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ORIGINAL ARTICLE

Molecular Detection of Panton-Valentine Leukocidin (PVL) and Methicillin Resistance in Staphylococcus aureus pathogen

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ABSTRACT

Key words:
CA-MRSA, HA-MRSA, PVL genes, mecA gene

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Background: *Methicillin resistance in S. aureus is caused by the acquisition of mecA gene, that encodes an additional ß-lactam-resistant penicillin-binding protein, termed PBP2a. PVL toxin is one of many toxins produced by S.aureus.* ***Objectives:*** *to evaluate the efficacy of phenotypic methods and detection of mec A gene with PCR for detection of MRSA and to assess the incidence of PVL gene in MRSA and all S. aureus isolates* ***Methods:*** *576 patients from Assiut University Hospitals were enrolled in this study were classified into community acquired infection group (CAI) and Hospital acquired infection group (HAI)culture and PCR were done for all samples* ***Results:*** *92 s.aureus isolates detected MRSA were 62 (67.4%) of all S.aureus infections, They were 30 (65.2 %) of CAI and 32 (69.6 %) of HAI, The prevalence of PVL genes, The prevalence of PVL genes in CAI isolates was 10.9%. None of HAI isolates had PVL gene.* ***Conclusion:*** *The presence of PVL gene cannot be used as a sole marker for CA-MRSA and further studies are required to find a reliable marker or combination of markers to facilitate the recognition of CA- MRSA strains.*

INTRODUCTION

S. aureus is a major pathogen that causes a wide spectrum of clinical manifestations and recognized to be causing nosocomial and community-acquired infection. MRSA was first reported, within a year of methicillin introduction, since then, MRSA strains have spread among hospitals and disseminated worldwide. MRSA has become a worldwide problem, although its prevalence varies considerably among countries as well as between different regions and hospitals within countries [7]

MRSA colonization/infection is encountered in small community hospitals, chronic care facilities and even within the community. Patients colonized in hospitals, when discharged, can spread strains throughout the community and thus colonize or infect non-hospitalized patients. Conversely, patients who have been colonized or infected in the community can introduce MRSA into a hospital, when they are admitted [15].

Although airborne transmission of MRSA is generally considered to be less frequent than transmission via direct contact, airborne MRSA is an important factor to be considered in otolaryngology-head and neck surgery units, because in-patients with malignancy and tracheal fenestration, who lack normal host defense mechanisms in the upper respiratory tracts, are easily infected with airborne MRSA [26].

MRSA also may spread from an initial site of colonization or infection to a site where they cause infection in the same patient (e.g., spread from the colonized nose to a wound), the resulting infection is described as 'endogenous' [2].

MRSA differ genetically from MSSA isolates by the presence of a large stretch of foreign DNA (40-60 Kb), referred to as the *mec* element [30].The origins and mechanism of transfer of SCC*mec* are still unclear and so far no bacterial isolates of any other genera have been reported to carry this element [12].

Seven main types of SCC*mec* (type I to VII) are recognized (Table1). The SCC*mec* type I, IV, V, VI and VII cause only β-lactam antibiotic resistance, while SCC*mec* type II and III cause resistance to multiple classes of antibiotics, due to the additional drug resistance genes integrated into SCC*mec*, as two transposons e.g Tn*554*, Tn554 and integrated plasmids as pUB110, pT181 and pI258 encoding resistance to several heavy metals and aminoglycosides as kanamycin, tetracycline and several heavy metals [18].

Different genotypes are also associated with different types of infections. Types I, II and III SCC*mec* are large elements that typically contain additional resistance genes and are characteristically isolated from HA-MRSA strains. Conversely, CA-MRSA is

associated with types IV and V, which are smaller and lack resistance genes other than *mecA*[17]

HA-MRSA infections occur most commonly in immune compromised individuals in hospitals and health care centers. MRSA are regarded as HA-MRSA when infections caused by them are likely to be acquired in health care settings when they emerge at least 48hours after admission in patients having particular risk factors such as prolonged hospital stay, care in ICUs, prolonged antibiotic treatment, surgical interventions, and/or close contact with MRSA-positive individuals [24]

The majority of CA-MRSA infections are non-life-threatening infections of the skin and soft tissues, these organisms are also capable of producing devastating disease in certain patients. Among these infections are necrotizing fasciitis, septic thrombophlebitis of the extremities, a 'pelvic syndrome' (septic arthritis of the hips, pelvic osteomyelitis, pelvic abscesses and pelvic septic thrombophlebitis), Waterhouse–Frederickson syndrome and rapidly progressive pneumonia [20].

