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Prevalence of Panton Valentine Leukocidin Gene in Methicillin Resistant and Sensitive *Staphylococcus aureus* Isolated from Egyptian Hospitals

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STAPHYLOCOCCUS aureus (S. aureus) carrying Panton-Valentine leukocidin (PVL) has become a serious global problem. Panton-Valentine leukocidin-positive *Staphylococcus aureus* can result in several infections. Although it is associated with community acquired methicillin resistant *S. aureus* (MRSA), several outbreaks due to methicillin-sensitive *Staphylococcus aureus* (MSSA) were reported. This study was conducted to determine the frequency of PVL-positive gene in methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* (MSSA) among isolates from Egyptian hospitals.

Various clinical samples were collected from two Egyptian hospitals, one in Cairo and the other in Zagazig Governorates. The samples were collected from January 2010 to December 2010 and subjected to culture then bacterial identification. *S. aureus* was identified by conventional methods then MRSA and MSSA isolates were identified using sensitivity test for both oxacillin and cefoxitin and the results were compared with Chrom ID MRSA (Chromogenic media for detection of MRSA). Polymerase chain reaction (PCR) was used to detect the PVL gene among 42 MRSA and 25 MSSA isolates.

Among PCR tested isolates, 11.9% of *S. aureus* isolates harbored the PVL gene (8/67). Five MRSA isolates were harboring the gene representing also 11.9% (5/42). Three among 25 MSSA isolates were PVL positive (12%). Accordingly, no significant difference was observed between MRSA and MSSA regarding the presence of the PVL gene. On the other hand, no PVL gene was detected among 10 Gram positive isolates other than *S. aureus* (3 of them were coagulase negative Staphylococci).

Staphylococcus aureus (*S. aureus)* is one of the major human pathogens that can cause community and hospital-acquired (HA) infections (Fey *et al*., 2003). This bacterium is the most prevalent isolate taken from hospitalized patients.

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The global emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) has turned into a serious public health problem. The bacterium is known as the most significant cause of nosocomial infections, which are resistant to different antibacterial classes. Antibiotic therapy has faced severe difficulties due to these strains (David *et al*., 2006 and Holmes *et al*., 2005).

The pathogenesis of *S. aureus* is caused by several virulence factors such as staphylococcal exoproteins (Gravet *et al*., 1998). Among the exotoxins produced by *S. aureus*, some can selectively destroy phagocyte cells such as polymorphonuclear cells and monocytes. These exotoxins belong to the bi-component leukotoxin family, including S and F proteins. Panton-Valentine leukocidin (PVL) is a member of the staphylococcus leukotoxin family (Choorit *et al*., 1995; Kaneko *et al*., 1997 and Havaei *et al*., 2010). It is a very important virulence factor in *S. aureus*, which is a pore-forming exotoxin and its toxic effects result from the synergistic performance of 2 separate proteins (LUK S-PV and LUK F-PV) (Supersac *et al*., 1993 and Ruimy *et al*., 2008).

Staphylococcus aureus strains containing the PVL gene have the potential to epidemiologically spread in the community (Hollis *et al*., 1995). In fact, the PVL gene was first reported among community-acquired (CA) MRSA strains (Miller *et al.*, 2005).

CA-MRSA strains are more likely to produce PVL (Supersac *et al*., 1993), but some studies refer to the prevalence of PVL-containing *S. aureus* isolates from the community to the hospital (O'Brien *et al*., 1999), this is a remarkable risk to public health. Moreover, the analysis of MRSA isolates in Holland (2003) showed that 8% of nosocomial isolates carry locus for PVL (Wannet *et al*., 2004). Therefore, the isolates do not only exist in communities but can also spread in hospitals (Wagenlehner *et al*., 2007). Consequently, early diagnosis and decolonizing carriers is inevitable and essential, since it can prevent person-to-person transmission of the isolates and, ultimately, fatal prevalence (Munckhof *et al*., 2009).

In the current study, the prevalence of PVL gene was determined using PCR technique among *S. aureus* isolated from clinical samples in two Egyptian hospitals. The isolates were identified as MRSA or MSSA.

