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MOLECULR CHARACTERIZATION OF ANTIBIOTIC RESISTANT GENE OF STAPHYLOCOCCUS AUREUS ISOLATED FROM HOSPITALS

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ABSTRACT

The Multiple Antibiotic Resistance of Methicillin Resistant Staphylococcus aureus (MRSA) has become a major clinical problem worldwide. With increasing incidence of resistance in microbes and decreasing rates of discovery of newer effective antibiotics, we are almost on the verge of an impending medical disaster. Microbial resistance is, therefore, a major cause of concern. It is no more a neglectable risk but a real threat to our survival on earth. Molecular methods for detection of MRSA is a potentially valuable development to reduce test time and increase sensitivity and specificity. It is most important that any method should accurately distinguish between MRSA and mixtures of methicillin-susceptible S. aureus with methicillin-resistant staphylococci present in the sample .Cost-effectiveness data are still awaited but we would anticipate a progressive introduction of this PCR-based technology into clinical practice at an early date..

KEYWORDS: *Staphylococcus aureus, MRSA, Antibiotics resistance gene, mecA gene.*

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INTRODUCTION

Introduction of penicillin in the year 1940 had greatly improved the prognosis for patients with severe staphylococcal infections, but after a few years of clinical use, resistance appeared owing to production of β-lactamases.[1] Methicillin was designed to resist β lactamase degradation, but Methicillin Resistant *Staphylococcus aureus* (RRSA) and Methicillin Resistant Coagulase Negative Staphylococci (MRCoNS) strains were soon identified. Since then, these strains have spread worldwide.[2] Until recently, Methicillin Resistant Staphylococci were predominantly nosocomial pathogens causing hospital acquired infections [3] but Methicillin Resistant Staphylococal (MRS) strains are now being increasingly isolated from community acquired infections as well.[4-5] B-lactam antibiotics such as methicillin inactivate Penicilling Binding Proteins PBP's 1, 2 and 3 in the bacterial cell wall which have an enzymatic role in the synthesis of Peptidoglycans. However , *S. aureus* acquires methicillin resistance by insertion of staphylococcal cassette chromosome (SCC*mec*), carrying the *mecA* gene, into chromosome. This gene encodes an altered penicillin-binding protein, PBP-2a, which is not inhibited by existing b-lactam antibiotics [6-7] thus conferring resistance to these organisms. MRSA prevalence increased from 12% in 1992 to 80.83% in 1999. Indian literature shows that MRSA incidence was as low as 6.9% in 1988 and reached to 24% and 32.6% in Vellore and Lucknow in 1994 and was of the same order in Mumbai, Delhi and Bangalore in 1996 and in Rohtak and Mangalore in 1999. However, in some of the centres it was as high as 87%. After the emergence of MRSA as a nosocomial pathogen in the early 1960s, there have been an increasing number of outbreaks of MRSA infections in hospitals reported from many countries.[8] Life-threatening sepsis, endocarditis, and osteomyelitis caused by MRSA have also been reported .[9] Since resistance to multiple antibiotics among MRSA isolates is very common, there is a possibility of extensive outbreaks, which may be difficult to control. MRSA is now one of the commonest nosocomial pathogens, and asymptomatically colonized healthcare workers are the major sources of MRSA in the hospital environment. Early detection of MRSA and formulation of effective antibiotic policy in tertiary care hospitals is of paramount importance from the epidemiological point. The higher price of vancomycin, its unavailability in many parts of the country, and also the possibility of emergence of resistance to the drug should at least make the clinicians look into the alternatives. Therefore, regular surveillance of hospital-associated infections including antimicrobial susceptibility pattern of MRSA and formulation of a definite antibiotic policy may be helpful in reducing the burden of MRSA infections in the hospital.[10] Among all known bacteria, *Staphylococcus aureus* is possibly the greatest concern of all health-care-associated pathogens due to its ability to cause a wide variety of life-threatening infections, *Staphylococcus aureus* has the ability to rapidly adapt to different environmental conditions.[11] In 2003, *S. aureus* was reported to be the leading cause of health-care-associated infections globally.[12] Numerous anti-staphylococcal agents exist, including linezolid, daptomycin, tetracyclines and fluoroquinolones, but these are rapidly becoming of less value due to the ability of the bacterium to develop efficient mechanisms to neutralize these agents . The methicillin resistance mechanism is the most recognized in methicillin-resistant *S. aureus* (MRSA) strains.[11] MRSA has been recognized as an important health-care-associated infection. Treatment as early as 1959 included semi-synthetic penicillin drugs such as methicillin for *S. aureus* infection.[13] The rise of MRSA strains became apparent as early as 1960, approximately a year after methicillin introduction.[11] In the early 1980's MRSA strains were identified as a major cause of nosocomial infections due to the increase every decade .[14] Since 1987, MRSA was increasingly found in the community and referred to as community-acquired MRSA (renamed community-associated MRSA) (CA-MRSA) .[15] The development of methicillin resistance in *S. aureus* strains can be ascribed to the altered penicillin-binding protein (PBP2a), which has a reduced affinity to penicillin and β-lactam antibiotics .[16] The *mec*A gene that encodes the altered protein (PBP2a) is not inactivated by methicillin during treatment.[17] The *mecA* gene resides on a genomic island termed the staphylococcal cassette chromosome *mec* (SCC*mec*).[18] MRSA strains that have been clinically identified as community associated have been shown to be more virulent with a high degree of severity of disease when compared with HA-MRSA .[19] This is due to the production of the Panton–Valentine leukocidin (PVL) toxin .[20] PVL is a toxin associated with deep skin infection, soft tissue infection and necrotizing pneumonia). PVL toxin stimulates pore formation in the leucocyte membrane resulting in the death of the cell, thus promoting tissue necrosis. Because, the PVL-associated genes of CA-MRSA are harboured by a bacteriophage, ϕSLT, these toxin genes may be transmitted easily to other HA-MRSA strains.[21] *N*PVL toxin has been identified as a genetic marker for CA-MRSA strains .[22] In comparison with previous detection methods such as Southern blotting and pulsed-field gel electrophoresis (PFGE), PCR assays such as multiplex-PCR (M-PCR), real-time PCR, hypervariable region (HVR) and *spa*-typing techniques can provide a rapid amplification, detection and typing tool for MRSA strains .[23] Healthcare providers should be aware of new technological approaches to the detection and confirmation of MRSA in clinical samples, and ensure that adequate health technology evaluation programmes are in place. S. aureus is a Gram-positive coccus where the round cells, approximately 1 μm in diameter, form grape-like (Greek staphyle) clusters indicative of the ability to divide in more than one plane. They are capable of both aerobic and anaerobic respiration and most strains ferment mannitol anaerobically. On blood agar they form characteristic golden (Latin aureum) or white colonies. They produce catalase, coagulase and an extracellular cell clumping factor, and some strains produce capsules.[24] Virtually all MRSA produce an additional penicillin-binding protein, PBP2a or PBP2′[25-26] which confers resistance to all currently available β-lactam agents. PBP2a is encoded by the mecA gene.[27] Although methicillin is now not used in treatment, it was the first penicillinase-resistant penicillin to be used in the 1960s and was recognized at that time as the most

