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Molecular characterisation of Methicillin resistant *Staphylococcus aureus* (MRSA) isolated from ocular patients

[1]Sandesh Suresh K., [2]R. Ragunathan and [2]Jesteena Johney

[1]Department of Biosciences, Mangalore University, Karnataka
[2]Centre for Bioscience and Nanoscience Research, Eachanari, Coimbatore, Tamilnadu

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ABSTRACT

Methicillin-resistant Staphylococcus aureus one of the main pathogen responsible for causing infections of skin and systemic infections. This methicillin-resistant Staphylococcus aureus may be resistant to β-lactam antibiotics as well as other antibiotics. The resistance of Methicillin-resistant Staprhylococci to various was revealed by antibiotic sensitivity test and the gene for antibiotic resistance was revealed by carrying out PCR using mecA and blaIMP primers. The molecular characterization and antibiotic sensitivity test was carried out for the three strains collected from the ocular patients. The MIC test was carried out for all the three strains of Staphylococci using vancomycin which revealed the minimum concentration of vancomycin required to inhibit the growth of this pathogen.

Key words: Methicillin, Multi drug resistant pattern *S. aureus*, DNA, PCR and MIC

INTRODUCTION

Infectious diseases are the world's leading cause of premature deaths, killing almost 50,000 people every day. In recent years, drug resistance to human pathogenic bacteria has been commonly reported from all over the world (1, 2). However, the situation is alarming in developing as well as developed countries due to indiscriminate use of antibiotics. The drug-resistant bacteria and fungal pathogens have further complicated the treatment of infectious diseases in immunocompromised, AIDS and cancer patients (3, 4). In the present scenario of emergence of multiple drug resistance to human pathogenic organisms, this has necessitated a search for new antimicrobial substances from other sources including plants. The ease with which bacteria become resistant to currently used antimicrobial agents has been and continues to be of concern to clinicians, public health officials, and researchers. Today, transferable drug resistance represents a major threat to the treatment of infectious diseases in both humans and animals, including farmed fish. The use of antimicrobial agents in both human and veterinary medicine exerts a strong selective pressure inducing resistance to antimicrobial agents among bacteria.(5).

Staphylococcus aureus is a gram positive, round shaped, small, non-motile bacteria belonging to the family Staphylococcaceae (6, 7) . Strains of *S. aureus* resistant to β-lactam antibiotics are known as methicillin-resistant *S. aureus.* It is also called multi-drug resistant(MDR) *S.aureus.* The emergence of multi-drug resistant bacteria is a major problem for the treatment of disease using antibiotics In recent years it has been reported that the clinical administration of antibiotics, against the pathogenic bacteria be gradually prohibited due to emergence of MDR bacterial strains including *S. aureus.* (8 , 9) . Methicillin-resistant *Staphylococcus aureus* (MRSA) account for a large part of nosocomial infections worldwide (10) and are associated with longer hospitalisation and higher lethality (11). The widespread use of antibiotics has been responsible for the development of numerous problems including the emergence of multidrug resistant bacteria. *Staphylococcus aureus* is one of the bacteria that have a dramatic increase in resistance to

antibiotics in the last decade. It often colonises the skin and nose in healthy individuals; however it can also cause severe disease (12 , 13). When methicillin and other antibiotics do not kill the bacteria causing an infection, it becomes harder to get rid of the infection. MRSA bacteria are more likely to develop when antibiotics are used too often or are not used correctly. *Staphylococcus aureus* continues to be dangerous pathogen for both community acquired as well as hospital associated infections. *S. aureus* resistant to methicillin was reported soon after its introduction in October 1960 (14). The emergence of high levels of penicillin resistance followed by the development and spread of strains resistant to the semisynthetic penicillins (methicillin, oxacilin and nalcillin) , macrolides, tetracycline and aminoglycosides has made the therapy of staphylococcus disease a global challenge. (15)

The *mecA* gene responsible for methicillin resistance is a part of mobile genetic element found in all MRSA strains. It was demonstrated that *mecA* is part of a genomic island designated staphylococcal cassette chromosome *mec* (SCC*mec*) (16). Methicillin-resistant *S.aureus* (MRSA) was considered as one of the most difficult bacteria to treat in patients. The difficulty in treating MRSA infections is compounded by the fact that many strains also possess efflux pumps, which export certain tetracyclines, macrolides, genes which confer resistance to antibiotics and antisepsis (17) . In Staphylococci, three genes (erm A, erm B and erm C) encoding methyl transferases are responsible for resistance to macrolide by the modification of the ribosomal target site in 23s rRNA (18 , 19)

The persent study is the molecular characterization of the gene which confers the resistance to antibiotics.

