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Phenotypic detection of resistance in *Staphylococcus aureus* isolates: Detection of (*mec A* and *fem A*) gene in methicillin resistant *Staphylococcus aureus* (MRSA) by Polymerase Chain Reaction

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Abstract:

Background:

The drug resistant phenomenon is being worldwide concern especially in the last 20 years. Among the most threatening antibiotic-resistant pathogens known are strains of methicillin-resistant S. aureus (MRSA), they are resistant to ß-lactams and other cell-wall-active agents. In many developing countries, the situation appears gloomy due to inadequate or poor implementation of policy on infection control, lack of political will, inadequate resources including shortage of skilled manpower, poor motivation of health care workers and researchers.

Objective:

The present study tries to describe an accurate and quick detection for of clinically relevant antibiotic resistance gene of Staphylococcus aureus using PCR technique.

Methodology:

The *mec A* gene was amplified to characterize MRSA isolates at species level. The S. aureus isolates were analyzed for their susceptibility to different classes of antibiotics using the disk diffusion method.

Results:

Of the total 429 *Staphylococcus aureus* isolated, 114 (26.54%) strains were MRSA. MSRA strains were selected for PCR assay. Eighty one MRSA strains (71.05%) were mecA gene positive and thirty three (28.95%) MRSA strains were mecA negative visualized on 1% agarose gel electrophoresis.

Conclusions:

The PCR assay was rapid and accurate procedure for the detection mec A gene of MRSA strains as compared to the conventional methods like cutler, biochemical and microscopically since the time was taken is less and can help efficiently in infection management.

Keywords: *mec A*, *fem A*, MRSA, SCC, PBP2a, PCR, genes, Staphylococcus aureus

Introduction

Staphylococcus aureus is a versatile, opportunistic pathogen able to cause a wide range of diseases in humans, from minor skin infections to severe illnesses such as septicemia, toxic shock, endocarditis and pneumonia. It is also able to colonize and infect a variety of other host species, including farm and companion animals and wildlife. The emergence and dissemination of methicillin-resistant S. aureus (MRSA) since the early 1960s has posed a major challenge to the treatment of S. aureus infections **(1, 2)**.

Methicillin resistant S. aureus (MRSA) has become a major public health problem worldwide **(3)**. The burden of MRSA continues to raise, with a growth rate of 14% of all S. aureus strains from clinically significant samples in New South Wales, Australia **(4)**. The rising colonization rates lead to the increasing of infection rates in the community and in hospitals. The consequence to the health care system is longer hospital stays and greater costs, which approximately double the expenditure per patient **(5)**.

The resistance in MRSA is due to the expression of Penicillin binding protein (PBP2a) encoded by mecA genes **(6,7)** which is located on the Staphylococcal cassette chromosome (SCC) **(8)**, a large genetic mobile element which differs in size and genetic composition among different strains of MRSA **(9)**. Different types of SCC mec cassettes were extensively studied by PCR techniques. The resistance mechanism involves changes or defects brought about by mutation on mecA gene which results in the organism's resistance to antibiotics. In addition, other antibiotic resistance genes may also be present in the cassette rendering resistance to multiple antibiotics.

Detection of mecA gene by Polymerase chain reaction is considered as the gold standard **(10, 11)** for methicillin resistance as these genes are highly conserved among Staphylococcal species. In the present study, the PCR method was used for the detection of mecA genes among the MRSA strains.

Among the most important of these pathogens are vancomycin-resistant enterococci (VRE) and methicillin-resistant Staphylococcus aureus (MRSA). As summery; the majority of MRSA strains have been associated with hospital-acquired colonization and infections. MRSA strains in nursing homes and long-term care facilities are usually of nosocomial origin , and most MRSA strains isolated from patients upon admission to hospitals or nursing homes can be traced to a previous stay in a similar setting **(12)**.

