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ORIGINAL ARTICLE

Detection of Panton-Valentine Leukocidin Gene in Clinical Isolates of Staphylococci at Assiut University Hospitals

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Key words:

PVL-gene, HA- MRSA, HA- MSSA, CA-MRSA, CA- MSSA

ABSTRACT

Background: *Panton-Valentine leukocidin (PVL) toxin is mainly associated with necrotic lesions involving the skin or mucosa .PVL has been linked by epidemiological studies to community-associated methicillin resistant Staphylococci (CA-MRSA) and a relatively few data about the incidence of this toxin in nosocomial infections .* ***Objectives:*** *This study aimed to investigate the incidence of PVL gene in nosocomial staphylococcal isolates at Assiut University Hospitals, and to investigate its association with various risk factors and different types of infections.* ***Methodology:*** *This study was a case control study carried on seventy eight patients with nosocomial infections admitted to Postoperative ICU, Trauma ICU, Chest ICU and Internal Medicine ICU; also 27 patients of community acquired infections were also included as a control group. The detection of the mecA gene and PVL gene were done by single target polymerase chain reaction (PCR).* ***Result:*** *The PVL-gene producing strains constituted (17.9%) of all nosocomial isolates. The PVL-gene were detected in all CA-MRSA (44.4%) and not detected in CA-MSSA.* ***Conclusion:*** *Detection of PVL-gene in both community and hospital isolates made this gene not a reliable genetic marker for CA-MRSA.*

INTRODUCTION

Panton-Valentine leukocidin (PVL) toxin is one of many toxins associated with *S. aureus* infection. PVL is a synergohymenotropic toxin that acts through the synergistic activity of 2 non-associated secretory proteins, component S and component F[1].

These two toxins, LukS-PV and LukF-PV act together as subunits. They fit together and form a ring with a central pore through the host defense cells in particular, white blood cells, monocytes and macrophages. This leads to the leak of cell contents[2].

PVL is encoded by two genes, lukS-PV and lukF-PV. They found in a prophage and integrated in the *S. aureus* chromosome[3]. Different PVL-positive *S. aureus* strains have been shown to carry different phage sequences. And one of the PVL-carrying phages has shown to infect PVL-negative *S. aureus* strains, resulting in PVL production [4].

PVL-carrying *S. aureus* strains was linked to furuncles, cutaneous abscesses, severe necrotic skin infections, life-threatening invasive diseases like bacteremia, necrotizing fasciitis, and necrotizing hemorrhagic pneumonia [5].

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PVL has been epidemiologically associated with virulent strains of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) [6]. The PVL genes were present more frequently in *S. aureus* strains that caused primary skin infections and primary community-acquired pneumonia [7]. The differentiation between CA-MRSA and hospital acquired MRSA (HA-MRSA) is becoming difficult to understand, since CA-MRSA could spread into hospitals [8].

To-date there is a relatively few data about the incidence of the toxin in HA-MRSA infections, but MRSA by itself is a prominent cause of nosocomial infections. The risk of the acquiring MRSA in the hospitals increased by severity of illness, length of stay and use of intravascular devices, therefore acquisition of the PVL genes by MRSA represents a challenge in disease management and infection control [9].

We conducted this study to investigate the incidence of PVL gene in nosocomial staphylococcal isolates at Assiut University Hospitals. To detect the linkage between the PVL gene and patient comorbidity factors and outcome. Also to detect the antibiotic sensitivity pattern of the PVL-producing isolates.