MATERIALS AND METHODS

Three strains of methicillin-resistant *Staphylococcus aureus* was collected from the ocular patients. The culture of three different strains of MRSA was sub-cultured on the Mannitol Salt Agar medium. The mother broth culture of all the strains of MRSA was also prepared.

Antibiotic sensitivity test: One of the antibiotic sensitivity test for done for three different strains of MRSA by using octadisc consisting of eight antibiotics - Oxytetractyclin (O), Cefataxime (Ce), Cephalexin (Cp), Co-Trimoxazole (Co), Chloramphenicol (C), Nalidixic acid (Na), Furazolidone (Fr), Norfloxacin (Nx). The culture was spread on the Mueller Hinton Agar medium with the help of cotton swab and octadisc was placed at the centre of the plate. In another test the antibiotic sensitivity test was done by placing ten different antibiotics in two different petriplates consisting of Mueller Hinton Agar media (5 in each plate).
Isolation of DNA and Plasmid: DNA and Plasmid was isolated from all the three strains of MRSA. The DNA was isolated by phenol-chloroform extraction method and the plasmid was isolated by mini prep method. The isolated DNA and RNA was then subjected to agarose gel electrophoresis and the bands were observed under UV transilluminater.

PCR and RAPD: The DNA and plasmid isolated from all the three strains of MRSA was subjected to Polymerase Chain Reaction (PCR) using mecA and bla IMP primers for the detection of genes encoding antibiotic resistance. The PCR mixture contained DNA 2µL, PCR mastermix 10µl, Primers 2µl each, Molecular biology grade water 6µl and 10x assay buffer 5µl. The amplified product was then subjected to RAPD analysis by running it in the agarose gel and visualizing the band under UV transilluminater.

Table 1: Primers used for the detection of genes encoding antibiotic resistance

Primer	Primer sequence 5'-3'
mecA	AACAGGTGAATTATTAGCACTTGTAAG ATTGCTGTTAATATTTTTTGAGTTGAA
blaIMP-F	GTTTATGTTCATACWTCG
blaIMP-R	GGTTTAAYAAAACAACCAC

Minimum Inhibitory Concentration(MIC): The Minimum Inhibitory Concentration (MIC) test was carried out for all the three strains of MRSA Inoculated in the nutrient broth to which different diluted concentration of vancomycin prepared from the stock (1mg/ml) was added and incubated for 24 hours. The Minimum Inhibitory Concentration was found colorimetrically.

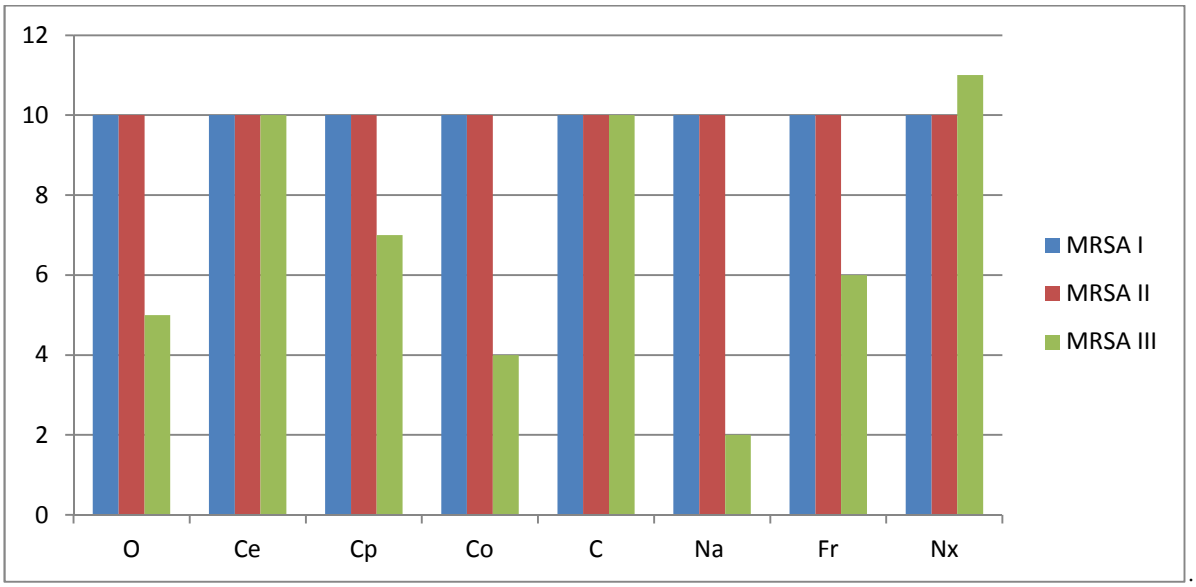
Isolation of Bacterial cell wall proteins: The bacterial cell wall protein was isolated from all the three strains of MRSA and the isolated proteins was subjected to SDS-PAGE.

