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CHARACTERIZATION OF PVL (PANTO-VALANTINE LEUCOCIDINE) GENE IN METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ISOLATES FROM SOME HOSPITALS IN BAGHDAD CITY

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ABSTRACT
The present study determined the prevalence of Methicillin-resistant *Staphylococcus aureus* (MRSA) from 210 clinical samples collected from three hospitals Al-kindy, Al-Yarmouk and Ibn Al-balady hospitals. From the beginning of January 2015 till the end of May2015, all samples were bacteriologically examined by Traditional based on cultural characteristics, biochemical reactions using API Staph. and molecular methods using PCR. The result of traditional methods revealed that 150 were *S. aureus* while 143 isolated depending on molecular methods by using *nuc* gene .In another hand Methicillin resistance was determined by Kirby-Bauer's disc diffusion method. The PCR was used for *MecA* gene detection from MRSA strains, 137were MRSA positive *MecA* gene and six MRSA strains were *MecA* negative while, 120 isolates were MARSA positive *PVL* gene when visualized on 2% agarose gel electrophoresis.

KEYWORDS: *Staphylococcus aureus*, *MecA*, *PVL gene*, Antibiotics, *Staphylococcal* infections, Methicillin resistance.

INTRODUCTION

Staphylococcus aureus is a most important human pathogen which causes numerous infections, both in the healthy individuals and in hospitalized patients. Over 60% of the clinical isolates of *S. aureus* were found to produce the penicillin binding protein 2a (PBP2a) the factor which is responsible for the methicillin resistance, which is encoded by the gene, *MecA*.[1] Resistance to antimicrobial agents is a major concern worldwide and is exemplified by the global spread of methicillin-resistant *Staphylococcus aureus* (MRSA).[1]

Panton-Valentine leucocidin (*PVL*) has gained much importance in the recent past due to its association with the community acquired methicillin-resistant *S. aureus* (CA-MRSA) infection. The *S. aureus* isolates with *PVL* are rapidly spreading and they cause serious skin and soft tissue infections such as abscesses, breast abscesses, necrotizing fasciitis and pneumonia in otherwise healthy individuals.[4-8] PVL is a phage encoded exotoxin of *S. aureus* and it has been found to be cytotoxic to rabbit and human neutrophils as it induces apoptosis.

MATERIALS AND METHODS

1- Clinical Isolates

Two hundred and ten clinical samples collected from three hospitals Al-kindy, Al-Yarmouk and Ibn Al-balady hospitals. From the beginning of January 2015 till the end of May 2015.

2-Isolation and identification of *S. aureus* by traditional methods

-Culturing on selective media

Direct smear examination by Gram's stain followed by culture on Mannitol salt agar at 37°C overnight incubation. The isolates were identified by characteristic colony morphology of *Staphylococcus*. Yellow-colored colonies were obtained on Mannitol salt agar, further confirmed by biochemical reactions using API Staph.,-antibiotic susceptibility test was performed by the modified Kirby-Bauer method (Benson, 2001).

3-Molecular identification of *S. aureus*

-Bacterial Genomic DNA Extraction

An overnight culture in brain heart infusion broth was collected by centrifugation and Extraction of DNA from isolated bacteria, carried out by using genomic DNA kit (Gene aid). Preserved DNA with 50-100µl of TE solution in Eppendorf tubes at 20-Cº.

-Detection of *S. aureus* by polymerase chain reaction (PCR)

1-Detection of *S. aureus* by *nuc* gene specific primer

PCR used for detection of the *nuc* gene for conformation the identification of the *S. aureus*, according to Brakstad *et al*., (1992). These primers synthesized by Cinna Gen Company (Table 1).

Table (1): The sequence and concentration of forward and reverse primers of *nuc* gene.

Primers Type	Primers Sequence	Concentration in picomoles	Product size
nuc Forward	5-GCGATTGATGGTGATACGGTT--3	30262.27	300bp
nuc Reverse	5-AGCCAAGCCTTGACGAACTAAAGC-3	35265.50	300bp

PCR reaction was conducted in master mix tube containing lypholyzied master mix table (2), with 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 3).

Table (2);-AccuPower® PCR Premix content.

Reaction size	Component
1.5 µM	$MgCl_2$
250 µM	dNTP (dATP, dGTP, dCTP, dTTP)
1 U	*Taq* DNA polymerase
30 µM	KCl
10 µM	Tric-HCl (pH 9.0)

Table (3): - The mixture of conventional PCR working solution for detection of, *nuc* gene in *S. aureus*.

Working solution	µl
Water	13 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA	5 µl
Final volume	20 µl

Amplification was conducted using a master cycler Eppendorf programmed with 35 cycler for Initial denaturation 95°C for 3 min., Denaturation for 94°C 1min., Annealing 55°C 30 sec., Extension 72°C 1.5min and final Extension 72°C 3.5min. (Table 4).