Material and Methods

Patient and sampling

The samples were collected from out patients and inpatients departments, (Intensive Care Unit, orthopedic) at Zagazig University hospitals, Egypt and from Kobbri El-Kobba Military Hospital in Cairo. The samples collected from January 2010 to December 2010. These cases were suffering urinary tract infection, cutaneous abscesses, wounds infection, pneumonia, furunculosis and several necrotic infections. These samples included mainly pus, urine, blood and sputum.

Bacterial cultivation and isolation

The collected samples were cultivated on Blood agar, Deoxyribonuclease, Mannitol salt agar and Meuller Hinton agar.

Bacterial Identification

A. *Morphology and microscopy:* Colonies were examined for morphology and Gram positive grape-like clusters of *staphylococci* (Kloos and Bannerman, 1995).
B. *Biochemical reactions:* Catalase test, tube and slide coagulase test, mannitol salt agar and deoxyribonuclease test were performed.

Antibiotic susceptibility test

Disc diffusion method : Sensitivity of the isolated strains to oxicillin (1 µg) and cefoxitin (30 µg) was performed on Meuller Hinton agar plates.

Chrom ID MRSA

The bacterial isolates were subjected to culture on a chromogenic media for detection of MRSA.

Multiplex polymerase chain reaction (PCR)

The primers (GeneBank accession no. AB245454) were designed according to Taneike *et al.* (2006) and Motoshima *et al.* (2010). The primer for the *luk-PV* gene that encoding Panton- Valentine Leucocidin is the forward primer GTG ATC GCG CTG AGG TAG TG and the reverse primer CCT CCT GTT GAT GGA CCA CT. PCR reaction was performed according to the following steps:

A. Dream Taq Green PCR Master Mix (2x) was gently vortexed and briefly centrifuged after throwing.
B. A thin-walled PCR tube was placed on ice and the following components were added for each 50µl reaction:

Dream Taq green PCR Master Mix (2x)	**25µl**
Forward primer	0.5 µM
Reverse Primer	0.5 µM
Template DNA	1 µg
Water, nuclease free	To 50 µl
Total volume	50 µl

C. Samples were gently vortexed & spin down.
D. Thermal cycling conditions of PCR reaction were as follows:

Steps	**Temp. ˚c**	**Time**	**No. of cycles**
Initial denaturation	94	3min	1
Denaturation	95	30 sec.	30
Annealing	54	30 sec.	
Automated Florescent extension	72	1min.	
Final extension	72	5min	1

E. Five µl of PCR product was loaded directly on agarose gel using DNA Ladder. (Taneike *et al*., 2006).

Statistical analysis

Data analysis was performed using Chi-squared test, Fisher's exact test and T-test.

Results

Table 1. shows the distribution of MRSA and MSSA among different types of specimens with no statistically significant difference.

Five out of 8 PVL carrying *S. aureus* isolates were MRSA and ten non *S. aureus* isolates were negative for the PVL gene, 3 of them were coagulase negative Staphylococci. The prevalence of the PVL gene among MRSA and MSSA isolates (Table 2) and different age of patients (Table 3), shows no significant difference. On the other hand among the 8 PVL strains, 7 were isolated from males, none from females and 1 without recovered gender, revealing a significant differnce(Table 6).

TABLE 1. Distribution of MRSA and MSSA isolated from different samples.

Types of samples	MRSA isolates		MSSA isolates	
	Number/ Total	%	Number/ Total	%
Urine	5/8	62.5	3/8	37.5
Pus	30/49	61.2	19/49	38.8
Blood	3/4	75	1/4	25
Peritoneal Fluid	0/1	0	1/1	100
Sputum	1/1	100	0/1	0
Sinusitis Fluid	0/1	0	1/1	100
Pleural Fluid	1/1	100	0/1	0
Not Reported	2/2	100	0/2	0

* $P > 0.05$ as comparison for all specimen types.

TABLE 2. Prevalence of PVL gene among MRSA & MSSA isolates.

AMONG ISOLATES	PVL ISOLATES/TOTAL	PERCENT
MRSA	5/42	11.9 %
MSSA	3/25	12 %
Total *S .aureus*	8/67	11.9%

TABLE 3. Patients' age among PVL harboring isolates.

