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RESEARCH ARTICLE

NON-ASSOCIATION OF THE PRESENCE OF PANTON-VALENTINE LEUKOCIDINGENE WITH ANTIMICROBIAL-RESISTANCE IN *STAPHYLOCOCCUS AUREUS* ISOLATES IN COTONOU, BENIN

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ABSTRACT

The objective of this study was to determine the prevalence of Panton Valentine Leukocidin (PVL) and *mec*A genes in *Staphylococcus aureus* in Cotonou and potential association between the presence of PVL gene and antibiotic resistance. Isolates of *S. aureus* consecutively recovered from various clinical samples in four laboratories in Cotonou were subjected to PCR of *nuc* gene (for identification of *S. aureus*), drug susceptibility testing to various antibiotics (by Kirby - Bauer method) and PCR for PVL and *mecA* genes. In total, 115 non-duplicate isolates of *S. aureus* were studied. The prevalence of PVL gene was 14.8% while that of *mec*A gene was 24.3%. The prevalence of PVL genes was higher in wound swabs than in other specimens. There was no statistical significant difference between the presence of PVL and *mec*A genes in *S. aureus* isolates (p = 0.70). Also, the association between PVL genes and phenotypic resistance to main groups of antibiotics was not statistically significant. In conclusion, in Cotonou, prevalence of PVL gene in *S. aureus* isolates was 14.8% while that of *mec*A gene was 24.3%. The presence of PVL gene was not significantly associated with antibiotic resistance.

INTRODUCTION

Staphylococcus aureus is one of the important causes of bacterial infections in hospitals and community settings. It can cause a wide range of infections varying from mild infections such as furuncles to life-threatening infections such as pneumonia and severe sepsis (Gayathri *et al*., 2009). *S. aureus* infections are worldwide in distribution and their incidence is high especially in low- resource countries where health hygiene is often compromised (Nickerson *et al*., 2009). In addition, the pathogen has the ability to develop resistance to commonly used antibiotics in clinical practice including methicillin. Methicillin resistance is mediated by the production of an altered Penicillin-Binding Protein (PBP) 2a coded by the *mec*A gene complex (Dumitrescu *et al*., 2010). Of particular concern is Methicillin Resistant *S. aureus* (MRSA) strains that can develop resistance to other groups of antibiotics leading to difficulties in treating such infections (Dumitrescu *et al*., 2010). *S. aureus* carries several virulence factors among which is Panton-Valentine Leukocidin (PVL), a toxin that has been shown to play important roles in the pathogenesis of staphylococcal infections and immune avoidance in human hosts (Foster *et al*., 2005; Shallcross *et al*., 2013). *S. aureus*stra in that harbours both *mec*A and PVL genes may constitute a threat, in that patients infected by such strains may be at high risk of developing severe infections with multiple antibiotic resistance. It is therefore important to know the actual prevalence of such strains in various settings. This information is scarce in low - resource countries, particularly in Africa. In Benin (West Africa), few studies are available on PVL and/or MRSA (Baba-Moussa *et al*., 2008 and 2011; Ahoyo *et al*., 2012; Sina *et al*., 2013). However, these studies were either performed in only one particular hospital or did not use molecular tests for accurate identification of MRSA. In this present study, we aimed at determining the prevalence of PVL and *mec*A genes in *S. aureus* isolates in four laboratories in Cotonou and also identify a possible association between the presence of PVL gene and antibiotic resistance.

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MATERIALS AND METHODS

Settings

Isolates were collected from medical microbiology laboratory of the University Teaching Hospital, Hubert Koutoukou-Maga

(the reference hospital for the country), medical laboratories of Saint Luc Hospital and Menontin Hospital, and that of the Ministry of Health, Cotonou. The first three laboratories receive specimens from out-patients as well as in-patients while the latter receive specimens mainly from patients outside the hospitals.

Laboratory investigations

Collection of Isolates

All *S. aureus* isolates collected were Gram-positive cocci occurring in pairs or clusters, have grown on mannitol salt agar, catalase-positive and further identified by PCR of *nuc* gene (Brakstad, 1992).