METHODOLOGY

1- Patients and specimen collection:

This study included 78 patients admitted to four adult Intensive Care Units at Assiut University Hospitals (Postoperative ICU, Trauma ICU, Chest ICU

and Internal Medicine ICU). After informed consent, basic demographic and comorbidity factors were recorded including age, sex, presence of diabetes mellitus, immunosuppression ,use of steroids and anticancer chemotherapy, or leucopenia < 1x10 ^{9}/L. Also data about extrinsic factors eg, external associated medical devices as urinary catheter, peripheral intravenous catheter, drainage tube and assisted nutrition were recorded. Patients involved in the study were daily monitored for development of nosocomial infections (development of any signs of infection 72 hours after admission), and clinically diagnosed as pneumonia, urinary tract infections, wound infections and bacteremia. The study also included 27 patients of community acquired infections and attended Outpatient Clinic of Chest and Internal Medicine Departments. Pyogenic skin infections were recognized and defined according to the methods of Roberts and Rook[10]; a gram stain of pus showed many polymorphonuclear leukocytes with extracellular gram-positive cocci and a culture yielded pure growth of *S. aureus* on blood agar plates. Pneumonia was defined according to Pingleton *et al* recommendations[11]; acute onset of respiratory symptoms, physical findings such as crackles and rhonchi, radiographic signs, and by bacterial diagnosis. Urinary tract infections in patients who had undergone instrumentation or catheterization were defined by a concentration of >50 leukocytes/mm^{3} and a pure culture of *S. aureus*, yielding >10^{5} cfu/mL [6].

2- Identification and phenotypic testing:

Initial screening and identification of Staphylococcal isolates were performed according to the standard laboratory protocols, including gram stain, mannitol fermentation, and catalase and slide coagulase tests[12].

3- Oxacillin Resistance Screening Agar Base (ORSAB):

Isolates gave positive growth on mannitol salt agar. They were subcultured on ORSAB medium (Oxoid) to detect Methicillin resistance. The lithium chloride added to the medium provided further suppression of nonpathogenic staphylococci[13].

4- Antibiotic susceptibility testing:

High density inocula equivalent to 0.5 MacFarland Barium Sulphate unit were prepared [16,] The following antibiotic discs (Hi-Media, Mumbai) were used; oxacillin (1 µg), vancomycin (30 µg), gentamicin (10 µg), linezolid (30 µg), imipenem (10 µg), tiecoplanin (30 µg), tetracyclins (30 µg), tigecyclins (15 µg) and cefotaxime (30 µg), The zone diameters were measured and interpreted according to the criteria established by CLSI (2010) [14].Tigecycline resistant strains were confirmed by determination of MIC E-Test (MTS, Italy) with a concentration range of 0.016 to 256 µg/ml. The proposed breakpoint for tigecycline is greater than 0.5 mg/l for *S. aureus* (both methicillin-resistant and methicillin-susceptible strains) [15].

5- Detection of the mecA gene and PVL gene by the PCR:

DNA extraction was made by commercially available QIAamp DNA mini kit[16].DNA purity and quantitation through absorbance value measured by a spectrophotometer (ABI) using wavelengths of 260 nm and 280 nm [17].Amplification of mecA using the following primers: mecA -1 (5'- AAA ATC GAT GGT AAA GGT TGGC-3') and mecA-2 (5'- AGT TCT GCA GTA CCG GAT TTG C-3').DNA amplification consisted of an initial cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 10 min [18].Amplification of PVL gene using the following primers : lukPV-1 (5′-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A3') and luk-PV-2 (5′-GCA TCA ACT GTA TTG GAT AGC AAA AGC3')[21].The samples were denatured at 94°C for 5minutes followed by 30 cycles of the following steps: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10minutes[6]. Detection of PCR product by agarose gel electrophoresis visualized with ethidium bromide on 1.5% agarose gels[18].

6- Statistical analysis:

Data collected and analyzed by computer program SPSS" ver. 21" Chicago. USA. Data expressed as mean, Standard deviation and number, percentage. Mann-whitney was used to determine significant for numeric variable. Chi. Square was used to determine significance for categorical variable. (P>0.05 considered not significant, and P<0.05 considered significant).

RESULTS

This study included 78 nosocomially infected patients admitted to four ICUs at Assisut University hospitals. They were 61 males and 17 females with their mean age (42.5±14.24 years). HA-MRSA, HA-MSSA were defined as one culture from clinical specimen obtained ≥72 hrs after patient's admission. These patients showed risk factors for infection eg, recent hospitalization, surgery, diabetes, immunodeficiency and cancer.

