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**PHENOTYPIC AND GENOTYPIC DEPICTION OF METHICILLIN-RESISTANT
Staphylococcus aureus ISOLATED FROM CANCER PATIENTS**

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ABSTRACT

PCR assay for detection of *mecA* gene in MRSA was compared with disc diffusion method. A total of 170 strains from cancer patients undergoing chemotherapy were examined and some concordance was found in phenotypic (disc diffusion) and genotypic (PCR) methods. Coagulase producing *S. aureus* were further studied by using phenotypic method (slide coagulase), which revealed 141 strains as coagulase positive, while 29 remained negative. Same samples subjected to PCR-based genotypic technique, which exhibited expression of coagulase gene in all strains. A typing method for *S. aureus* based on PCR amplify the variable region of the coagulase gene followed by *Alu I* restriction enzyme digestion and analysis of restriction fragment length polymorphism (RFLP). Among genotypically positive strains of MRSA, RFLP patterns were further investigated, which showed 5 distinct electrophoretic types. Thus, this study suggests that genotypic techniques are more accurate than phenotypic methods for identification and typing of MRSA.

Keywords: MRSA, Polymerase chain reaction, Restriction fragment length polymorphism,
Staphylococcus aureus

INTRODUCTION

Infections triggered by *Staphylococcus aureus* are a primary source of sickness predominantly among the immunosuppressive cancer patients [1]. Maximum strains of *S. aureus* which were initially sensitive to penicillin and later to methicillin finally reported to develop resistance to these drugs [2, 3]. The phenotypic methods of MRSA determination may be affected by heterogeneous expression of resistance, variations in inoculum's size, incubation time and pH of medium [4, 6]. National Committee for Clinical Laboratory Standards (NCCLS) have provided guidelines for antibiotic resistance, but problems occur when microorganisms show their zone of inhibition and minimum inhibitory concentration (MIC) near standard break points [7], thus misleading to proper diagnosis and ultimately results in poor prognosis [5]. That is why phenotypic methods have been overtaken by molecular methods [4, 7]. Among the molecular techniques, bacteriophage typing is difficult to perform in the laboratory due to lack of availability of reagents [8]. Moreover, plasmid analysis has also its own complications, as plasmids may be absent

from isolates, vary in size and may be lost during replication [9]. Pulse field gel electrophoresis (PFGE) and multi locus sequence typing are technically complex and time consuming but appear to be more efficient [10]. The production of coagulase remains prime criterion for identification and typing of *S. aureus* strains from nosocomial infections [11, 12]. A variety of allelic forms of *S. aureus* coagulase are characterized and each isolate has the tendency to produce one or more of these enzyme variants [13]. Its discriminatory power relies on the heterogeneity of region containing 81bp tandem repeats at 3' end coding region of coagulase gene, which differ both in number of tandem repeats and the location of *Alu* I restriction sites among different isolates [14, 15].

In order to select appropriate antibiotic to treat particular strain of *S. aureus* in immuno-compromised cancer patients; it is essential to identify the strains on the basis of their genotyping. Thus, this study was designed to investigate the advantage of genotypic methods based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) over conventional phenotypic techniques.

MATERIAL AND METHODS

S. aureus strains, the isolates of *S. aureus* were collected from Institute of Nuclear Medicine and Oncology, Lahore (INMOL). The *S. aureus* strains were identified on the basis of their morphology and Gram stain characteristics and then characterized by biochemical (catalase and coagulase) tests. Phenotypic characterization was done in previous study by Waqas [24]. **Isolation of genomic DNA**, Genomic DNA was isolated by using the method described by Sambrook from MRSA samples collected from the cancer patients undergoing chemo- and radio-therapies [16]. **Oligonucleotides for *mec* gene**, For PCR amplification of *mec* gene, forward (GTAGAAATGACTGAACG TGGGATAA) and reverse (CCAATTCCACATTGTTTC GGTCTAA) set of primer was used [10]. Amplification of *mecA* with these primers displayed a 310 bps specific product. **PCR Amplification and gel electrophoresis**, PCR master mixture (16 µl) was prepared by adding, PCR buffer 2 µl, MgCl₂ 5 µl, deoxynucleoside triphosphate 3 µl, *mecA* 1 µl, *mecA*2 1 µl, H₂O 3.8 µl and 1.5U (0.2 µl) of DNA Taq Polymerase. 4 µl DNA sample was added in the tube and then placed in thermal cycler (Perkin Elmer, UK), amplification was performed

according to following thermal profile, initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 40 sec, annealing at 50°C for 45 sec, extension 72°C for 50 sec, repetition of denaturation, annealing and extension for 30 cycles and final extension at 72°C for 7 min. The PCR products were separated by gel electrophoresis using 1 % agarose gel run at 120V for 20 min and visualized by ethidium bromide staining. After electrophoresis the fragments of amplified product were visualized directly under the gel documentation system (Vilber Laurmet, France), in which the gel was directly exposed to UV light with a wavelength of 23 nm.