RESULTS

Antibiotic sensitivity

Table 2: The zone of inhibition for each antibiotics of the octadisc was measured. The result obtained is as follows:

Name of Antibiotics	Zone of inhibition for *S.aureus* I	Zone of inhibition for *S.aureus* II	Zone of inhibition for *S.aureus* III
Oxytetracyclin (O)	10mm (Resistant)	10mm (Resistant)	5mm (Resistant)
Cefataxime (Ce)	10mm (Resistant)	10mm (Resistant)	10mm (Resistant)
Cephalexin (Cp)	10mm (Resistant)	10mm (Resistant)	7mm (Resistant)
Co-Trimoxazole (Co)	10mm (Resistant)	10mm (Resistant)	4mm (Resistant)
Chloramphenicol (C)	10mm (Resistant)	10mm (Resistant)	10mm (Resistant)
Nalidixic acid (Na)	10mm (Resistant)	10mm (Resistant)	2mm (Resistant)
Furazolidone (Fr)	10mm (Resistant)	10mm (Resistant)	6mm (Resistant)
Norfloxacin (Nx)	10mm (Resistant)	10mm (Resistant)	11mm (Resistant)

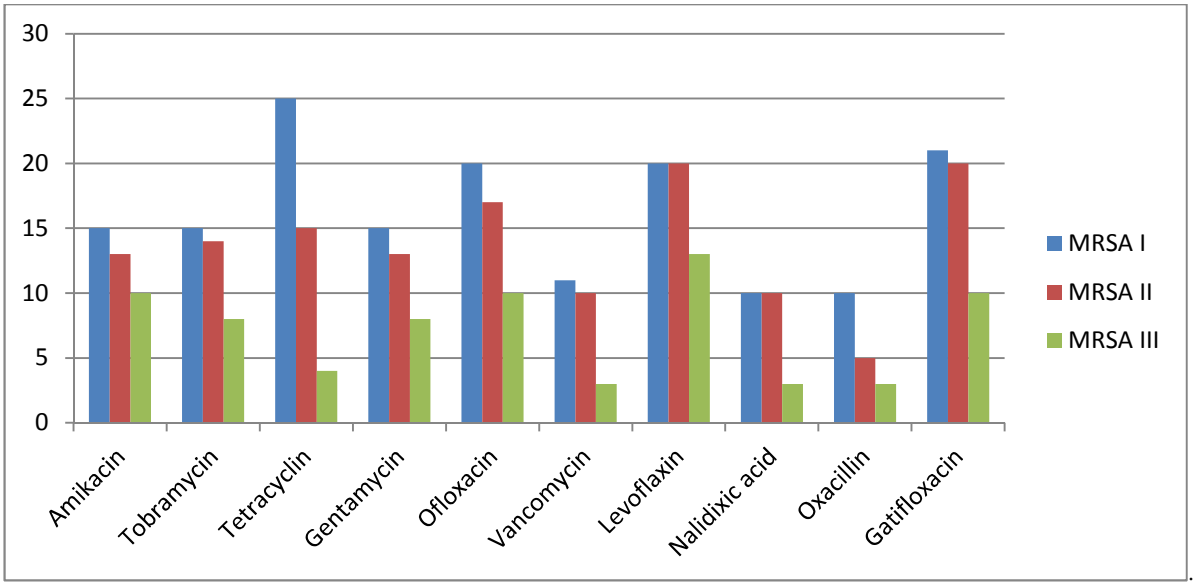


(Graph showing the antibiotic resistance in MRSA strains)

All the three strains of methicillin-resistant *Staphylococcus aureus* (MRSA) are resistant to all the antibiotics of octadisc. The zone of inhibition measured for all the eight antibiotics was same with respect to MRSA I and MRSA II whereas MRSA III are much more resistant to Oxytetracyclin, Cephalexin, Co-Trimoxazole, Nalidixic acid and Furazolidone, the zone of inhibition were smaller.