Isolates	Age Mean ± SD	P value
PVL positive	26.1 ± 13.2	> 0.05
PVL negative	38.3 ± 18.1	

The type of sample shows no statistical association with the detection of the PVL gene in the isolated *S. aureus* strains (Table 4). However, 5 out of 8 PVL

carrying isolates were from pus samples. Among the 8 PVL gene carrying strains, there were five isolates from different wounds or abscesses, one from blood infection and one from lung infection (pleural fluid) (Table 5).

TABLE 4. Detected PVL gene among different types of samples.

Types of samples	PVL negative isolates		PVL positive isolates	
	Number/ Total	%	Number/ Total	%
Urine	8/8	100	0/8	0
Pus	44/49	89.8	5/49	10.2
Blood	3/4	75	1/4	25
Peritoneal Fluid	1/1	100	0/1	0
Sputum	1/1	100	0/1	0
Sinusitis Fluid	1/1	100	0/1	0
Pleural Fluid	0/1	0	1/1	100
Not Reported	1/2	50	1/2	50

TABLE 5. Sources of the isolates carrying the detected PVL gene.

Isolate	2	5	11	20	25	34	37	78
Specimen	Pus	Pus	Pus	Blood	Pleural fluid	Pus	Pus	NR
Source	Abscess after appendicitis	Abdominal infection after surgery	Foot wound	Blood	Pleural fluid	wound infection	wound infection	NR

TABLE 6. Patients' gender of PVL positive isolates.

Isolates	Number/Total (%)		P value
	Male	Female	
PVL positive	7/38 (18.4%)	0/27 (0%)	< 0.05
PVL negative	31/38 (81.6%)	27/27 (100%)	

Tables 7 and 8. show that among 42 isolates reported as MRSA by the conventional sensitivity tests (for oxacillin and cefoxitin), only 27 isolates were recognized as MRSA by the Chrom ID MRSA test. This figure shows a high significant difference between the two methods that the Chrome ID MRSA test shows a specificity of 96% and sensitivity of 64.3% respectively.

Figure 1. Gel electrophoresis images of the PCR products for the PVL gene. Eight isolates were shown to be positive for PVL gene and gave a band at 352 bp. First lane, molecular size marker and the second lane, positive control (a standard strain NCTC 13300).