Table (4):- PCR program for *nuc* gene amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94 °C	3 min
Denaturation	94 °C	1 min
Primmer annealing	55 °C	30 sec.
Primmer extension	72 °C	1.5 min
Final extension	72 °C	3.5 min
Cycles number : 35 cycle		

1-Detection of *Mec A* gene in *S. aureus* by specific primer

PCR used for detection of the *MecA* gene for conformation the identification of the *S. aureus*, according to Martineu *et al*., (2000). These primers synthesized by Cinna Gen Company (Table 5).

Table (5): The sequence and concentration of forward and reverse primers of *Mec*Agene.

Primers Type	Primers Sequence	Product size
mecA Forward	5-AACAGGTGAATTATTAGCACTTGTAAG-3	170 bp
mecA Reverse	5-ATTGCTGTTAATATTTTTTGAGTTGAA-3	170 bp

PCR reaction was conducted in 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 6).

Table (6): - The mixture of conventional PCR working solution for detection of, *Mec*Agene in *S. aureus*.

Working solution	µl
Water	13 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA	5 µl
Final volume	20 µl

Amplification was conducted using a master cycler Eppendorf programmed with 35 cycler for Initial denaturation 95°C for 4 min., Denaturation for 94°C 30 sec., Annealing 55°C 1min., Extension 72°C 1min and final Extension 72°C 5min. (Table 7).

Table (7):- PCR program for *Mec*Agene amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94°C	4 min
Denaturation	94°C	30 sec.
Primmer annealing	55°C	1 min
Primmer extension	72°C	1 min
Final extension	72°C	5 min
Cycles number : 35 cycle		

Detection of *PVL* gene in S. *aureus*: - (Cabrera *et al*., 16)

Table (8): The sequence and concentration of forward and reverse primers of *PVL* gene.

Primers Type	Primers Sequence	Product size
pvl Forward	ATCATTAGGTAAAATGTCTGGACATGATCCA	433 bp
pvl Reverse	GCATCAASTGTATTGGATAGCAAAAGC	433 bp

PCR reaction was conducted in 20µl of reaction mixture containing, 1 µl of each primer, 5 µl DNA template and 13 µl of deionized water (Table 9).

Table (9): - The mixture of conventional PCR working solution for detection of, *pvl* gene in *S. aureus*.

Working solution	µl
Water	13 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA	5 µl
Final volume	20 µl

Table (10):- PCR program for *pvl* gene amplification by the conventional methods.

Thermocycler conditions	Temperature (°C)	Time (min)
Initial denaturation	94°C	5 min
Denturation	95°C	0.5 min
Primmer annealing	60°C	45 sec
Primmer extension	72°C	1 min
Final extend	72°C	7 min
Cycles number : 35 cycle		

Gel Electrophoresis

PCR products and the ladder marker were resolved by electrophoresis on 2% w/v agarose gels. DNA samples were loaded in the tray of Gels and 100 bp marker was included in every gel and run in TBE (1X) buffer, Gels were stained with ethidium bromide (0.5 µgml-1) and analyzed using UV eliminator The molecular weight determination of resolved band was based on their correspondence to the ladder bands.

RESULT AND DISSECTION

1-Clinical Samples

-Identification of *S. aureus* by traditional methods

A total number of 210 clinical samples were collected from different three hospitals in Baghdad city Al-kindy, Al-Yarmouk and Ibn Al-balady hospitals. The specimens included nasal swab, wound swab, burn swab, abscess and pus, sputum, ear swab, urine and blood culture. (150) isolates identified as *Staphylococci* on a mannitol salt agar depending on yellow color of the colonies, the

media considered a selective and differential growth medium which is used for encouraging the growth of Staphylococci and inhibit others by containing high concentration of NaCl and phenol red as an indicator fig. (1) (Leboffe and Pierce, 2010).150 isolates gave a positive result and were identified as *S. aureus*. due to the production of catalase enzyme which is distinguished them from Streptococcus spp. (Benson, 2001; Brooks *et al*., 2007). Finally, the API Staph. System was used for accurate identification of the isolates at generic and species level. The test was applied on all isolates, which previously identified by conventional biochemical tests the results gained from API Staph system were in covenant with those obtained from biochemical identification.

Figure (1): Appearance of *S. areas* isolates on mannitol salt agar.

Identification by Molecular methods

All isolates were submitted to conventional PCR for further identification on molecular level by using specific primers for detection *nuc* gene, from (150) S. *aureus* isolates recognizing depending on traditional methods (143) isolates were positive for *nuc* gene with product size 300pb fig. (2).

The *nuc* primer set recognized all tested isolates belonged to *S. aureus*, but not other bacteria tested Published data indicate that treatment with antibiotics does not interfere with the detection of the *nuc* gene as long as minimum quantities of the target DNA sequences are still present in the clinical specimens.