The zone of inhibition obtained for ten different antibiotics placed in Mueller Hinton Agar media for all the three strains of MRSA is as follows:

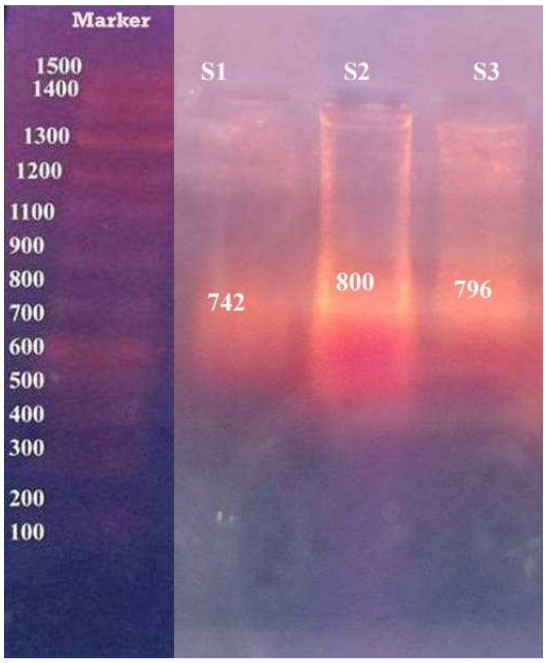
Name of antibiotics	Zone of Inhibition for *S.aureus* I	Zone of Inhibition for *S.aureus* II	Zone of Inhibition for *S.aureus* III
Amikacin	15mm (Intermediate resistant)	13mm (Resistant)	10mm (Resistant)
Tobramycin	15mm (Intermediate resistant)	14mm (Intermediate resistant)	8mm (Resistant)
Tetracyclin	25mm (Sensitive)	15mm (Intermediate resistant)	4mm (Resistant)
Gentamycin	15mm (Intermediate resistant)	13mm (Intermediate resistant)	8mm (Resistant)
Ofloxacin	20mm (Sensitive)	17mm (Sensitive)	10mm (Resistant)
Vancomycin	11mm (Resistant)	10mm (Resistant)	3mm (Resistant)
Levoflaxin	20mm (Sensitive)	20mm (Sensitive)	13mm (Resistant)
Nalidyxic acid	10mm (Resistant)	10mm (Resistant)	3mm (Resistant)
Oxacillin	10mm (Resistant)	5mm (Resistant)	3mm (Resistant)
Gatiflaxin	21mm (Sensitive)	20mm (Sensitive)	10mm (Resistant)



(Fig.1 . Antibiotic resistance pattern of MRSA strains)

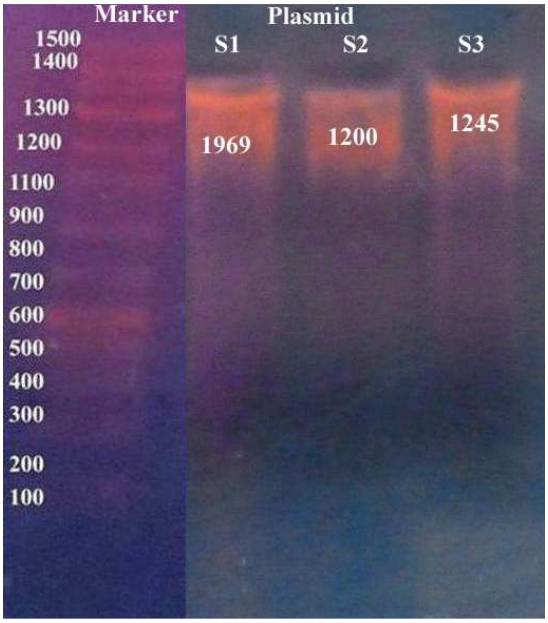
Among the three strains of MRSA, MRSA I is intermediate resistant to Amikacin, Tobramycin and Gentamycin and is sensitive to Tetracyclin, Ofloxacin, Levoflaxin and Gatifloxacin. MRSA II is intermediate resistant to Tobramycin, Tetracyclin and Gentamycin and is sensitive to Ofloxacin and Gatifloxacin whereas MRSA III is resistant to all the antibiotics used.