Also 27 patients attended the Outpatient Clinic of Internal Medicine and Chest Departments were enrolled.CA-MRSA and CA-MSSA was defined as strains isolated from outpatients who had no history of hospitalization within the past year and presented no other established risk factors for infection.

The nosocomial isolates were 53 (67.9%) HA-MRSA, 9 (11.5%) HA-MSSA and 16 (20.5%) HA-MRCNS isolates. The PVL-gene producing strains constituted 17.9% (14/78) of all nosocomial isolates (Fig. 1 &2).

The distribution of the PVL-gene in nosocomial isolates from the different ICUs is showen in table 1.The PVL-gene were detected in all CA-MRSA 12 (44.4%) and not detected in CA-MSSA.

Patients with PVL-producing strains tended to be male subjects with two or more comorbidity factors. Intravenous catheterization and immunodeficiency were relatively high; 11 (78.6%) and 5 (53.7%) respectively, although this was not statistically significant, (Table 2).

The morbidity rate attributed to infection with PVL-producing strains was low. Only two patients died; one with necrotizing pneumonia and the other with acute myeloid leukemia. Two important manifestations of patients with PVL-producing isolates were pneumonia and bacteremia 7 (50%) and 4 (28.6%) respectively.

PVL-producing isolates were sensitive to vancomycin (12 (85.7%)), tigecycline (9 (64.3%)), linzolid (11 (18.6%)) and Teicoplanin (6 (42.9%)) as showen in table 3. No detectable difference was noted in the resistance pattern between PVL-positive and PVL-negative isolates, (Figure 3).

Table 1: Distribution of PVL-gene producing nosocomial isolates of MRSA, MSSA and MRCNS in different ICUs at Assiut University Hospitals.

Nosocomial isolates / ICUs	MRSA (n= 53)		MSSA (n= 9)		MRCNS (n= 16)	
	PVL+	PVL-	PVL+	PVL-	PVL+	PVL-
Postoperative	10(23.3%)	4(40.0%)	1(14.3%)	1(50.0%)	4(28.6%)	-
Trauma	2(4.7%)	1(10.0%)	-	-	2(14.3%)	-
Internal medicine	13(30.2%)	1(10.0%)	3(42.9%)	-	3(21.4%)	1(50%)
Chest	18(41.9%)	4(40.0%)	3(42.9%)	1(50.0%)	5(35.7%)	1(50%)

Table 2: Demographic data, risk factors and type of infection for patients with nosocomial staphylococcal infections.

Nosocomial isolates / Characteristics	MRSA (n= 53)		MSSA (n= 9)		MRCNS (n= 16)	
	PVL+	PVL-	PVL+	PVL-	PVL+	PVL-
Demographic data						
Age	43.97±15.13	35.1±13.02	40.00±14.53	48.00±11.3	44.35±12.28	38.00±16.97
Sex:						
Female	10 (23.3%)	2(20.0%)	1(14.3%)	1 (50%)	3(21.4%)	-
Male	33(76.7%)	8(80.0%)	6(85.7%)	1 (50%)	11(78.6%)	2(100%)
	P= 0.597 NS		P= 0.417 NS		P= 0.650 NS	
Risk factors						
Mechanical vent.	5(11.6%)	-	-	-	1(7.1%)	-
	P= 0.335 NS				P= 0.87 S	
Diabetes	15(34.9%)	1(10.0%)	-	-	-	-
	P= 0.120 NS					
Surgery	9(20.9%)	4(40.0%)	2(28.6%)	1 (50.0%)	5(35.7%)	-
	P= 0.193 NS		P= 0.583 NS		P= 0.458 NS	
Malnutrition	13(30.2%)	3(30.0%)	-	2(100%)	3(21.4%)	1(50.0%)
	P= 0.653 NS		P< 0.02*		P= 0.450 NS	
Catheter I.V	27(62.8%)	8(80.0%)	3(42.9%)	2(100%)	9(64.3%)	1(50.0%)
	P= 0.259 NS		P= 0.278 NS		P= 0.625 NS	
Catheter urine	12(27.9%)	5(50.0%)	-	1(50%)	5(35.7%)	1(50.0%)
	P= 0.165 NS		P= 0.222 NS		P= 0.458 NS	
Artificial nutrition	12(27.9%)	5(50.0%)	1(14.3%)	-	4(28.6%)	-
	P= 0.165 NS		P= 0.778 NS		P= 0.550 NS	
Immunodeficiency	18(41.9%)	4(40.0%)	4(57.1%)	-	4(28.6%)	1(50.0%)
	P= 0.602 NS		P= 0.278 NS		P= 0.542 NS	
Type of infection						
Wound and skin infection	3(7.0%)	2(20.0%)	1(14.3%)	1(50.0%)	1(7.1%)	-
Urinary tract infection	2(4.7%)	-	-	-	1(7.1%)	-
Bacteremia	12(27.9%)	3(30.0%)	3(42.9%)	-	2(14.3%)	1(50.0%)
Pneumonia	26(60.5%)	5(50.0%)	3(42.9%)	1(50.0%)	10(71.4%)	1(50%)
	P= 0.554 NS		P= 0.413 NS		P= 0.661 NS	

