



pvl-carried methicillin resistant *Staphylococcus aureus* isolated from hospitalized patients in Baghdad, Iraq

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Abstract

Out of 150 different specimens, 67 *S. aureus* isolate were isolated. However, *16sRNA* gene was located only in 60 isolates. Moreover, *mecA* gene was located in 48 isolates; thereby MRSA covered 80% of all *S. aureus* isolates. Of considerable interest, *pvl* gene was detected in only six isolates (10%). Hence, the present work emphasizes the notion suggested that *pvl* is not an indicative of CA-MRSA.

Keywords: *pvl*, MRSA, PCR, hospitalized patients

العنقوديات الذهبية المقاومة للمثبيلين الحاملة لجين *pvl* المعزولة من المرضى الراقدين في المستشفى في بغداد ، العراق

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الخلاصة

من مجموعه 150 عينة سريرية مختلفة تم عزل 67 عزلة من العنقوديات الذهبية. في حين تم الكشف عن 60 عزلة منها حاوية على جين *16SrRNA*. علاوة على احتواء 48 عزلة على جين *mecA* و بهذا تكون نسبة العنقوديات الذهبية المقاومة للمثبيلين حوالي 80%. من الجدير بالاهتمام، تم الكشف عن وجود جين *pvl* ست عزلات (10%). ومن هنا بينت الدراسة الحالية ان جين *pvl* لا يعد دليلا كاشفا على العنقوديات الذهبية المقاومة للمثبيلين المرتبطة بالمجتمع.

Introduction

Staphylococcus aureus is a persistent pathogen for human and animals, which is responsible for a wide range of infections. The pathogenesis of *S. aureus* infection is a multifaceted process comprising a varied repertoire of either secreted or surface-associated virulence determinants that are synchronously up-regulated at different infection stages [1].

MRSA embraces those *S. aureus* strains being harboured *mecA* gene rendering them resistance to methicillin and basically all other beta-lactam antibiotics. Once methicillin was presented as a medicine to treat staphylococcal strains that resist penicillin in 1961, MRSA strains were reported [2]. Soon after this group of bacteria has emerged as a great challenge in human medicine, especially as a hospital acquired pathogen. However, nosocomial strains have developed resistance towards almost all common antibiotics; hence treating this group of staphylococci constitutes a problematic concern. Furthermore, since the 1990s, MRSA is considered as a concern in those who have not been hospitalized or recently had intensive surgery; hence such strains are known as community-acquired or community-associated MRSA [3].

Panton-Valentine leukocidin (PVL) is one of the pore-forming cytotoxin with a substantial role in MRSA pathogenesis by targeting all leukocytes types except for lymphocytes. Moreover, epidemiological investigations have demonstrated that the PVL genes are harboured mostly in CA-MRSA strains. Nevertheless, these genes carried by HA-MRSA has been reported as well [4].

The prevalence of HA-MRSA harbouring *pvl* gene underlies a serious risk and potentiates a problematic concern that leads to the emergence of strains with enhanced virulence [5]. Upon that, the

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present work undertakes to investigate the prevalence of HA-MRSA bearing the *pvl* gene amongst patients attending Baghdad hospitals.

Materials and Methods

Staphylococcus aureus isolation and identification

One hundred and fifty-seven specimens were collected during a month between November and December 2016. These specimens included anterior nares swabs (n=13) were taken from hospitalized patients referring Al-Yarmouk teaching Hospital and Baghdad Medical City (Madinet Al-Teb) in Baghdad, Iraq.

All these specimens were cultured onto plates of Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hr. Colonies that appeared from primary cultures were re-inoculated by sub-culturing on BHI agar, then re-cultured onto MSA and incubated at 37°C for 24 hr to obtain purified bacterial isolates.

Detection of *16SrRNA*, *pvl* and *mecA*

Extraction of Bacterial DNA

Genomic DNA was extracted using Presto™ Mini gDNA Bacteria (Geneaid, Thailand). Upon the procedure itemized by the manufacturing company, DNA was extracted from overnight cultures of the carefully chosen staphylococcal isolates

Purified DNA concentration was measured at the suitable excitation and emission wavelengths (504 nm_{Ex}/531nm_{Em}) using Quantus™ Fluorometer (Promega, USA). 17 µl of QuantiFluorR dsDNA Dye was added to 3.5 µl of 1X TE buffer Mix.