DNA and Plasmid Isolation:



(Fig.2 Agarose gel electrophoresis of DNA isolated from the three strains of MRSA 1, Marker; 2, DNA of MRSA I; 3, DNA of MRSA II; 4, DNA of MRSA III)

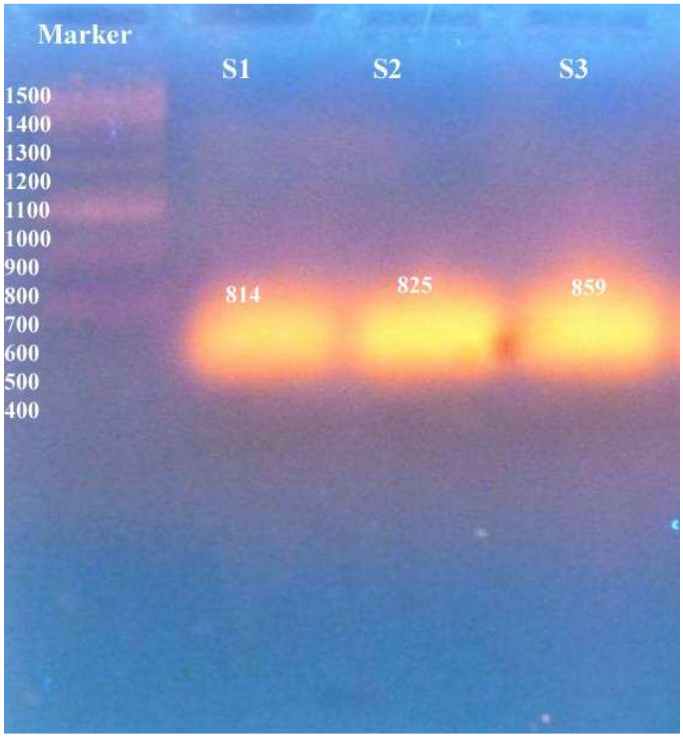
The DNA isolated by phenol-chloroform was subjected to agarose gel electrophoresis and the bands were obtained. The size of the DNA was determined by running the marker along with the DNA of our interest. DNA of molecular weight 742bp was obtained from MRSA I, DNA of molecular weight of 800 bp was obtained from MRSA II and DNA of molecular weight 796kp was obtained from MRSA III.



(Fig. 3 Agarose gel electrophoresis of plasmid isolated from all the three strains of MRSA; 1, Marker; 2, Plasmid of MRSA I; 3, Plasmid of MRSA II; 4, Plasmid of MRSA III)

The plasmid isolated by miniprep method was subjected to agrose gel electrophoresis. The molecular weight of plasmid was determined by running the marker along with the isolated plasmid. Plasmid of 1969bp was obtained from MRSA I, plasmid of 1200 was obtained from MRSA II and plasmid of 1245bp was obtained from MRSA III.

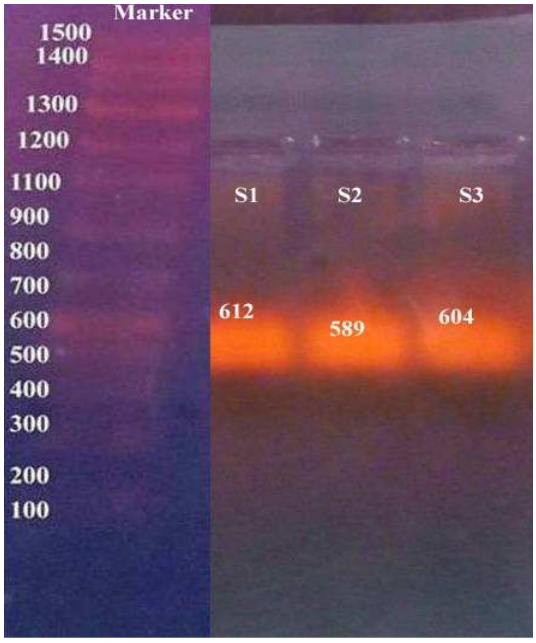
Analysis of PCR amplified product:



(Agarose gel electrophoresis of PCR amplicon of *S.aureus* of mecA and blaIMP gene; 1, DNA marker ; 2, amplified gene of *S.aureus* I DNA; 3, amplified gene of *S.aureus* II DNA; 4,amplified gene of *S.aureus* III DNA) 5, amplified gene of *S.aureus* I plasmid; 6,amplified gene of *S.aureus* II plasmid; 7, amplified gene of *S.aureus* III plasmid)

The molecular weight of the amplified DNA was found out by running the marker DNA along with the amplicon. The molecular weight of the amplified gene of MRSA I DNA was 814bp, the molecular weight

of the amplified gene of MRSA II DNA was 825bp and the molecular weight of the amplified gene of MRSA III DNA was 859bp.



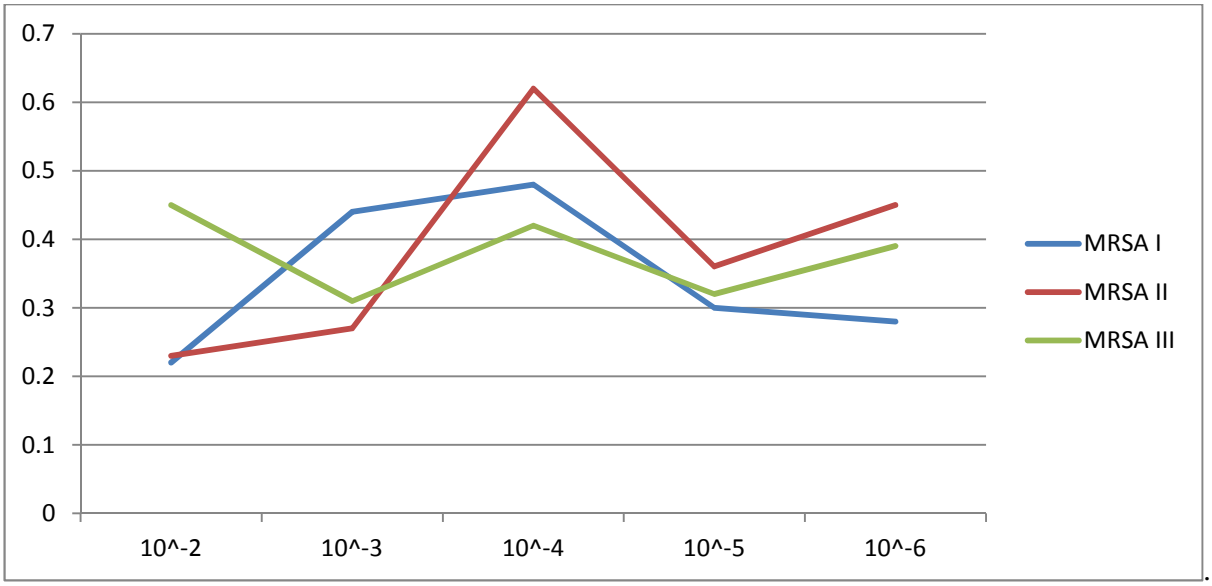
(Agarose gel electrophoresis of PCR amplicon of *S.aureus* of mecA and blaIMP gene; 1, Marker DNA; 2, amplified gene of *S.aureus* I plasmid; 3,amplified gene of *S.aureus* II plasmid; 4, amplified gene of *S.aureus* III plasmid)

The molecular weight of the amplified gene of plasmid was found out by running the marker DNA along with the amplicon. The molecular weight of the amplified gene of MRSA I plasmid was 612bp, the molecular weight of the amplified gene of MRSA II plasmid was 589bp and the molecular weight of the amplified gene of MRSA III plasmid was 604bp.

Minimum Inhibitory Concentration:

Table 3: The absorbance of the nutrient broth media inoculated with MRSA strains to which different diluted concentration of vancomycin added is as follows

Dilution	Abosrbance of media containing S.aureus I	Absorbance of media containing S.aureus II	Absorbance of media containing S.aureus III
10^-2	0.22	0.23	0.45
10^-3	0.44	0.27	0.31
10^-4	0.48	0.62	0.42
10^-5	0.30	0.36	0.32
10^-6	0.28	0.45	0.39



(Graph 2 showing the MIC for different dilution concentration of vancomycin with respect to all the three strains of MRSA)

Among different dilution of vancomycin antibiotic, the MIC for MRSA I and MRSA II strains was obtained for 10^-4 dilution and the MIC for MRSA III strain was obtained for 10^-2 dilution.