Table 3: Antibiotic sensitivity pattern of PVL-producing nosocomial staphylococcal isolates.

Antibiotic / Strains	PVL-producing nosocomial staphylococcal isolates n= 14		
	R	**I**	**S**
Oxacillin	12 (85.7%)	-	2 (14.3%)
Tigecycline*	1 (7.1%)	4 (28.6%)	9 (64.3%)
Teicoplanin	3 (21.4%)	5 (35.7%)	6 (42.9%)
Vancomycin**	2 (14.3%)	-	12 (85.7%)
Linzolid	3 (21.4%)	-	11 (18.6%)
Gentamicin	10 (71.4%)	-	4 (28.6%)
Cefotaxime	13 (92.9%)	-	1 (7.1%)
Imipenem	8 (57.1%)	2 (14.3%)	4 (28.6%)
Tetracycline	11 (78.6%)	-	3 (21.4%)

*Tigecycline resistance was confirmed by MIC E-Test (MTS, Italy) [15].
**Vancomycin resistance was confirmed by detection of *VAN-A* gene by PCR [19].

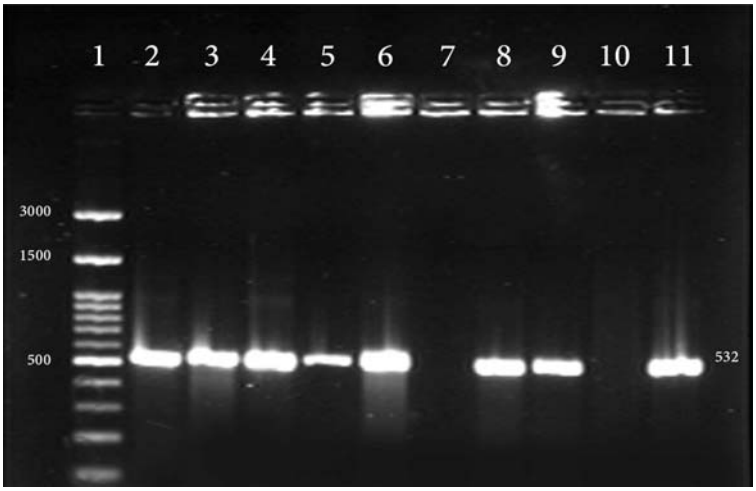

Fig. 1: Gel electrophoresis for detection of mecAgene. Lane 1: DNA marker (100-3000bp); lane 7: negative sample, and lanes:2,3,4,5,6,8,9,11are mecAgene positive samples (532bp), lane 10: negative control.