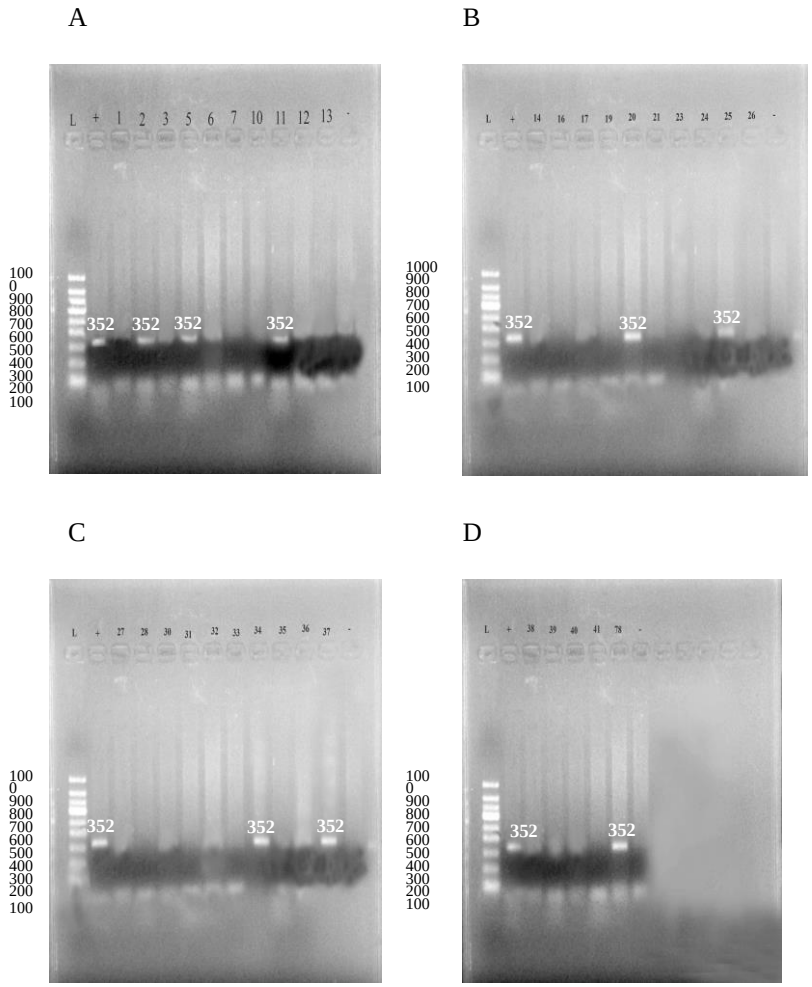

Fig. 1. Detected bands of PVL as shown on agarose gel electrophoresis of the PCR products for different bacterial isolates.

A. Lane 1: molecular size marker & lane 2: Positive PVL gene carrier strain (a standard strain NCTC 13300) & Lane 13: negative control (water) & Lanes 4, 6, 10: PVL gene positive isolates number 2, 5 & 11, other lanes: negative PVL gene isolates.

B. Lane 1: molecular size marker & lane 2: Positive PVL gene carrier strain (a standard strain NCTC 13300) & Lane 13: negative control (water) & Lanes 7, 11: PVL gene positive isolates number 20, 25 & other lanes: negative PVL gene isolates.

C. Lane 1: molecular size marker & lane 2: Positive PVL gene carrier strain (a standard strain NCTC 13300) & Lane 13: negative control (water) & Lanes 9, 13: PVL gene positive isolates number 34, 37 & other lanes: negative PVL gene isolates.

D. Lane 1: molecular size marker & lane 2: Positive PVL gene carrier strain (a standard strain NCTC 13300) & Lane 8: negative control (water) & Lane 7: PVL gene positive isolate number 78 & other lanes: negative PVL gene isolates.

TABLE 7. Detection of MRSA by conventional antibiotic sensitivity and chrome ID MRSA tests.

Isolates	Number/Total (%)		P value
	By Antibiotic Sensitivity	By Chrome ID MRSA	
MRSA	42/67 (62.7%)	28/67 (41.8%)	< 0.001
MSSA	25/67 (37.3%)	39/67 (58.2%)	

TABLE 8. Sensitivity and specificity of the chrome ID MRSA test as compared to antibiotic sensitivity.

		Number of isolates by Chrome ID MRSA		Total
		MRSA	MSSA	
Number of isolates by antibiotic Sensitivity	MRSA	27	15	42
	MSSA	1	24	25
Totals		28	39	67
Sensitivity of Chrome ID MRSA		64.3%		
Specificity of Chrome ID MRSA		96%		

Discussion

Panton-Valentine leukocidin (PVL) is a cytotoxin produced by *Staphylococcus aureus* that causes leukocyte destruction and tissue necrosis (Genestier *et al*., 2005). Although produced by < 5% of *S. aureus* strains according to some studies, the toxin is detected in large percentages of isolates that cause necrotic skin lesions and severe necrotizing pneumonia (Lina *et al.*, 1999). It is commonly associated with community-acquired methicillin resistant *S. aureus* (CA-MRSA) (Vandenesch *et al.*, 2003). However, several outbreaks due to methicillin susceptible *S. aureus* (MSSA) have also been reported (Boubaker *et al*., 2004 ; Österlund *et al*., 2002 and Le Thomas *et al.*, 2001).

The prevalence of the PVL gene among MRSA, MSSA and all *S. aureus* isolated in this study, showed similar percentages (around 12%). This agreed with a study reporting that the rate of MSSA harboring the PVL gene was (11.3%) (Shallcross *et al.*, 2009). While, the prevalence of the PVL gene is estimated to be some 2–35% among MRSA strains as previously reported by other studies (Santucci *et al.*, 2003 and Melles *et al.*, 2004). Previous reports indicated that PVL is produced by < 5% of *S. aureus* strains (Lina *et al*., 1999). On the other hand, Azar *et al.* (2012) showed that the prevalence of the PVL gene in MRSA strains was (7.23%), while this prevalence was (33.3%) for in MSSA strains. Another study shows that the prevalence of PVL-positive MRSA was 10.7% and among PVL-positive isolates 75% were MRSA (6 MRSA out of 8 PVL-positive) (Solmaz *et al*., 2012), which is close to our study as 5 out of 8 PVL positive isolates were MRSA (63%). A recent Indonesian study reported that 1.5% of the studied patients carried Panton-Valentine leukocidin (PVL)-positive methicillin-sensitive *S. aureus* (MSSA) (Santosaningsih *et al.*, 2014).