HA-MRSA and CA-MRSA isolates are found to be genetically different. HA-MRSA has been associated with SCC*mec* I, II, or III. These HA-MRSA SCC*mec* types may contain resistance elements for non–β-lactam antibiotics including macrolides, Lincosamides, aminoglycosides, fluoroquinolones, Tetracyclines, and sulfonamides. In contrast, CA-MRSA is characterized by the presence of SCCmec IV or V. These CA-MRSA SCC*mec* types contain primarily *mec*A, *ccr* and on some occasions genes that encode for various toxins, commonly the *pvl* gene. The *pvl* gene codes for PVL cytotoxin, which inserts itself into the host's plasma membrane to form a pore [27]

Resistance to β-lactam antibiotics based on the inability of these agents to bind to the new PBP-2a of *mec*A gene which is located on a staphylococcal chromosome cassette (SCC*mec*) [3]

At least 5 SCC mec types (types I-V), varying in size from ~20 kb to 68 kb, have been identified. The smallest of these -SCCmec are types I, IV and V that contain only recombinase genes together with the structural and regulatory genes for resistance to methicillin, but lack the transposable elements and genes encoding resistance to non- β- lactam antibiotics carried by types II and III [10]

Panton – Valentine Leukocidin (PVL) is one of many toxins associated with S. aureus infection. PVL causes leukocyte destruction and tissue necrosis. It was named after Sir "Philip Noel Panton" and "Francis Valentine" when they associated it with soft tissue infections in 1932 [6].

The work on PVL began in 1894, but the story is far from over. The precise role of PVL in the pathogenesis of severe *S. aureus* infection is still not known. The incident of PVL associated *S. aureus* infections will continue to increase in coming years as *S. aureus* strains bearing the PVL genes continue to spread worldwide and the work still continue on the "anti-toxin" or "toxoid" concept of treatment and prophylaxis [9]

PVL is encoded in a prophage designated as O-SLT Staphylococcal Leukocytolytic Toxin), which is a virus integrated into the *S. aureus* bacterial chromosome. Its genes secrete two protein toxins designated LukS-PV and LukF-PV, 33and 34 Kda in size, respectively [21]

The two components bound protein of PVL genes , LukS-PV and LukF-PV, are secreted before they assemble into a pore-forming heptamer on PMNs Leukocytes membranes leading to PMN lysis [16].

Depending on the concentration of PVL toxin . It can cause either lysis (necrosis) of the leukocyte or apoptosis. High concentrations of PVL cause the lysis of the leukocyte due to the assembly of many pores on the PMNs membrane. Leading to an influx of extracellular substances as ethidium ions**,** On the other hand the low PVL concentration causes PMNs apoptosis via pathway that involves PVL, medical pore assembly on the mitochondrial membrane. Consequently, cytochrome C is released, inducting apoptotic proteins-caspases 3 and 9 that lead to DNA fragmentation and PMNs apoptosis [22].

Aim of the work

The aim of this work to evaluate the efficacy of phenotypic methods and detection of mec A gene with PCR for detection of MRSA. And to assess the incidence of PVL gene in MRSA and all S. aureus isolates.

PATIENTS AND METHOD

1) Patients:

The current study was carried out on 576 patients either from outpatients' infection or from patients admitted to Assiut University Hospitals. Patients were classified according into two groups**:**

Group I: community acquired infection group (CAI) included 304 patients they were either admitted inpatients less than 48 hours from different wards and ICUs of Assiut University Hospitals, with no history of hospitalization, surgery, dialysis, or residence in a long-term care facility within 1 year of the MRSA culture date and has no permanent indwelling catheter or percutaneous medical device (e.g., tracheostomy tube, gastrostomy tube, or Foley catheter) or outpatient from different outpatient clinics.

Group II: Hospital acquired infections (HAI) it included 272 inpatients they were admitted more than 48 hours from different wards and ICUs of Assiut University Hospitals they had different nosocomial infections.