Gene amplification protocol

Conventional multiplex PCR technique was carried out to amplify fragments of *pvl* (433bp), *16SrRNA* (756bp) and *mecA* (310bp) genes. Two microliters of each primer (Table-1), different concentrations of DNA (depending on DNA yield) extracted from each *S. aureus* isolate and deionized D.W. were added to PCR premix tubes in order to reach 20 µl as a final volume. The multiplex PCR conditions were as follows: 2 µl of template DNA in a 20 µl final reaction volume containing 0.2 µM for the primers specific for the *pvl*, *16SrRNA* and *mecA* genes with the thermocycling conditions set at 94°C for 10 min, followed by 10 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 75 s and 25 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 75 s [6]. PCR products were visualized using 2% agarose gel stained with diamond nucleic acid dye (Promega, USA).

Table 1-Primers used for the amplification of genes in *S. aureus* [6]

Primer Name	Primer Sequence 5'→3'	Target gene	Amplicon size (pb)
Staph756F	AACTCTGTTATTAGGGAAGAACA	<i>16SrRNA</i>	756
Staph750R	CCACCTTCCTCCGGTTTGTCCACC		
Luk-PV-1	ATCATTAGGTAAAATGTCTGGACATGA TCCA	<i>pvl</i>	433
Luk-PV-2	GCATCAAGTGTATTGGATAGCAAAAGC		
MecA1	GTAGAAATGACTGAACGTCGGATAA	<i>mecA</i>	310
MecA2	CCAATCCACATTGTTTCGGTCTAA		

Sequencing of *16SrRNA* PCR product

PCR product of *16SrRNA* was sent to MacroGen DNA Sequencing company (Seoul, Korea), analyzed, then each sequence was aligned and compared with already known *16SrRNA* gene sequences using basic local alignment search tool (BLAST) program.

Result and discussion

Staphylococcus aureus isolation and identification

One hundred and fifty-seven different clinical specimens were collected from patients attending hospitals in Baghdad and streaked on MSA. Sixty-seven isolates appeared as round yellow colonies; therefore, they are primarily identified as *S. aureus*.

DNA extraction and preparation

After DNA extraction by Presto™ Mini gDNA Bacteria Kit, DNA concentration was between 10 and 87 ng/ml; whereas, purity was about 1.88 - 1.99. A ratio of 1.8 -2.0 is generally accepted as “pure” for DNA. If the ratio is appreciably lower than the indicated ratio, it may specify the presence of

protein, phenol or other contaminants that absorb strongly at or near 280 nm [7]. Gel electrophoresis was done to confirm the purity of extracted DNA.

Detection of *16SrRNA*, *mecA* and *pvl* genes by polymerase chain reaction

In this study, multiplex PCR technique was applied to confirm the presence of *16SrRNA*, *mecA* and *pvl* genes. The existence of genes was detected by presence of single band at a given molecular weight. (viz. 756 bp, 433 bp, and 310 bp for *16SrRNA*, *pvl*, and *mecA*, respectively) of marker that be used as in Figure-1.

The current results revealed that 60 out of 67 *S. aureus* isolates harbored *16SrRNA*. Even though, seven isolates were identified using traditional methods as *S. aureus*, they did not have this gene; hence, they were excluded from the study. This finding highlights the inaccuracy, albeit they are quiet essential, of traditional methods for the identification of *S. aureus*. In order to avoid false-positive results, all the traditional identification should be confirmed by means of molecular methods.

Sequencing results revealed that GenBank accession numbers for the nucleotide sequences of the *16SrRNA* gene fragments were called [JN315154.1](#), [KT369584.1](#), [MF784283.1](#), [KX583574.1](#), [MF784283.1](#), [JN084552.1](#), [MF385269.1](#), [KF733730.1](#), [KR265361.1](#), and [CP012976.1](#).