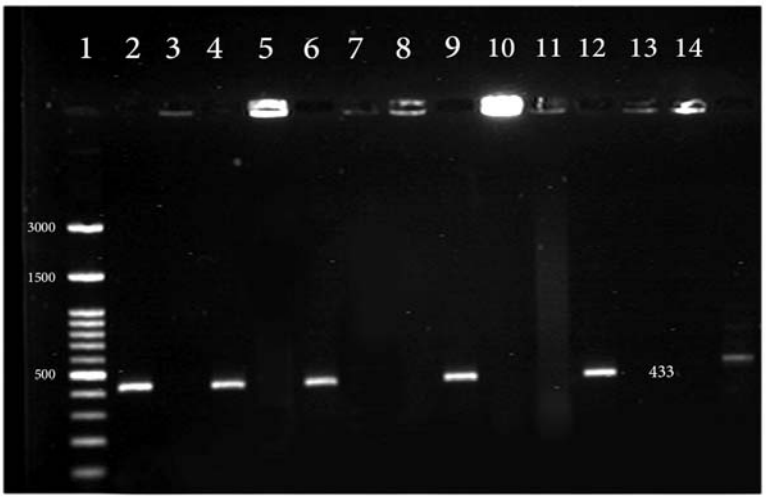

Fig 2: Gel electrophoresis for detection of PVL-gene. Lane 1: DNA marker (100-3000bp); lanes 3, 5, 7,10 are negative sample, and lanes: 2, 4 are PVL-gene positive community acquired strains (433bp), lanes: 6,9,12 are PVL-gene positive hospital acquired strains, lane 13: negative control.

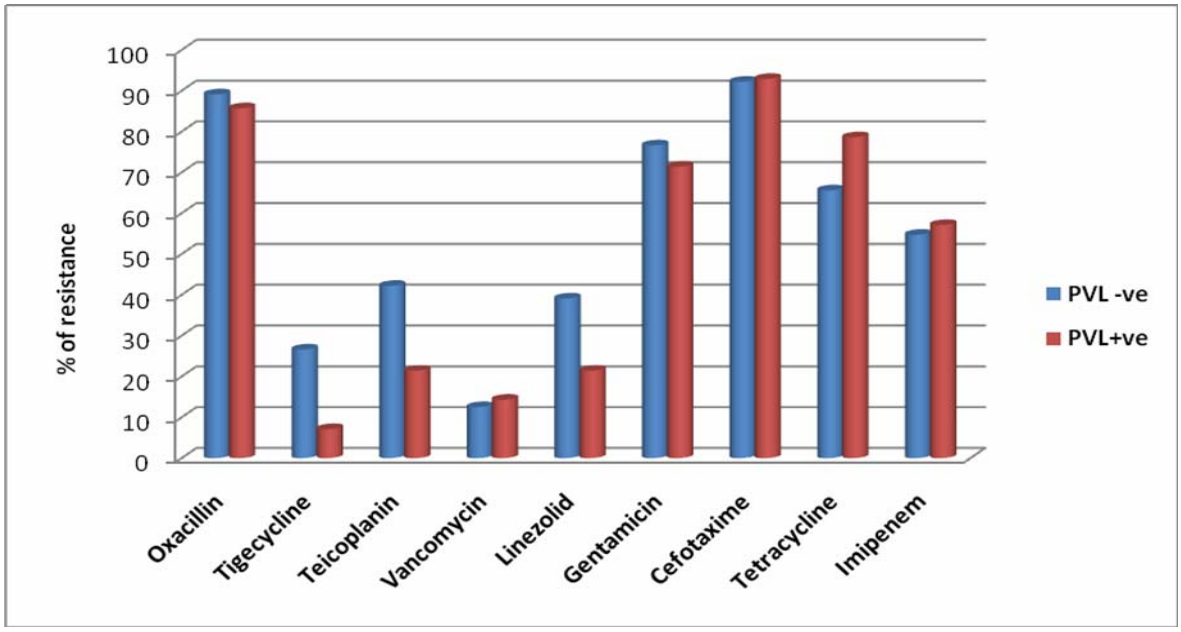

Fig. 3: Resistance pattern of PVL positive and PVI negative strains to different antibiotics.