- Verbal consent was taken from each patient before entering the study.
- Different samples were collected from all group patients.
- Full history was taken from each patients including: Name, age, gender, socioeconomic status and residence cause of admission, duration of admission and admission to wards and/or ICUs.

2) Samples

A- Specimens of blood, sputum, urine, and wound were collected under aseptic precautions and transported immediately to microbiology laboratory to be processed and examined.

3) Methods

1. Isolation of staphylococci

By culturing of the samples on: Nutrient agar, Blood agar, Mannitol salt agar, Muller-Hinton agar, Oxacillin resistance screening agar (ORSAB), The antibiotics contained in ORSAB are oxacillin at 2 mg / liter to inhibit methicillin- sensitive staphylococci and Polymyxin B for the suppression of other bacteria able to grow at such a high salt concentration**.** Maximum 2 hours.

The selected colonies stored in brain heart broth containing 5% glycerol at - 20 C °

4) Detection of MRSA

1. Phenotypic detection

a- Disk diffusion susceptibility method:

In all confirmed S. aureus isolates, Oxacillin and Cefoxitin disc diffusion methods were performed for the identification of MRSA according to CLSI guidelines as follows: Resistance (≤ 10 mm), moderately sensitive (11-12 mm) and sensitive (≥ 13 mm), whereas Cefoxitin ≥ 22 mm as sensitive and ≤ 21 mm as resistant

b- Screening of MRSA by ORSAB :

ORSAB is based on traditional MSA with a reduction in salt concentration from 75 g/L (7.5%) to 55g/L (5.5%). This lower level of salt is still sufficient to inhibit most bacteria other than S.aureus but allow growth of MRSA.

2. Molecular detection of MRSA and PVL: detection of mec A gene and (luk F-PV/luk S-PV) fragments gene by PCR after DNA extraction.

- Primers (Qiagen, Germany):

PVL (F): 5-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCCA-3

PVL (R): 5-GCA TCA AGT GTA TTG GAT AGC AAA AGC-3

MecA (F): AAA ATC GAT GGT AAA GGT TGG C

MecA (R): AGT TCT GCA GTA CCG GAT TTG C

- Taq PCR Master Mix (Qiagen, Germany)

For mec A the amplification products (533 bp) were detected by gel electrophoresis

For PVL the amplification products (433 bp) were detected by gel electrophoresis

- PCR products were electrophoresed with 1.5% agarose gel and visualized with ethidium bromide under ultraviolet light.

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation, Chi-square, and Analysis of variance [ANOVA] tests by SPSS V16.

RESULTS

- Detection of MRSA isolates.

Phenotypic detection tests: A total of 57 out of 92 *S. aureus* isolates were considered MRSA by Oxacillin disc method, while 62 were considered MRSA by Cefoxitin disc and ORSAB methods

PCR for detection of mec A gene.

All S. aureus isolates (92) were analyzed by PCR to detect *mecA* gene. 62 (67.4 %) S. aureus isolates had mec A gene they were 30 (65.2%) in CAI isolates and 32(69.6%) in HAI isolates, as shown in table (1)

Table (1): PCR for detection of MRSA isolates in HAI and CAI

Methicillin susceptibility testing	CAI (n = 46)		HAI (n = 46)		TOTAL (n = 92)	
	No.	%	No.	%	No.	%
Methicillin-resistant	30	65.2	32	69.6	62	67.4
Methicillin-sensitive	16	34.8	14	30.4	30	32.6

PCR for detection of PVL gene.

The prevalence of PVL genes in staphylococcal infections isolates, was 10.9 % in CAI isolates and None of hospital acquired S.aureus isolates had PVL gene

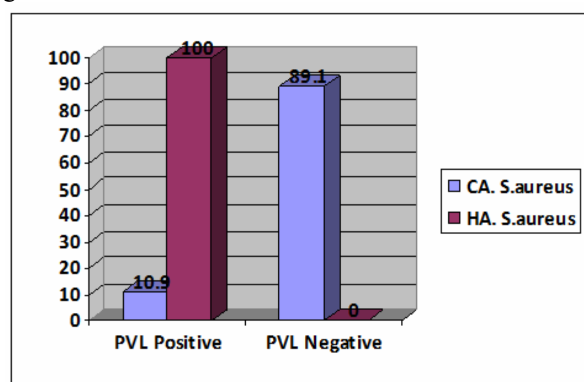

Fig. 1: Distribution of PVL gene in CA- and HA-S. aureus isolates

The existence of PVL genes in MRSA isolates was determined. It was 8% as shown in table (2) , All S. aureus contain PVL genes were CA-MRSA and their percentage was 16.6% .There is significant difference (p value <0.05).