However, the differences in the rate of prevalence are possibly due to different geographical areas and the type of assay used to diagnose the gene.

The age of patients had no significant effect on the prevalence of PVL according to the current study. Similarly, data analysis of Azar *et al.* (2012) who studied isolates from burns, revealed that there was no significant correlation between the presences of genes *mecA,* PVL and patients' age or degree of burn (P> 0.5). Lopez-Aguilar *et al.* (2007) also showed no significant effect of patient age. However, Shallcross *et al.* (2010) reported that compared to individuals aged <20 years, the odds of PVL-SA-related infection were highest in subjects aged 20–39 years and reduced thereafter with increasing age (OR 3.2, 95% CI 1.1–8.7, p 0.02).

On the other hand the 7 PVL isolates in this study were from males and not from females and 1 without recorded gender with a statistically significant difference. The higher prevalence of PVL in isolates from males is not clearly explained but the relative activity of males and possible exposure to different types of injuries and community infections may provide a possible explanation especially since most of these isolates were from wounds. Although, Lopez-Aguilar *et al.* (2007) showed no statistically significant effect of patient gender, the 5 PVL positive isolates were exclusively from males while PVL negative isolates were 12 were males and 7 were from females. On the other hand Shallcross *et al.* (2010) showed that men had approximately twice the odds compared to women of having PVL-*S. aureus*-related disease.

The type of sample shows no statistical association with detection of the PVL gene in the isolated *S. aureus* strains. However, 5 out of 8 PVL carrying isolates were from pus samples. Among the 8 PVL gene carrying strains, there were five isolates from different wounds or abscesses, one from blood infection and one from lung infection (Pleural fluid). Gerard *et al.* (1999) reported that a collection of 172 *S. aureus* strains were screened for PVL genes by polymerase chain reaction amplification. PVL genes were detected in 93% of strains associated with furunculosis and in 85% of those associated with severe necrotic hemorrhagic pneumonia (all community-acquired). They were detected in 55% of cellulitis strains, 50% of cutaneous abscess strains, 23% of osteomyelitis strains, and 13% of finger-pulp–infection strains. PVL genes were not detected in strains responsible for other infections, such as infective endocarditis, mediastinitis, hospital-acquired pneumonia, urinary tract infection, and enterocolitis, or in those associated with toxic-shock syndrome. It thus appears that PVL is mainly associated with necrotic lesions involving the skin or mucosa. Solmaz *et al.* (2012) reported that out of 56 isolates, 14.3% were PVL positive with 37.5% were from abscess and 62.5% from wound. Among all of these isolates 67.8% were MRSA.

It is noted in various reports that a patient with abscess or recurrent furuncle should be primarily suspected of PVL related *S. aureus* infection (Department of health, 2007). Shallcross *et al.* (2010) showed that PVL-SA infection was strongly associated with skin and soft tissue disease and these samples were four-

fold more likely to contain the PVL genes compared to all other sample types. Didisheim *et al*. (2014) reported 3 cases of osteoarticular infections by *S. aureus* PVL positive sensitive to methicilline, which illustrates the difficulties encountered in the management and treatment, as well as the potential for serious orthopedical complications.

Accurate and rapid detection of methicillin-resistant *Staphylococcus aureus* is very important in a clinical laboratory setting to avoid treatment failure. Velasco *et al.* (2005) stated that in the absence of availability of molecular biology techniques, the cefoxitin disc was the best predictor of methicillin resistance in *S. aureus* among the techniques tested. Other authors also reported that the results of cefoxitin disc diffusion test one in concordance with the PCR for *mecA* gene and give better results compared to the oxacillin disc diffusion method. Thus, cefoxitin disc diffusion test can be an alternative to PCR for detection of MRSA in resource constraint settings (Anand *et al.*, 2009). However, Adaleti *et al.* (2008) compared conventional methods (oxacillin agar screening (OAS), oxacillin disc diffusion (ODD) and cefoxitin disc diffusion (CDD) methods) against the gold standard polymerase chain reaction (PCR) technique to determine the best combination of the routine procedures. They concluded that combining the ODD and CDD methods could be a good choice for detecting methicillin resistance in *S. aureus* strains where *mecA* PCR cannot be performed. By consideration of this report, combining the ODD and CDD methods was used in the current study to identify MRSA strains.