The study aimed to set up a rapid and accurate detection procedure for methicillin resistance among Staphylococcal isolates through the amplification of specific gene determinants by PCR in order to treat clinical condition and in eradication of the pathogen.

MATERIALS AND METHODS

Clinical Isolates

A total of 114 clinical samples of Staphylococci were isolated from 429 patients. After obtaining an informed consent clinical sample which includes pyogenic materials, aspirates and wound secretions from patients who attended various departments at Al-Hillah Educational Hospital, were collected. All *S. aureus* isolated from nasal swab, boil & urine and identified primarily by routine laboratory procedures which included the cultural and microscopically morphology and biochemical tests including β-hemolysis on blood agar, catalase 3%, oxidase, growth on Mannitol salt agar, urase, DNase, coagulase and Api staph **(13)**.

The specimens were collected under sterile aseptic conditions using sterile test tubes and swabs then transported immediately to the Microbiology laboratory for processing.

Antibiogram:

Susceptibility test was done on all 114 *S. aureus* isolates. Antibiotics were determined by disc diffusion method. Prepared by paper discs impregnated with antibiotic solutions (Himedia, India) placed on the surface of the plate (Molar Hinton agar) inoculated all over with bacterial culture incubated at 37°C overnight and the inhibition zone around the disc was measured **(14)**.

The Kirby-Bauer's disc diffusion method using oxacillin 1 µg disc on 5% Muller-Hinton agar incubated overnight at 35C was done for the detection of MRSA according to the guidelines recommended by the Clinical Laboratory Standards Institute (CLSI).

Genomic DNA isolation

Total genomic DNA was isolated using the Wizard genomic DNA purification kit (Promega Corporation, Madison, WI, USA). A single colony was taken from a nutrient agar, which had been incubated overnight and emulsified into 1000 µL distilled water. Centrifuged at 14000g for 2 min, supernatant was discarded then extracted as Promega purification kit protocol instructions.

PCR Protocol

A three step PCR method reported by Oliveira et al **(15)**, in this study we used the **MultiGENE** conventional PCR which provided with optimal program. The primers used for amplification was derived from the region of the mecA gene. Primers used in this study was; femA F: 5' – AAAAAAGCAC ATAACAAGCG – 3' R: 5' – GATAAAGAAGA AACCAGCAG – 3' (**132bp**); and for mecA F: 5'-TGCTATCCACC CTCAAACAGG-3' R: 5'-AACGTTGTAAC CACCCCAAGA-3' (**286bp**). Under the following cycling conditions one cycle of initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30s, annealing for 30s at 54°C, and extension at 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplified products were separated on a 1% low melting agarose gel (Promega). They were electrophoresed (1X TBE buffer at 170V and for 30 minutes), stained with 0.5% ethidium bromide, visualized and recorded by using gel documentation system (Cleaver Scientific). A 100bp ladder (pioneer) was run as a molecular marker.

RESULTS and DISCUSSION

Of the total 429 Staphylococcal aureus clinical isolates studied, 114 (26.54%) isolates were MRSA (inhibition zone of 10mm or less by Kirby-Bauer's disc diffusion method using 1µg oxacillin disc on 5% Mueller-Hinton agar). *Staphylococcus aureus* is recognized as one of the most important bacterial pathogen that seriously contribute to the problem of nosocomial and community acquired infections **(16)**.

The high prevalence among hospitalized patients is expected due to the long hospital stay, ward conditions such as bed making, changing of clothes, sneezing, nose picking and other personal habits like poor hygiene, which pollute every patient in the wards. Transmission of MRSA occurs primarily from colonized or infected patients or staff to other patients or vice versa. Among the resistant pathogens, MRSA is of great concern because of its particular importance in causing various clinical conditions. Therefore, the risk of acquiring *S. aureus* infection

is increased in the wards in the presence of other hospitalized 'shedders' who may be possibly infected with the antibiotic resistant strains **(17)**.

There was a high prevalence of *S. aureus* among female (70 cases) compared to the 44 cases among the males as showed in table 1.