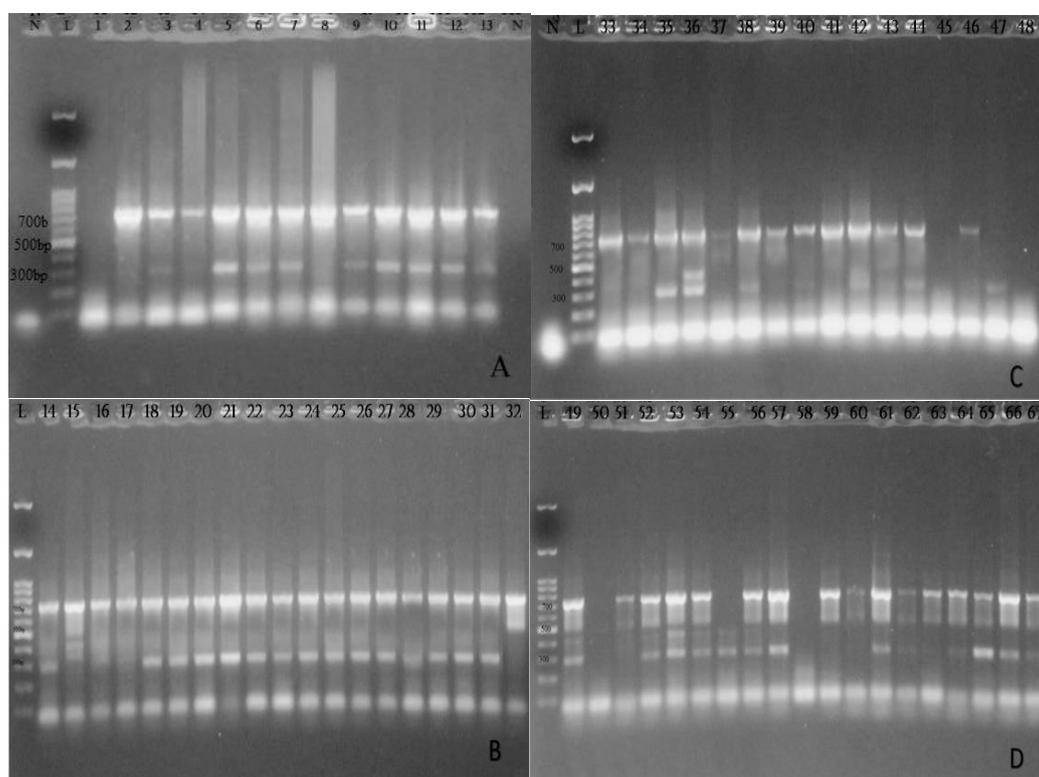


Figure 1- Visualization of *16SrRNA* (756 bp), *pvl* (433) and *mecA* (310) genes by 2% agarose gel analysis. The shown bands are representative of PCR products amplified from 67 *S. aureus* isolates (lanes 1 – 67, respectively), lane N: negative control, lane L represents 100 bp DNA ladder.

Sequence analysis of the *16SrRNA* gene has been widely used to identify bacterial species. Bacterial *16SrRNA* genes generally contain nine “hypervariable regions” that demonstrate considerable sequence diversity among different bacterial species and can be used for species identification. Moreover, genus-specific *16SrRNA* sequence used as an internal amplification control for staphylococcal DNA [8].

Karmakar *et al.* [9] mentioned that among 165 samples, 100 strains (60.60%) were isolated from a selective MSA media and then these isolates were identified as *S. aureus* by different biochemical tests. Gram staining, catalase, coagulase, and thermonuclease were important phenotypic identifying markers of *S. aureus*. They found that 100%, 92%, and 84% isolates were positive for catalase, coagulase, and heat-stable nuclease, respectively. The results of present study agreed with Another study done by Rusenova and Rusenov [10] that total of 156 isolates suspicious for *S. aureus* were detected by a conventional biochemical method. The majority of *S. aureus* strains gave typical

biochemical reactions with the exception of 30 (19.2%) and 25 (16%) that were VP negative and weak positive in fermenting mannitol respectively. Twelve strains were found to be non-haemolytic (7.7%). However, precise detection of *S. aureus* was done by combination of conventional and molecular methods.