Molecular tests: PCR detection of mecA and PVL genes

DNA extraction. DNA extraction from each isolate was carried out as previously described but with minor modifications (Mayoral, 2005). Briefly, colonies were emulsified in 500µl of sterile distilled DNA-free water. The mixture was boiled at 100^0C for 15 minutes, cooled on ice, and then centrifuged at 13,000 rpm for 5 minutes. The supernatant containing the DNA was stored at 4^oC before use.

DNA amplification

For *mec*A gene, amplification procedures were carried out as previously described (Sakoulas, 2001)**.** Primers used were *mecA1: 5'-GTAGAAATGACTGAACGTCCGATAA-3' and mecA2: 5'-CCAATTCCACATTGTTTCGGTCTAA-3'*(Eurogentec, Belgium). The 50µl mix reaction contained 200µM for each dNTP (Sigma, USA); 0.4µM of each primer, 1.25U of Jump Start Taq polymerase (Sigma, USA) and 5µl of DNA extract. PCR amplification programme was as follows: initial denaturation at 94^oC for 5 minutes, 37 cycles of denaturation at 94^oC for 1 minute, annealing at 54^oC for 1 minute and 30 seconds, extension at 72^oC for 1 minute; and final extension at 72^oC for 10 minutes. The amplification products (310 bp) were detected by gel electrophoresis. For PVL gene, amplification procedures were carried out using a method described by (McClure *et al*., 2006). Primers used were*pvl1, 5'-ATCATTAGGTAAAATGTCTGGACATGATCC*-3' and *pvl2*, 5'-*GCATCAAGTGTATTGGATAGCAAAAGC*-3' (Eurogentec, Belgium). The 50µl mix reaction contained 200µM for each dNTP (Sigma, USA); 0.8µM of each primer, 1.25U of Hot Start Taq polymerase (Promega, France) and 5µl of DNA extract. PCR amplification programme was as follows: initial denaturation at 94^oC for 5 minutes, 37 cycles of denaturation at 94^oC for 1 minute, annealing at 50^oC for 1 minute and extension at 72^oC for 1 minute; and final extension at 72^oC for 10 minutes. The amplification products (433 bp) were detected by gel electrophoresis.

Antimicrobial disc susceptibility testing

A 0.5 McFarland standard suspension of each isolate was prepared. A 1:100 dilution of the suspension was used to culture on Mueller Hinton (MH) agar plate. The following antibiotic disks (Biorad, France) were used to determine the susceptibility pattern of the isolates: fusidic acid (10 µg), erythromycin (15 µg), fosfomycin (50 µg), gentamycin (10 µg), lincomycin (15 µg), ofloxacin (5 µg), penicillin G (6 µg), pristinamycin (15 µg), rifampicin (30 µg), spiramycin (100 µg), tobramycin (10 µg) and vancomycin (30 µg). Disks were applied on the plate and incubated for 24 hours. The inhibition zone diameter for each isolate was measured and compared with interpretative standards (CA-SFM, 2012).

Quality control

For molecular tests, to minimize cross contamination, standard microbiological procedures were strictly followed. DNA extraction and PCR-amplifications were done in molecular laboratories that are separate from the clinical microbiology laboratory where cultures were done. The PCR laboratory has designated sections for pre-amplification, DNA extraction and amplification/post-amplification with a uni-directional movement of staff. Methicillin resistant *S. aureus* strains ATCC 43300 and a known methicillin susceptible *S. aureus* were used as positive and negative controls respectively for antimicrobial disk susceptibility testing.

Data analysis

Data were analysed using Microsoft Excel and Epi info software from Centre for Disease Control and Prevention, Atlanta Georgia, USA. Chi square test and t-test were used to determine the statistical significance. The p- value less than 0.05 was considered to be significant.