The lesions sampled included: Otitis Media, Septic burns and wounds, abscesses, Septic surgical wounds, Chronic wounds, Breast abscesses, UTIs and Osteomyelitis as revealed in table 2.

Of the 26 antibiotics tested, **Amoxicillin** showed the highest overall resistance followed by Methicillin, Cefoxitin, Ceftizoxime and Rifampim respectively (Figure 1).

PCR results indicated that eighty one (71.05%) out of 114 MRSA isolates were positive for mec A genes as indicated by 132 and 286 base pairs regions whereas (28.95%) of isolates were mec A gene negative (absence of the corresponding band).

The genus, *Staphylococcus* comprises about 34 different species and methicillin-resistance was reported in most of the species, which included the most pathogenic species *S. aureus* and other commensal species.

Early and accurate diagnosis of MRSA is crucial in effective management and control of spread of MRSA infections. PCR-based assays are considered as the gold standard for the detection of MRSA, due to the heterogeneous resistance by various phenotypic detection methods displayed by many clinical isolates. Genotypic methods are more accurate in detecting methicillin resistant Staphylococci as compared to conventional susceptibility methods **(18)**.

In this study, the MRSA resistance pattern was studied against the 12 antibiotics including penicillin and vancomycin, many isolates proved to be resistant to the some antibiotics (fig 1). Regarding to the disc diffusion method for the detection of MRSA busy laboratories processing, screening and reading of the specimen results are time-consuming, all isolates are confirmed with tube coagulase and susceptibility testing and keeping agar plates for an extended period (48 h) or may be more, increases the workup of suspicious colonies significantly, with a small increase in MRSA detection this agreed with Diederen et al. (2006) **(19)**. In conclusion, molecular techniques remains the most sensitive method in detecting S. aureus at both genus and species level and with 100% accuracy in detecting MRSA, when compared with the classical identification method and this agreed with Martineau et al. (2001) **(20)**. In addition, for greater detection rates, molecular methods have the shortest turnaround time. Although, molecular testing remains expensive relative to conventional agar based detection, there is an overall cost savings, especially if molecular testing is directed at high-risk populations.

The repression of mecA gene and the resulting absence of MR in some of the isolates could be due to several factors. Both genetic and environmental factors play a significant role in the expression of MR. The genetic factor could be repression of mecA by mecI and mecR1, which are its co-repressors **(21)**. The induction of mecA gene occurs through a signaling pathway initiated by the interaction of β-lactams with mecR1, a trans-membrane protein. Therefore, selective pressure generated by indiscriminate use of antibiotic therapy is an important environmental factor in the induction of mecA gene **(22)**.

FemA was amplified in all the standard strains of S. aureus , while no amplification was seen in *S. epidermidis*. All the methicillin-resistant Staphylococci, which included were found to be positive for the mecA gene, whereas both the MSSA isolates were negative for the mecA gene, so it used in this study **(23)**.

The use of rapid molecular methods, which included PCR for the specific identification of *S. aureus* and the detection of methicillin-resistance, had been described previously **(24)**. Most of the previous studies had used anyone of the following genes viz., nuc, 16S rDNA, coag and femA with mecA for the rapid and the specific detection of MRSA at the species level. The emergence of highly pathogenic CA-MRSA infections in healthy individuals **(25)** and their recent emergence as nosocomial pathogens **(26)** with multidrug resistance had necessitated the development of a rapid diagnostic technique for the specific detection of MRSA **(27)**.