The current study shows that among 42 isolates reported as MRSA by the conventional sensitivity tests (for oxacillin and cefoxitin), only 27 isolates recognized as MRSA by few Chrom ID MRSA test. This figure shows a high were significant difference between the two methods and shows sensitivity and specificity of Chrom ID MRSA of 64.3% and 96% respectively as compared to antibiotic sensitivity methods. These results disagree with other reports by Kumar *et al*. (2013) who stated that the MRSA ID chromogenic medium has a sensitivity of 96% and a specificity of 99.5% after 24 hr of incubation but it compared to PCR as a gold standard. This variation in specificity may (in addition to the different gold standard reference methods) due to cultivation conditions such as incubation time as Van Vaerenbergh *et al.* (2010) compared two chromogenic media for the detection of MRSA: BBL CHROMagar MRSA II (BD) and MRSA ID agar *(bioMe´rieux)*. After 24 hr, the sensitivities of both media were high and comparable. Both media showed an important decrease in specificity after 48 hr of incubation (decreases of 8% for MRSA II and 10% for MRSA ID), but MRSA II was significantly more specific at both time points.

PVL- carrying isolates do not only exist in communities but can also spread in hospitals (Wagenlehner *et al.*, 2007). To counter this emerging global threat to Public health, systematic surveillance of both hospital and community isolates is required, together with measures designed to limit their spread. Consequently, early diagnosis and decolonizing carriers is inevitable and essential, since it can prevent person-to-person transmission of the isolates. In suspected cases, PVL

and MRSA detection could be included in one panel for rapid response to the detected isolates. Such panel could be a multiplex PCR for PVL and *mecA* genes.

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دراسة على عزلات إكلينيكية من ستافيلوكوكس اوريوس الشرسة المقاومة للميثيسيللين والتي تحمل جينات البانتون- فالنتين لوكوسيدين

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تعتبر البكتيريا العنقودية من نوع اوريوس (ستافيلوكوكس اوريوس) الحاملة لسم البانتون – فالنتين لوكوسيدين ميكروب رئيسي مسئول عن إصابات مختلفة ، وقد وجد أن سم البانتون فالنتين لوكوسيدين موجود في أنواع الباكتريا العنقودية من نوع الأوريوس المقاومة للمضاد الحيوي الميثيسيللين وأيضا في الأنواع الحساسة له.
تم في هذه الدراسة تحديد نسبة جين البانتون – فالنتين لوكوسيدين في أنواع الكتريا العنقودية المقاومة للميثيسيللين والأنواع الحساسة له من خلال عزلات تم تجميعها من المستشفيات المصرية.

تم تجميع عزلات الستافيلوكوكس من مرضى من مستشفى كوبري القبة العسكري ومستشفى الزقازيق الجامعي مع تسجيل المعلومات من قبيل نوع العينة وتشخيص المريض وتاريخ دخوله المستشفى والقسم واسم المريض وسنه وجنسه والمضادات الحيوية التي يعالج بها وغير ذلك.

الزرع على الأوساط الغذائية الانتقائية والتشخيصية للتفريق بين الأنواع المقاومة للميثيسيللين والأنواع الحساسة له ، وتم عمل اختبار الحساسية باستخدام الأوكساسيلين والسيفوتوكسين وتم مقارنة النتائج بنتاج اختبار الكروم أجار ، وتم الكشف عن جينات البانتون فالنتين لوكوسيدين باستخدام طريقة تفاعل البلمرة التسلسل بين ٤٢ نوع علة مقاومة للميثيسيللين و ٢٥ عزلة حساسة له.

اتضح بعد اجراء اختبار تفاعل البلمرة المتسلسل ان ١١,٩% من العزلات حاملة لجين البانتون – فالنتين لوكوسيدين (خمسة عينات من النوع المقاوم للميثيسيللين (١١,٩%) وثلاث عينات من النوع الحساس له (١٢%).