	MRSA		Total
	CA (n 30)	HA (n 32)	
PVL Negative	25 (83.4%)	32 (100%)	57 (92%)
PVL Positive	5 (16.6%)	0 (0%)	5 (8%)
Total	30 (100%)	32 (100%)	62 (100%)

Fig. 2: Gel electrophoresis of PCR amplification for mec A gene in S. aureus isolates (533 bp): Lane 1: marker (100bp) – Lane 3,5,6: positive cases - Lane 6: negative case- Lane 2: negative control.

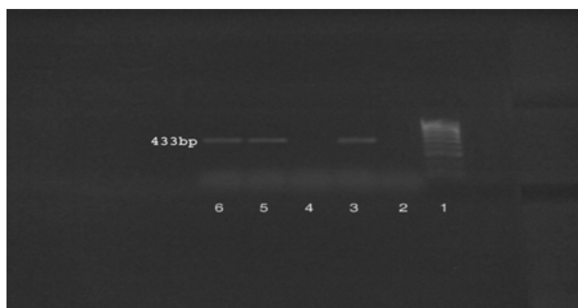

Fig. 3: Gel electrophoresis of PCR amplification for PVL gene in S. aureus isolates (433bp): Lane 1: marker (100bp) – Lane3, 5,6: positive cases - Lane 4: negative case-

DISCUSSION

S. aureus remains one of the most frequently isolated pathogens in hospital and community settings. Infections caused by *S. aureus,* especially MRSA, are emerging as a major public health problem, interestingly; the global epidemiology of MRSA infections is changing significantly ***(Yao et al., 2010).***

In the present study, it was found that HA- MRSA infection was (51.6%), while CA- MRSA infection was (48.4%).

Cefoxitin disc diffusion test had a sensitivity and specificity of 100%.[1,29]

This study revealed that MRSA was (100%) identified by oxacillin screen agar (62 of 62), but (91.9%) by oxacillin disc diffusion (57 of 62). MRSA was identified in (34.9%) by oxacillin screen agar and (37.3%) by oxacillin disc diffusion[19], (75.26 %) of S. *aureus isolates*, were MRSA[14]

In this study, the presence of PVL genes among S. aureus isolates was 5.4%. Prevalence of PVL among *S. aureus* isolates was 3% in a Spanish study between 2005 and 2008[4]. Whereas in Malaysia, the presence of PVL among carriage and invasive S. aureus isolates. All strains were subjected to PCR to detect PVL genes. 4.5% of the carriage and 5% of the invasive isolates were PVL positive.[22]

This low PVL positivity could be explained by the fact that only a few S. aureus strains are susceptible to infection with PVL- converting phages. This phage had shown to be infect only 3% of PVL negative S. aureus strains[13]

In a study in Bilbao, Spain found the prevalence of PVL is only 2.3% among MRSA isolates[4]. Low PVL level among MRSA isolates which was 2% in a French study using RT-PCR[23] . In addition, MRSA with PVL was rare (4.5%) in a study conducted at the Royal Free Hampstead Hospital in North London, England [25].

CONCLUSION

- The best phenotypic method for detection of MRSA is the combination of the Cefoxitin disc diffusion method and ORSAB media. It is simple, rapid, more sensitive, easy, and can help in screening of patients and staff members.
- PVL positive CA-MRSA is more prevalent in younger males with skin and soft tissue infections which have distinct pattern of susceptibility to certain non-β-lactam antimicrobial drugs, and can be effectively cured by incision and drainage, if indicated. Further studies needed to assess the validity of this distinct susceptibility pattern as one of the characteristic defining criteria for identification of CA-MRSA
- Wider application of molecular typing in hospitals is recommended that should shed light to the epidemiology of hospital acquired infections and, therefore, allow for more effective control and prevention strategies.

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