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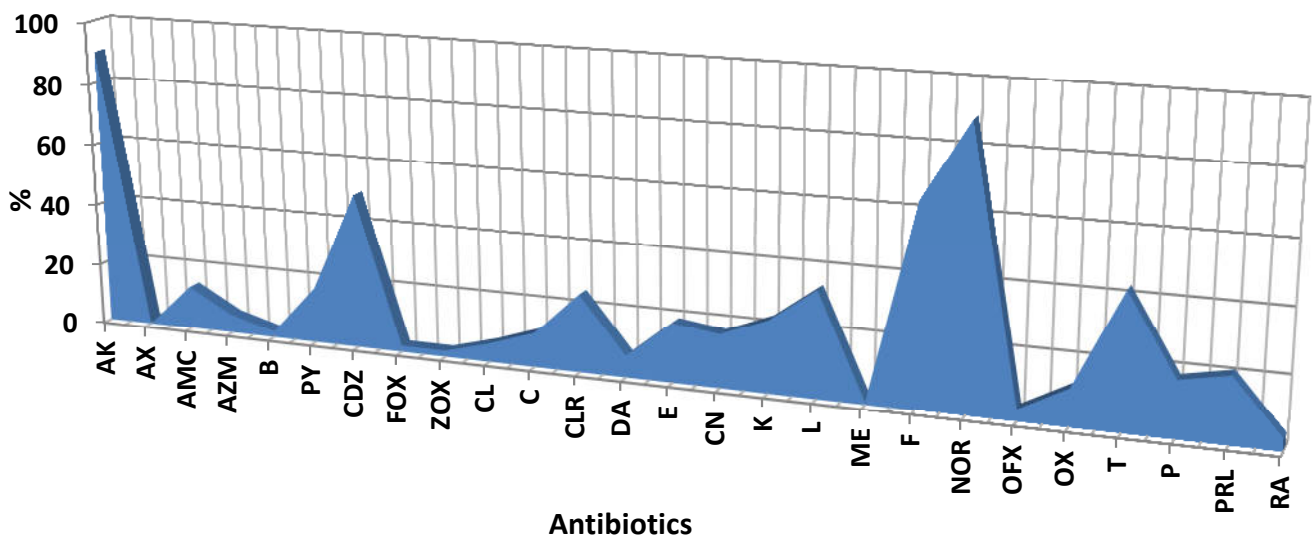
Table1: Age distribution of the study population with MRSA infection

Age (y.)	Female	Male
0-9	9	5
10-19	13	3
20-29	17	8
30-39	19	12
40-49	4	11
50-59	8	5
Total	70	44

Table2: The prevalence of MRSA in the different lesions sampled

Disease	percentage
Otitis Media	7.27
Septic burns and wounds	20.84
abscesses	8.77
Septic surgical wounds	13.36
Chronic wounds	13.90
Breast abscesses	0.84
UTIs	3.72
Osteomyelitis	31.32
Total	100

figure 1: antibiotic sensetivity related to MRSA isolates



AK \Amikacin, AX \Amoxicillin, AMC \Amoxicillin + Clavulanic acid, AZM\Azithromycin, , B \Bacitracin, PY \Carbenicillin, CDZ \Cefodizime, FOX \Cefoxitin, ZOX \Ceftizoxime, CL \Cephalexin, C \Chloromphenicol, CLR \Clarithromycin, DA \Clindamycin, E \Erythromycin, CN\Gentamycin, K\Kanamycin, L\Lincomycin, ME\Rifampim, F\Nitrofurantoin, NOR\Norfloxacin, OFX \Ofloxacin, OX\Oxacillin, T\Oxytetracyclin, P\Penicillin G, PRL \Piperacillin, RA\Rifampim.