reliable agent for routine susceptibility testing. Hence resistant strains were termed 'methicillin-resistant S. aureus' (MRSA). Later use of oxacillin as an alternative to methicillin in susceptibility tests resulted in the term 'oxacillin-resistant S. aureus' (ORSA). These designations are used interchangeably in the literature and are synonymous.[28] Methicillin-resistant Staphylococcus aureus (MRSA) were first reported in 1961 and have since become a major nosocomial pathogen worldwide.[29-30] The incidence of *S. aureus* bacteremia (SAB), particularly bacteremia caused by methicillin-resistant *S. aureus* (MRSA) strains, has increased dramatically in recent years in the United States and in some European countries.[31] An additional concern is the emergence of vancomycin-intermediate S. aureus (VISA) and more recently vancomycin-resistant S. aureus (VRSA). [32] The reservoir of MRSA is infected and colonized patients,[31] and the major mode of transmission from patient to patient is on the contaminated hands of healthcare workers.[33-34] It is axiomatic that the sooner an MRSA infection is diagnosed, and the susceptibility to antimicrobial agents established, the sooner appropriate therapy and control measures can be initiated. Laboratory diagnosis and susceptibility testing are crucial steps in treating, controlling and preventing MRSA infections. Guidelines for the control of MRSA infections in the UK have been previously published by a joint Working Party of the British Society for Antimicrobial Chemotherapy (BSAC), and the Hospital Infection Society (HIS) in 1986 [35], 1990 [36] and together with the Infection Control Nurses Association (ICNA) in 1998.[37] The Department of Health Special Advisory Committee on Antimicrobial Resistance (SACAR) asked the three Societies to revise the guidelines. Unlike the previous reports, which focussed on the prevention and control of MRSA infections, SACAR requested that guidelines should be extended to cover prophylaxis and therapy of MRSA infections and also the laboratory diagnosis and susceptibility testing of MRSA. Members of the Working Party were representatives of the BSAC, HIS and ICNA. This report deals with the laboratory diagnosis and susceptibility testing of MRSA in the UK.[38]