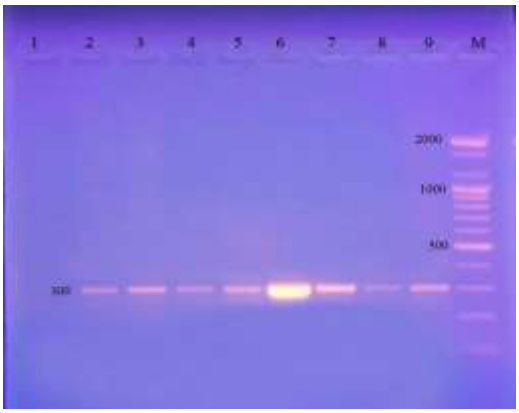

Fig (2): Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *nuc* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder).; Lanes (2-9) positive amplification of 300 bp for *nuc* gene.

The isolates that were previously identified by morphological, biochemical characteristics and molecular level by *nuc* gene primers as *S. aureus* were tested for antibiotic susceptibility using Methicillin antibiotic discs (5µg/disc) by applying the antibiotic disc diffusion method.

The results of this study confirmed that out of (143) tested *S. aureus* isolates that were (137) isolated exhibited a high level of resistance to Methicillin, the target antibiotic, which is reflected MRSA. This result is agreed with the outcome obtained by (Al-Ruaily and Khalil, 2011) in Saudi Arabia.

Rapid and accurate detection of methicillin resistance in *S. aureus* is essential for the use of appropriate antimicrobial therapy and for the control of nosocomial spread of MRSA strains. Thus evaluation the efficiency of the disk diffusion method is important. All the positive isolates (137) that were characterized as MRSA by the (methicillin disc test) were subjected to PCR to detect the presence of *mecA* gene, all of them gave positive results with 170pb PCR product fig (3). The acquisition of *mec A* gene is considered to be the first genetic requisite for methicillin resistance in *Staphylococci* (Nobumichi *et al.*, 1998).

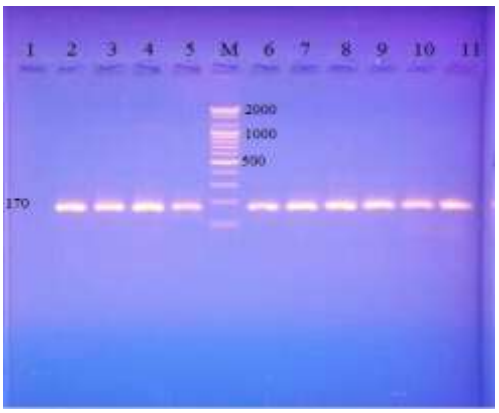

Fig (3):- Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *mecA* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder).; Lanes (2-11) positive amplification of 170 bp for *mecA* gene.

MRSA developed resistance to β-lactam antibiotics through the acquisition of the *mecA* gene that encodes penicillin-binding protein 2a (PBP2a), which has a significantly reduced affinity for β-lactam antibiotics, thereby conferring β- lactam resistance (Lee, 2010). The detection of *mecA* by the Polymerase Chain Reaction (PCR) is considered a gold-standard technique for methicillin resistance detection (Anand *et al.*, 2009). This is mainly because other methods such as the phenotypic methods may be difficult to interpret and

some isolates do not express their *mecA* gene unless selective pressure via antibiotic treatment is applied (Martineau *et a*l., 2000).

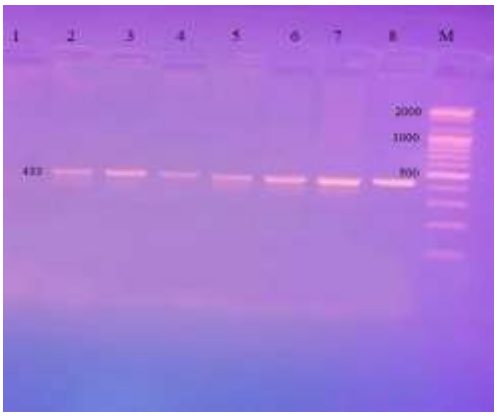

Fig (4):-Agarose gel electrophoresis of PCR amplification products of *S.aureus*, *PVL* gene (2% agarose, TBE buffer (1X), 5V/Cm, 2hr.). M: The DNA molecular Wight marker (100 bp ladder).; Lanes (2-8) positive amplification of 433 bp for *PVL*gene.

The results of this study confirmed that out of (137) tested *S. aureus* isolates that were (120) isolated exhibited *PVL* gene. The screening of *PVL* gene among MRSA has increased importance in recent years due to high involvement of PVL toxin in CA-MRSA Infections, Recent studies have shown that the *PVL* locus is a stable genetic marker of community-acquired MRSA infection code for the production of cytotoxins that cause tissue necrosis and leukocyte destruction by forming pores in cellular membranes (Kunsang *et al*., 2012).

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