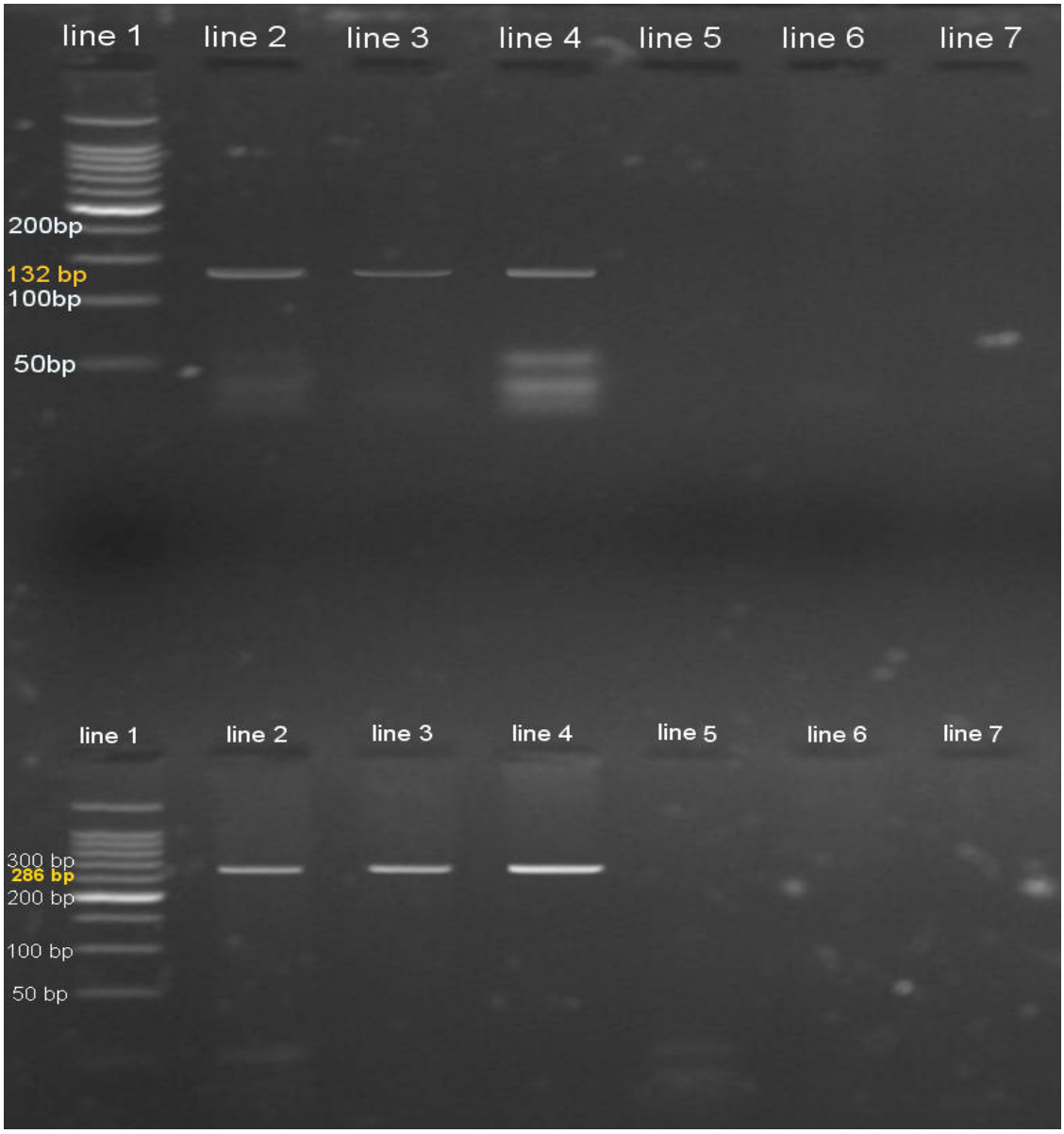


Figure 2. Genomic analysis of DNA for MRSA. The line1 represent ladder (L) lanes contain the 50bp DNA Step Ladder, 4% NuSieve® 3:1 agarose gel in 1X TBE buffer containing 0.5µg/ml ethidium bromide

- lane 2, 3, 4 shows the Sample-DNA from MRSA represent positive femA (132bp) for the upper electrophoresis and mecA (286bp) for the lower electrophoresis
- line 5, 6 shows the amplification products from MRSA represent negative femA (132bp) for the upper electrophoresis and mecA (286bp) for the lower electrophoresis
- Line 7 shows control negative.