The presence of the *mecA* gene in the isolates was confirmed by using multiplex PCR as a gold standard method. Single band was observed at given molecular weight (310 bp). The results of current study illustrated in Figure-1 demonstrated that out of 60 *S. aureus* isolates recovered from different specimens, 48 (80%) isolates were determined as MRSA.

The *mecA* gene synthesizes penicillin binding protein (PBP2a) and it is the cause of methicillin resistance in MRSA. This protein is able to reduce affinity for β -lactam antibiotics. This gene resides on the staphylococcal cassette chromosome (SCC). Staphylococcal cassette chromosome is a large genetic mobile element which varies in size and genetic composition among the strains of MRSA [9]. To treat staphylococcal infections, various classes of antibiotics including beta-lactams, glycopeptides, lipopeptide, oxazolidones, aminoglycosides, macrolides, and fluoroquinolones [11].

The results of current study are disagreed with studies done by Karmakar *et al.* [9] and Boucher and Corey [12], in which they separately reported that the frequency of MRSA infections increases continuously in hospital-associated infection.

During the past decades, MRSA has spread throughout the world and has become highly endemic in many geographical areas. Due to the changing pattern of antibiotic resistance in *S. aureus* and the prevalence of multidrug resistance in MRSA, some investigators have suggested that the resistance patterns should be evaluated periodically and antibiotic therapy should be guided by susceptibility testing [13].

The present study disagreed with a local study performed by Al-Dahbi and Al-Mathkhury [14] as they mentioned that the incidence of MRSA among *S. aureus* was 94.3% (higher than the present study). However, it is compatible with another local study performed by Karam and Al-Mathkhury [15] demonstrated that 20 isolates of *S. aureus* developed 80% Methicillin resistance.

To investigate the distribution of methicillin resistance staphylococci among the patients, Muhammad and Al-Mathkhury [16] performed the antibiotic sensitivity test to 137 *Staphylococcus* isolates using cefoxitin (30 μ g/disk) disk diffusion method. The results revealed that 68% of *S. aureus* isolates developed methicillin resistance.

Owing to unnecessary and unrestrained use of antibiotics, the bacterial species developed multidrug resistance; hence narrowing the therapeutic choices for the treatment [17]. MRSA originated from nosocomial infections highpoints this species as a potential pathogen; which have the capacity to cope with different antibiotics [12].

In the present study the detection of the exotoxin Pantone-Valentine leukocidin gene (*pvl*) was detected by multiplex PCR, a single band appeared at nearly 433 bp as it is illustrated in Figure-1. Only six isolates (10%) out of 60 isolates gave a positive result. Accordingly, the current study has found that 87.5% of all MRSA isolates were determined as HA-MRSA; whereas 12.5% as CA-MRSA.

However, two hundred and eighty-six *S. aureus* isolates were collected from separate places of the holy shrine in Najaf city, Iraq. Phenotypic and genotypic examination for community associated methicillin resistant *S. aureus* (CA-MRSA) isolates was carried out. The CA-MRSA isolates were examined using PCR primers for *pvl* gene. About 54 (18.8%) of the entire *S. aureus* examined isolates gave positive results to the gene [18].

A genetic study was accomplished by Al-Hassnawi *et al.* [19] in Babylon province demonstrated that 19 (79%) out of 24 isolates had positive result for *pvl* toxin gene.

The present work is incompatible with a study done by Weese *et al.* [2] who did not detect *pvl* positive isolates when they investigated raw meat in Canada using the real-time PCR technique. Also Velasco *et al.* [20] stated that *pvl* gene was not detected in any of 77 raw meat samples using the conventional culture and PCR techniques as well as the real-time PCR assay. Same authors reported that CA-MRSA strains are seemingly have the ability to harbour the *pvl* toxin. Such strains have been correlated to life-threatening infections and deaths, predominantly severe skin infections as well as tissue necrosis. Additionally, Rusenova and Rusenov [10] demonstrated that *pvl* exotoxin kills leukocytes (neutrophils and macrophages) in different mammals (*viz.* rabbits, cattle, and humans).

Conclusion

pvl gene was detected in only six isolates (10%). Therefore, the present study accentuated the unreliability of *pvl* as an indicator for CA-MRSA.

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