DISCUSSION

The evolution of increasingly antimicrobial resistant bacteria species stems from a multitude of factors that includes the widespread and sometimes inappropriate use of antimicrobials (20). MRSA is an important nosocomial pathogen which emerged as a result of a chromosomal mutation shortly after methicillin became available (21 , 22). This organism is essentially resistant to all beta-lactam antibiotics and a number of other agents (21). The frequencies of bacterial strains resistant to antimicrobial agents have increased dramatically in the environment as a consequence of the widespread use of these drugs (23 , 24). Methicillin-resistant *Staphylococcus aureus* (MRSA), besides having established itself as a major hospital pathogen , is now beginning to prevail in the wider community as well (25 , 26 , 27). The number of available antibiotics with proven efficacy for treating infections caused by these organisms has not been similarly expanded. Therapeutic trails with cephalosporins and the newest beta-lactam antibiotics, such as the carbapenems, have met with similar clinical failures despite good in vitro results (28).

Our present study shows that all the three strains of MRSA is resistant to almost all the β-lactam antibiotics. When antibiotic sensitivity test was carried out using octadisc , all the three strains of MRSA was found to be resistant to all the antibiotics of octadisc (Oxytetracyclin, Cefataxin, Cephalexin, Co-Trimaxozole, Chloramphenicol, Nalidixic acid, Furazolidone and Norfloxacin). And the antibiotic sensitivity test carried out using antibiotic disc shows the resistaance of MRSA to almost all the antibiotics. There are several factors that can make *S. aureus* to be resistance to antibiotics. Over prescribing of antibiotics by clinician is one way that can lead to resistance of *S. aureus***.** When there is over usage and incomplete course of antibiotics by patients **,** example when appropriate antibiotics are given to the animals, the owner may only give part of the course of antibiotic and not finish the remainder, possibly leaving bacteria partially treated. This will result in an increase resistance towards the antibiotics. The availability of antibiotics which is this is a big concern internationally where many antibiotics are available without prescriptions. Many pharmacists in these countries act as the caregiver and give out antibiotics based on patients' complaints without adequate diagnosis or testing and lead to the resistance of the bacteria towards antibiotic. High cost and lack of adequate medications also can lead to the resistance to antibiotic. In several lesser developed countries, many antibiotics are very expensive. This may contribute to only partial use of an antibiotic (29).

Our data demonstrates the PCR amplicon of all the three strains of MRSA contains genes for antibiotic resistance. The PCR carried out using two primers - mecA and blaIMP led to the amplification of the genes encoding resistance to β-lactam antibiotics.

The Minimum Inhibitory Concentration for three different strains of MRSA using different dilution of Vancomycin shows 10^-2 dilution is the Minimum Inhibitory Concentration for *S. aureus* I and 10^-4 dilution for *S. aureus* II. There has been concern about the development of vancomycin resistance in multidrug-resistant strains of *S. aureus,* especially since the demonstration of successful transfer of the *vanA* gene from enterococci to *S. aureus* under laboratory conditions (30). Acquisition of the enterococcal vancomycin-resistance mechanism by staphylococci has not yet been observed in clinical isolates. On the other hand reduced susceptibility to vancomycin has now been described in both Japan and the United States in association with the failure of vancomycin treatment of MRSA infections. Although the mechanism of staphylococcal resistance to vancomycin is not clear, a mechanism involving alterations in the bacterial cell wall and capture of antibiotic molecules at a distance from the sites of cell-wall synthesis has been proposed on the basis of properties of a vancomycin-resistant laboratory mutant (31 , 32). Clearly, elucidation of the mechanism of staphylococcal resistance to vancomycin is urgently needed, since current efforts in drug development are directed against the enterococcal mechanism of vancomycin resistance, which is distinct from that of staphylococci. (33)

CONCLUSION

In the present study the MRSA strains were obtained from ocular patients were characterised using molecular techniques. The MDR pattern was studied and found that all the three strains were showed the multidrug resistant pattern. The DNA and Plasmids were also isolated and confirmed the MDR and MRSA pattern. The PCR was also performed to identify the gene responsible for the MR character and the PCR amplified product confirmed the resistant pattern.

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