MATERIALS AND METHODS

Discarded bandages from infected burn wounds were collected into polythene bags wearing hand gloves from Osmania General Hospital and immediately transferred to the Laboratory.The morphological characterization and biochemical tests are done.[39] After that, the molecular characterization is done.

Serial dilutions and plating

The first step was making a serial dilution.Then 0.5 ml from each dilution was inoculated by pour plate technique in nutrient agar medium and all the plates are incubated at 30 ^{0}C for 24 hrs.

Morphological characterization

Among 35 colonies obtained on 10-^{5} plate by serial dilution and plating, two isolated colonies with different colony morphology were selected and pure cultures of these isolates were obtained by repeated streaking on nutrient agar slants. Finally, pure cultures were stored on nutrient agar slants at 4^{0}C in a refrigerator. These cultures were first identified by preliminary morphological characterization by doing:

1.1- GRAMS STAINING
1.2- ENDOSPORE STAINING
1.3- CAPSULE STAINING
1.4- MOTILITY TEST: (Hanging drop method)

Biochemical tests

The biochemical tests which are done :

Imvic tests

A- INDOLE PRODUCTION TEST
B- METHYL RED TEST
C- VOGES-PROSKAUER TEST
D- CITRATE UTILIZATION TEST
E- HYDROGEN SULFIDE (H_2S) PRODUCTION TEST
F- CARBOHYDRATE FERMENTATION
G- NITRATE REDUCTION TEST
H- CATALASE TEST
I - UREASE TEST

Detection of methicillin resistance

Oxacillin and cefoxitin disc diffusion method

The test organism was subcultured into nutrient broth & incubated for 2-4hrs at 37^{o}C. The turbidity was matched with 0.5 Mcfarland. A sterile cotton swab was dipped into the inoculum, it was rotated firmly against the inner wall of the test tube to remove excess fluid. The test strain was swabbed over Mueller Hinton agar (MHA) with 4 % Nacl for oxacillin and MHA without Nacl for cefoxitin. After 3-5 mins, 1 µg oxacillin and 30µg cefoxitin discs were applied on the corresponding plates. The plates were incubated at 35^{O}C for 18-24 hrs.Zone diameters were measured with a scale. Interpretation was done as per CLSI guidelines.[40]

Oxacillin dics diffusion test

Staphylococcus aureus was considered as sensitive if zone diameter was ≥ 13mm. Diameter of 11-12 mm was considered intermediate. The strain was considered as resistant if the zone diameter was ≤ 10 mm. MRSA NCTC 12493 was used as control.

Cefoxitin disc diffision test

As per CLSI guidelines 2005[41], *Staphylococcus aureus* strains were considered as sensitive if zone diameter was ≥ 20 mm and resistant if zone diameter was ≤ 19 mm.

Molecular tests

Isolation of bacterial genomic dna

The bacterial genomic DNA was isolated and visualized using 1% agarose gel electrophoresis

Detection of staphylococci by pcr technique

Materials required

The following reagents (in the table 1) are enough to perform 10 amplifications. All the reagents carefully stored as per the storage temperature indicated on the label.

