages until healed to prevent the spread of infection to others. Some of the remedial measures include performing hand hygiene after touching blood, wearing gloves when anticipated with blood and should wear a gown to protect skin .Nanoparticles (NP's) provide a versatile platform for therapeutic applications on their physical properties nanoparticle size range is commensurate with bio molecular and bacterial cellular systems, providing additional interactions to small molecule antibiotics.

Gold nanoparticles (GNP) can be used as a potent antimicrobial agent that can be tailored through surface hydrophobicity, providing new aspect to design antimicrobial nanoparticles[13]. Silver nanoparticles (SNP) also have become an important approach for applications in the development of antibiotic treatment of different bacterial infections. AgNPs possess high electrical and thermal conductivity, catalytic activity, and antibacterial properties. AgNPs are arising as new bacteriostatic agents, because they are comparable in efficacy and even more potent antimicrobial compounds than conventional antibiotics[14]. Chitosan is a nanoparticle N- acetyl glucosamine and glucosamine units. Chitosan nanoparticles (CNP) exhibit higher antibacterial activity than chitosan based on the special character of the nanoparticles[15].

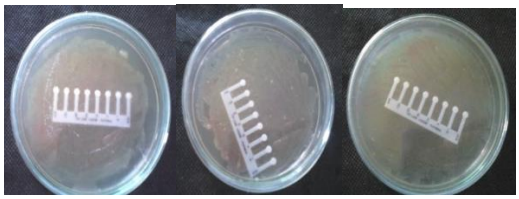
Table 3 Formation of zone of inhibition

Strains	SNP	GNP	CNP
S1	13	14	13
S2	11	13	12
S3	13	14	13

RESULTS

Minimum inhibitory concentration

MIC test was carried out for our strain of MRSA are resistant to the antibiotics. Zone of inhibition was not collected in the plate, due to antibiotic (Methicillin) which does not kill the bacteria.



a) b) c)

Figure 1 Hi-comb MRSA strips placed on Muller Hinton agar with: a) Strain S1 b) Strain S2 c) Strain S3

DNA isolation

The isolated DNA was subjected to agarose gel electrophoresis and the different bands were obtained. The DNA was determined by running the standard marker along with the DNA.

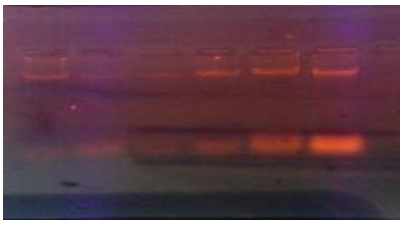


Figure 2 Isolated DNA in electrophoresis unit showing bands

Plasmid isolation and PCR amplification

The isolated plasmid was subjected to agarose gel electrophoresis. The presence of plasmid was determined by running the standard marker along with the isolated plasmid. The DNA was amplified using PCR (Polymerase Chain Reaction). The plasmid was found by running the DNA marker along with the PCR.

Figure 3 Amplified plasmid DNA in electrophoresis unit

Restriction fragment length polymorphism

The RFLP-PCR,one of the most widely used genotyping methods,has been applied to investigate the spread of *S.aureus.*With the isolated DNA restriction endonuclease analysis was carried out and hence the results were obtained.

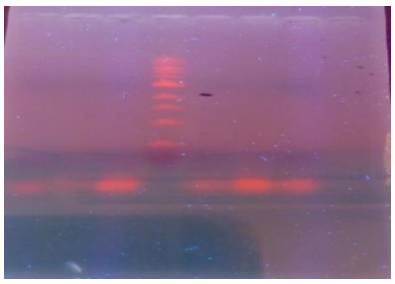


Figure 4 RFLP analysis of the obtained strains.

Remedial measures

Remedial measures was carried out in the plates for all the three strains by adding different nanoparticles (CNP,GNP,SNP) using well diffusion method and hence the zone of inhibition was obtained.