Oligonucleotides and PCR amplification of coagulase gene, For PCR amplification of coagulase gene, and internal primers for 3' end hyper variable region containing 81bp tandem repeats were used. Following set of forward (CCAGACCAAGATTCAACAAC) and reverse (AAAGAAAACCACTCACATC A) primers were used in the study [17]. PCR amplification was performed using the protocol as described above. **Restriction enzyme digestion with *Alu* I and gel electrophoresis** 10 µl of PCR product was digested overnight with 10 µl of the restriction endonuclease *Alu* I. 20 µl of mixture was incubated at 37°C for 16hrs

in a water bath. The restriction digest products were detected by 1% agarose gel electrophoresis following the same procedure as described earlier.

RESULTS

MRSA was isolated and characterized from 170 blood samples of cancer patients who are undergoing chemo- and/or radio-therapies. MRSA characterization was performed according to NCCLS (21), and biochemical characterization was carried out following the methods mentioned in Cheasbrough [23]. Though all samples showed positive reaction to catalase, but 141 strains found coagulase positive and 29 strains remained coagulase negative.

Detection of *mec* gene by PCR

mecA gene was detected by PCR in all 170 strains and characterized by presence of band at approximately 313 bps size (Figure 1).

Detection of Coagulase gene by PCR

Coagulase producing *S. aureus* was screened on the basis of coagulase gene by PCR, and the specific amplified band is shown in Figure 2. Detection by PCR of coagulase gene was carried out in all MRSA samples. After gel-electrophoresis, most of isolates expressed at 980 bps size. 29 of the 170 strains classified as coagulase negative by slide coagulase test, however were found coagulase positive by PCR.

Typing of *S. aureus* using Restriction Fragment Length Polymorphism (RFLP)

Molecular typing of 16 strains of MRSA was carried out by RFLP with *Alu* I (Figure 3). In lane 10 and 14, presence of three bands depicts that coagulase gene has been restricted by *Alu* I at two sites. Two bands in lane 1 show a single restriction site while, remaining samples with one band, exhibit no restriction site for *Alu* I.

Figure 1: (Detection of *mec* Agene by PCR)

Figure 2: (Detection of *coagulase* gene by PCR)

Figure 3 Molecular typing of MRSA using RFLP

DISCUSSION

Different strains of *S. aureus*, resistant to methicillin are considered as the leading cause of nosocomial infections, particularly among immuno-compromised patients [1]. The prevalence of MRSA is constantly rising in many parts of the world and in some hospitals it is more than half of all *S. aureus* infections [18].

In the present study, PCR products of all the *S. aureus* strains showed the expression of *mecA* gene. Determination of *mecA* gene at PCR level identifies intrinsic resistance in a comparatively shorter time and reliable manner. Current data show that molecular methods (PCR-based) are more reliable as compared to phenotypic techniques as, three strains of MRSA were found negative by slide-coagulase test, while their PCR products contrastingly showed presence of coagulase. This is in agreement with Himabindu [19], which showed the importance of molecular techniques in investigating *S. aureus* in patients suffering from the infection. Molecular typing plays an important role to treat MRSA-induced infections by targeting exact strains [20]. Coagulase production is the principal criterion for typing *S. aureus*;

numerous allelic forms of *S. aureus* coagulase exist [17]. Analysis of DNA sequences of cloned coagulase gene revealed that variable sequences exist within 3' end coding region of the allelic gene [21, 22]. The isolated *S. aureus* in the present study are classified into 5 distinct groups (Table 1) based on their PCR-amplified coagulase gene products and unique *AluI* RFLP profiles, which were in multiple of 81bp. This would constitute a minimum number of coagulase gene variants because of the limited number of bacterial isolates used for this preliminary investigation. ET3 and ET4 were most prevailing types, which exhibited same RFLP pattern. Our results are supported by Goh [11], Himabindu [19] and Hookey [23], who reported 19, 13 and 31 distinct RFLP strains respectively. So it seems that in current samples collected from cancer patients of INMOL hospital Lahore, ET3 is the most prevalent type which is followed by ET4. In PCR products, 740bp fragment was the most common and found in 8 samples, while 920bp fragment expressed in 5, exhibiting 40% and 25% frequency respectively.

Table 1: Electrophoretic types

Electrophoretic types	No. of isolates	PCR products approximately Base pair	%age of electrophoretic types
1	2	240/480/740	12.5%
2	1	240/550	6.25%
3	6	740	37.5%
4	5	920	31.25%
5	1	240	6.25%

Table 2: Showing electrophoretic types of *S.aureus* on the basis of PCR products

ET	No of isolates	PCR products approximately (bp)	Strains no
1	1	520/240	06
2	5	720	33,34,37,67,76
3	1	680	27
4	2	720/480/240	35,66,
5	4	900	69 ,77,05,28
6	1	900/1100	30
7	1	240	51

CONCLUSION

This study clearly demonstrates that genotypic detection of drug resistance is essentially important for reliable diagnosis in the clinical laboratory. Determination of resistant *S. aureus* strains needs comparatively much shorter time duration as compared to phenotypic laboratory techniques. Furthermore, PCR-RFLP method for the investigation of nosocomial out breaks due to MRSA can be a useful tool to therapeutically target exact strains of *S. aureus*.

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