Protocol for dna amplification

Primer sequences

F –- 5' – GTA GGT GGC AAG CGT TAT CC – 3'

R –- 5' – CGC ACA TCA GCG TCA G – 3'

For the reaction the following reagents are added to a PCR tube

Table 1
Reagents of PCR

10X assay Taq Pol Assay buffer 15 mM $MgCl_2$	5µl
DNTP Mix	3µl
Template DNA (ng/µl)	1µl
Forward primer(250ng/µl)	1µl
Reverse primer (250ng/µl)	1µl
Taq DNA Polymerase (3 µ/µl	1µl
Sterile water	38µl
Total reaction volume	50µl

Mix the contents gently and layer the reaction mix with 50 µl of mineral oil.

Carry out the amplification using following the reaction condition for 30 cycles.

Table 2
PCR conditions

PCR steps	Temperature	Time
Initial denaturation	94^0C	1minute
Denaturation	94^0C	30 secs
Annealing	48^0C	30 secs
Extension	72^0C	1minute
Final extension	72^0C	2 minutes

After the reaction is over 10µl of the aqueous layer was run in 1% agarose gel for 1 to 2 hours at 100 volts. Along with marker and locate the amplified product by comparing with the 0.8 kb fragment of the marker.

RESULTS

Morphological characterization

The morphological characterization is illustrated in the table below

Table 3
Summary of the results on morphological tests

S.no	Test	Result
1	Gram's staining	Positive
2	Endospore staining	Negative
3	Capsule staining	Positive
4	Motility	Negative

Biochemical tests

The biochemical tests are illustrated in the table 4 below

Table 4
Summary of the results on biochemical tests

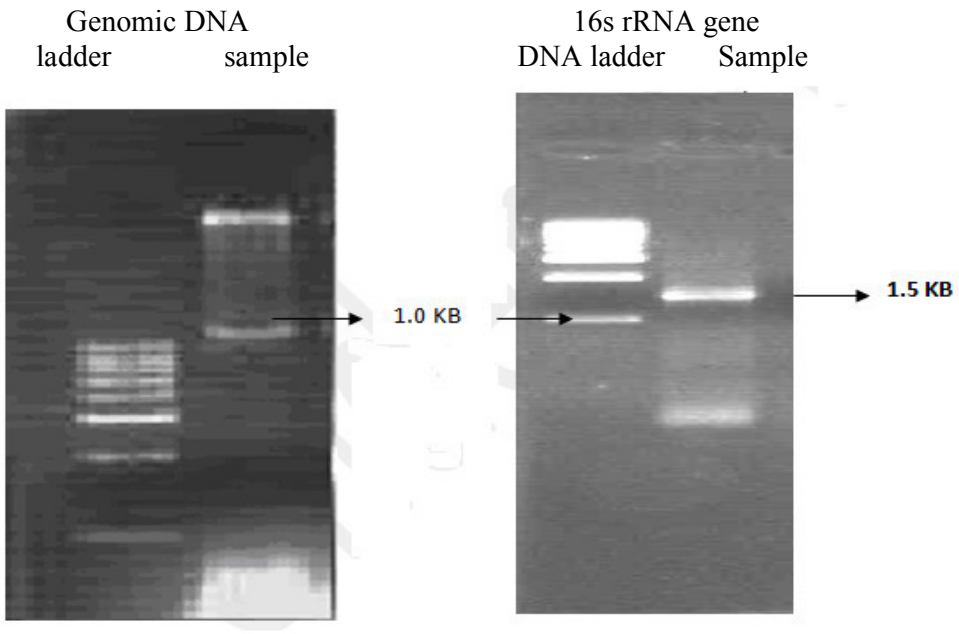
S.no	Test	Result
1	Indole production test	Negative
2	Methyl red test	Positive
3	Voges-proskaure	Negative
4	Citrate utilization test	Negative
5	H_2s production	Negative
6	Cho fermentation	Positive
7	Nitrate reduction	Positive
8	Catalase test	Positive
9	Urease test	Positive

The organism isolated from burn wounds was identified as staphylococcus aureus from morphological and biochemical tests according to bergey's manual.[40]

Molecular tests

Isolation of bacterial genomic dna

After running the PCR, we got the product near 1.5kbp region. That is, the fragment size after amplification is found to be1.5kbp. The result showed in figure 1



The arrow (⟶) indicates 1.5 KB, which represents 16S rRNA Gene in the Microorganism.