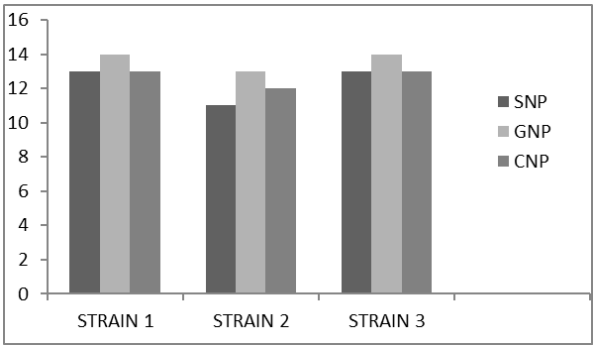


Figure 5 Formation of zone of inhibition using nanoparticles (in mm)

DISCUSSION

The extensive use of broad-spectrum antibiotics leads to the occurrence of noscomial infections by multi-drug resistant micro organisms[16]. Among them one such is MRSA; it is one the important noscomial pathogen that emerged due to the chromosomal mutations after methicillin came into existence.MRSA normally does not cause disease unless it enters an opening in the skin. Both methicillin-sensitive S.aureus (MSSA) and MRSA cause similar infections, ranging from minor infections of the skin to more serious infections such as septicaemia, pneumonia and major wound infection. Serious MRSA infections more often occur in people in hospitals and other types of healthcare facilities. The hospitalised patients are significantly associated with colonisation and serious infections of MRSA[17].

Our present study was to determine the antibiotic susceptibility of three different strains of MRSA. MRSA shows resistant to almost all the beta-lactam antibiotics[18].Three strains of MRSA were used for antibiotic susceptibility test which was carried out using MET disc. All the three strains were found to be resistant to the antibiotics of METdisc. There areseveral factors that can make *S. aureus*to be resistance to antibiotics. The serious infections of MRSA can be due to the over usage or the incomplete use of prescribed antibiotics to the patients. Both methicillin-sensitive Staph aureus (MSSA) and MRSA cause similar infections, ranging from minor infections of the skin to more serious infections such as septicaemia, pneumonia and major wound infection. Serious MRSA infections more often occur in people in hospitals and other types of healthcare facilities.ThePCR analysis of all the three strains of MRSA in our study, showed the presence of genes responsible for

antibiotic resistance. The PCR analysis was carried out using two primers - mecA and AmpC led to the amplification of thegenes encoding resistance to β-lactam antibiotics.

In our study the use of nanoparticles in antibiotic susceptibity against MRSA showed zone of inhibition in all the three strains. Nanoparticles (NP) due to their unique physio-chemical properties find its wide application in the field of medicine[19]. The small size and large surface ratio makes nanoparticles to interact with microbes to carry out broad range of antimicrobial activities. The inorganic metallic nanoparticles like Gold (Au) and Silver (Ag) and organic nanoparticle- Chitosan were used. Gold nanoparticle showed slightly higher effect than the other two nanopartilces. However studies revealed that the prolonged use of inorganic nanoparticles can affect the biological behaviour at the organ tissue, cellular, subcellular and protein levels[20,21]. So the organic nanoparticle chitosan, a biocompatible polymer obtained from chitin was used, which showed considerable antimicrobial effect in the strains of MRSA.

CONCLUSION

MRSA is found to be more prevalent in the hospital setting. This not only creates lot of problems in treatment aspect, but also pressurizes the need for taking control measures to prevent the spread of MRSA strains in the community. In the present study, MRSA strains obtained from the pus samples were characterised using molecular techniques. This study shows that all the three strains were multidrug resistant.Zone of inhibition was not observed in the MIC testfor all the three MRSA strains,which showed the resistance of MRSA to antibiotic (Methicillin) that does not kill the bacteria. The chromosomal DNA and Plasmid DNA were also isolated which confirmed the presence of MRSA. PCR was also performed, to identify the gene responsible and the PCR amplified product confirmed the resistant pattern.DNA was isolated and amplified by RFLP-PCR. In the present study, the remedial measures against MRSA strains were done by nanoparticles- Silver, Gold, Chitosan in which the zone of inhibition was observed. To conclude, the current advancements in nanoparticles and nanotechnology will be efficient in reducing the multidrug resistant pattern of bacteria.

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