DISCUSSION

This is the first study on PVL-harboring staphylococci at Assiut University Hospitals. PVL-gene gained a great attention as a key genetic marker for CA-MRSA and very limited studies reported the presence of this gene in nosocomial isolates. The incidence was very high representing 14 (17.9%) of all hospital acquired staphylococci, comparatively, the presence of PVL-positive staphylococci were 12.8% in China [20] and much more lower in France 5%[21].

The CA-MRSA isolates carrying the PVL-gene were 12 (44.4%), similar to an Indian study that reported (25.33%) of CA-MRSA harbored the PVL-gene [22]. In Egypt, Nagwa *et al* [23].reported that none of the HA-MRSA isolated from Alex. Main University Hospital gave positive result for PVL gene. While the same study reported high prevalence of PVL-gene in CA-MRSA.

These findings makes the differentiation of clinically distinct isolates depending on the PVL-gene is not reliable, and the PVL-gene is present in both community and hospital isolates.

The distribution of PVL-gene in HA-MRSA (18.9%), HA-MSSA (22.2%) and (12.5%) HA-MRCNS were not statistically significant (P-value = 0.793), but comparable results obtained in Nepal, where PVL-gene in the MRSA & MSSA represented (26.1% and 51.9%) respectively[24].The PVL-gene was detected mainly in isolates from patients suffered from Bacteremia (50%), Pneumonia (28.6%), and only 3 (21.4%) patients suffered from skin and soft tissue infections. Many studies showed strong association between PVL containing isolated with the abscesses, skin cellulites and boils, Nagwa *et al* [23]. reported that (27.8%) of PVL-positive MRSA isolates recovered from skin and soft tissue. Higher result (71%) reported in Germany[25].This difference any attribute to small study populations.

The PVL-producing isolates were high in male subjects (78.6%), in addition, the age of PVL-positive patients with MRSA, MSSA and MRCNS were not statistically significant. Previous studies showed strong association of PVL-positive isolates with younger patients[24]. This difference may be due to the investigation of nosocomial isolates in our study, which can infect any patient with one or more risk factors for acquiring an infection in the hospital regardless to age.

The outcome of the PVL positive patients was good and comparable to the PVL-negative patients. Only two cases were died. A case of acute myeloid leukemia, 55 years old male patient, presented with bone marrow failure, high grade fever, echymosis, purpuric eruption, bleeding per gum with recurrent cough and expectoration. Blood count showed pancytopenia with neutrophilic count $1.5x10^9$/L. The second case was young male, 35 years, with acute necrotizing pneumonia on artificial ventilation, presented with high grade fever cracks and rhonchi on the left infraclavicular and left mammary areas with increase T.V.F and bronchial breathing on the same areas with evidence of air spaces and cavitations on the left upper and middle lung zone on the left side.

Recently, treatment of serious MRSA infections has been based on the use of glycopeptides; vancomycin and teicoplanin. However, increasing rates of resistance to glycopeptides has urged the development of newer agents as tigecycline (the first member of the glycylcyclines; active against gram-positive, gram-negative, and anaerobic micro-organisms, including multidrug-resistant strains. It exhibits generally

bacteriostatic action by reversibly binding to the 30S ribosomal subunit and inhibiting protein translation[26]. The interesting point in our results is the antibiotic resistance pattern of the PVL-positive strains which was not greatly different from those PVL-negative strains, with the exception of tigecycline, teicoplain, and vancomycin. The PVL-positive strains are more sensitive to them, (figure 3). Shrestha *et al* [24], reported that no substantial difference could be noticed in the resistance pattern of both types of strains with the exception of tetracyclines and clindamycin. They observed that PVL-positive strains were more sensitive to them.

In conclusion, high incidence of PVL-gene in nosocomial staphylococci isolated from Assiut University Hospitals. This suggests the interhospital spread of these strains and so we recommend the need of implementation of patient safety measure and strict infection control practice. Also, PVL-gene detected in both community and hospital isolates and so this is not a reliable genetic marker for CA-MRSA. Finally, the treatment of such cases is not different from other nosocomial staphylococcal infection.

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