Figure 1
Genomic DNA and 16s rRNA gene
DNA ladder is of the size 1.0KB.

Detection of mec a gene by pcr for mrsa

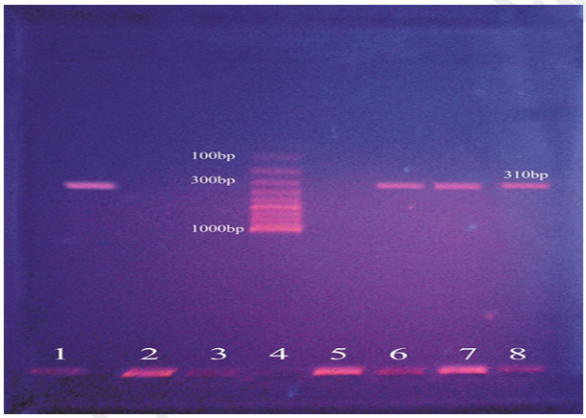
To isolate the plasmid L.B (Luria Bertani) medium was inoculated with one test colony and incubated for 16 hours before carrying out the plasmid isolation. PCR was run to amplify the mecA gene followed by gel electrophoresis to confirm the presence of mecA gene.

Molecular identification of mrsa strain

Genomic DNA isolation-LB broth was inoculated with above test colony and incubated for 20-24 hours at 30^0C and molecular identification of MRSA was done by amplifying 16s rRNA using PCR. A 310 bp amplicon corresponds to *mecA* gene which is shown in figure 2. After running the PCR, the PCR product was analyzed by1% agarose gel electrophoresis and was photographed under U.V light

Primer	Sequence	Position	Product size (bp)
Forward	5′-GTA GAA ATG ACT GAA CGT CCG ATA A-3′	318-342	310
Reverse	5′-CCA ATT CCA CAT TGT TTC GGT CTA A-3′	603-627	

Figure 2
Primers and sequences of mecA gene



2,3,5: Normal Staphylococcus Without mecA Gene
1,6, 7, 8: MRSA with mecA gene.
4: marker of 100bp to 1000bp

Figure 3
Amplifying 16s rRNA using PCR.

DISCUSSION

Methicillin-resistant Staphylococcus aureus (MRSA) are a type of staphylococcus bacteria that are resistant to many antibiotics. MRSA infections are more difficult to treat than ordinary staph infections. This is because the strains of staph known as MRSA do not respond well to many common antibiotics used to kill bacteria.Effective, Rapid Laboratory Diagnosis and susceptibility testing is critical in treating, managing and preventing MRSA infections.Diagnostic Microbiology Laboratories and reference laboratories are key for identifying outbreaks of MRSA. New rapid techniques for the identification and characterization of MRSA have been developed. This notwithstanding, the bacterium generally must be cultured via blood, urine, sputum, or other body fluid cultures, and cultured in the lab in sufficient quantities to perform these confirmatory tests first. Consequently, there is no quick and easy method to diagnose a MRSA infection. Therefore, initial treatment is often based upon 'strong suspicion' by the treating physician, since any delay in treating this type of infection can have fatal consequences. These techniques include Real-time PCR and Quantitative PCR and are increasingly being employed in clinical laboratories for the rapid detection and identification of MRSA strains .[42-43] Present Studies were focused on amplification of DNA encoding for ribosomal RNA genes (16S rRNA). The 16 S sRNA genes have been the most commonly employed genes for identification purposes in pathogenic bacteria. 16S rRNA genes are highly conserved and are found in all bacteria. Highly variable zones of 16S rRNA genes sequences provide unique signatures to any bacterium giving useful information about their identity. Similarly MRSA were detected by using primers for mecA gene. Molecular detection of pathogenic bacteria have several advantages for their adoption into routine clinican and diagnostic bacteriology. Although perceived to be little expensive, the overall quality of the test, and faster detection leading to early institution of therapy may overweigh the cost factor.

CONCLUSIONS

Molecular Diagnostic Techniques are expected to play a significant role in clinical and diagnostic bacteriology. Although their adoption may never replace the conventional methods their efficiency, quality, quickness and their role in the detection of slow growing organisms cannot be overlooked.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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