Table 1. Antimicrobialsusceptibility patterns

	MSSA			MRSA			Total *S. aureus*		
Antibiotic	R/I	S	*% R/I*	R/I	S	*% R/I*	R/I	S	*% R/I*
FAD	1	86	*1.2%*	00	28	*0%*	1	114	*0.9%*
E	8	79	*9.2%*	8	20	*28.6%*	16	99	*13.9%*
FSF	3	84	*3.5%*	1	27	*3.6%*	4	111	*3.5%*
GMI	6	81	*6.9%*	2	26	*7.1%*	8	107	*7.0%*
LCN	2	85	*2.3%*	0	28	*0%*	2	113	*1.7%*
OFX	17	70	*19.5%*	9	19	*32.1%*	26	89	*22.6%*
PEN	84	3	*96.6%*	28	0	*100%*	112	3	*97.4%*
PTN	0	87	*0%*	0	28	*0%*	0	115	*0%*
RAM	2	85	*2.3%*	0	28	*0%*	2	113	*1.7%*
TMN	6	81	*6.9%*	2	26	*7.1%*	8	107	*7.0%*
VAN	0	28	*0%*	0	28	*0%*	0	115	*0%*

FAD: Fusidic acid; E:Erythromicin; FSF:Fosfomycin; GMI:Gentamycin; LCN:Lincomycin; OFX:Ofloxacin; PEN:Penicillin G; PTN:Pristinamycin; RAM:Rifampicin; TMN:Tobramycin; VAN:Vancomycin ; MSSA= Methicillin susceptible *Staphylococcus aureus ;* MRSA *:* Methicillinresistant*Staphylococcus aureus ; S=susceptible ; R=resistant ; I=intermediate*

RESULTS

A total of 115 *S. aureus* isolates recovered from various clinical samples during the study period were included. Of these, 56 (48.7%) were from urine specimens, 40 (34.7%) from wound swabs, 13 (11.4%) from genital fluids while 6 (5.2%) were from blood cultures. Of the total 115 *S. aureus* isolates studied, 28 (24.3%) were positive for *mec*A gene representing the prevalence of MRSA in this population. Concerning the antimicrobial susceptibility testing, almost all isolates were resistant to Penicillin G while none was resistant to vancomycin (Table 1). MRSA were four times more likely to be associated with resistance to macrolids but was not to resistance to other antibiotics groups such as fluoroquinolons and aminosids (Table 2).

Table 2. Association between the presence of *mec*A gene and phenotypic resistance to main groups of antibiotics

Groups of antibiotics	MRSA		
	Odds-ratio	95% CIodds-ratio	p-value
Aminosids	1.04	0.20-5.46	0.96
Macrolids	3.95	1.32-11.82	0.01
Fluoroquinolons	1.95	0.75-5.06	0.17
Multiresistance	1.9	0.67-5.36	0.22

MRSA: Methicillin resistant S. aureus ;Multiresistance : resistance to at least two groups of antibiotics other than -lactams; CI : confidence interval

Table 3. Prevalence of PVL geneaccording to type of specimens

Type of specimens	PVLgene		
	Positive N *(%)*	Negative N *(%)*	Total N *(%)*
Urine	3 (5.4%)	53 (94.6%)	56 (100%)
Woundswabs	11 (27.5%)	29 (72.5%)	40 (100%)
Genitalfluids	2 (15.4%)	11 (84.6%)	13 (100%)
Blood	1 (16.7)	5 (83.3%)	6 (100%)

p=0.03 ; PVL : Panton Valentine Leukocidin

Table 4. Prevalence of *mec*A and PVL genes in *S. aureus*isolates

PVL	*S. aureus*		
	MRSA N *(%)*	MSSA N *(%)*	Total N *(%)*
Positive	3 (10.7%)	14 (16.1%)	17 (14.8%)
Negative	25 (89.3%)	73 (83.9%)	98 (85.2%)
Total	28 (100%)	87 (100%)	115 (100%)

p = 0.70 ; MSSA= Methicillin susceptible *S. aureus ; MRSA : Methicillin resistant S. aureus ; PVL : Panton Valentine Leukocidin; Multiresistance : resistance to at least two groups of antibiotics other than -lactams*

Table 5. Association between PVL gene and phenotypicresistance to main groups of antibiotics

	S. aureus with PVL+		
Group of antibiotics	Odds-ratio	95% Clodds-ratio	p-value
Aminosids	0.81	0.09-7.06	0.85
Macrolids	1.40	0.35-5.55	0.62
Fluoroquinolons	0.41	0.09-1.93	0.24
Multiresistance	1.02	0.26-3.95	0.97

Multiresistance : resistance to at least two groups of antibiotics other than -lactams; CI : confidence interval, PVL : Panton Valentine Leukocidin

In addition, MRSA was not associated with multiple antibiotic resistance, defined as resistance to at least 2 different groups of antibiotics other than β-lactams. Seventeen of the isolates were positive for PVL gene; thus the prevalence of PVL gene in isolates was 14.8%, 95% CI [9.4% – 22.4%]. The prevalence of PVL gene was higher in wound swabs than in other specimens (Table 3). There was no statistical significant difference between the presence of PVL and *mec*A genes in *S. aureus* isolates (p = 0.70) (Table 4). Also, the association between PVL genes and phenotypic resistance to main groups of antibiotics was not statistically significant (Table 5).

DISCUSSION

In this study, *mec* A gene prevalence was 24.3%.This finding was higher than 14% reported by Baba-Moussa in 2008, less than 45.5% reported by Affolabi in 2012 but in agreement with 25% documented by Sina in 2013 (Baba-Moussa *et al*., 2008; Affolabi *et al*., 2012; Sina *et al*., 2013). These disparities may be due to differences in the design and year of studies, the type of the specimens and patients and also methods used to detect MRSA. In fact, previous studies were mainly based on phenotypic detection of MRSA while the reference method of PCR detection of *mec*A was used in this study. Furthermore, PCR detection of *nuc* gene was carried out in this study, necessitating use of well-characterized *S. aureus* strains, which was not available in previous reports. Phenotypic tests are commonly used in routine practice but as reported in several studies, these tests may lack sensitivity and specificity (Boutiba-Ben Boubaker *et al*., 2004; Baddour *et al*., 2007; Datta *et al*., 2011). We observed that MRSA strains were more likely to be associated with resistance to macrolids (CI = 1.32-11.82, odds-ratio = 3.95) but not to other groups of antibiotics and multiple antibiotic resistance. Such association may render treatment of patients who are infected with macrolid- resistant pathogens more complicated. There is insufficient data on MRSA in Africa, as recently highlighted by Falagas (Falagas *et al*., 2013).

In Benin however, studies available were mainly on specific types of patients/ specimens and/or based in only one hospital (Baba-Moussa *et al*., 2008; Affolabi *et al*., 2012; Ahoyo *et al*., 2012; Sina *et al*., 2013). In contrary, this present study included tertiary as well as secondary hospitals in Cotonou, the main city in Benin. Including a wide range of patients/ specimens/hospitals gave a more accurate rate of the prevalence of MRSA in the city. Indeed, only tertiary hospital-based studies are more likely to recruit patients more at risk of harbouring MRSA which may lead to over-estimation of its prevalence (Wegner *et al*., 2013). The prevalence of PVL gene in isolates was 14.8%. This was lower than what were documented in previous studies in Benin (Ahoyo *et al*., 2012; Sina *et al*., 2013). The difference may be due to types of patients included in previous studies. From this study, it was observed that prevalence of PVL gene varied from one type of specimen to another (Table 3). Indeed, the prevalence of PVL was higher in wound specimens than in others, though present in all specimens. Several studies have shown that the presence of PVL gene is more associated with necrotic wound lesions (Shallcross *et al*., 2013). However, its presence in other specimens needs further evaluation. In this study, we found no association between MRSA and the presence of PVL gene in *S. aureus* isolates (Table 4). This is in agreement with the submissions of Alli in South Western Nigeria (Alli *et al*., 2012). However, the presence of both PVL and *mec*A genes in 3 MRSA isolates (Table 4) is worrisome as, such isolates may potentially cause severe and resistant infections. Fortunately,

this assumption was dampened by the fact that there was no statistically significant difference between the presence of PVL gene and resistance to main antibiotic groups and multiple antibiotic resistance (Table 5). In conclusion, from this study, prevalence of PVL gene in *S. aureus* isolates was 14.8% while that of *mec*A gene was 24.3%. PVL gene was not significantly associated with antibiotic resistance.

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