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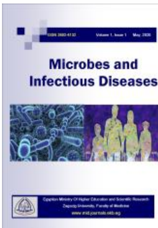

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Original article

Detection of PBP2a and *PVL* genes among *Staphylococcus aureus* and their methicillin-resistant strains isolated from a hospital in Sokoto Town

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ABSTRACT

Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) has increasingly been implicated to be a causal organism of nosocomial infection. This study aimed at detecting PBP2a and *PVL* gene in *Staphylococcus aureus* (*S. aureus)* and MRSA isolated from in-patients and patients' caregivers in a Sokoto Hospital. **Methods**: This cross-sectional study involved 201 samples comprising 129 swabs from patients with surgical wounds on admission and 72 swabs from their caregivers/relatives. The samples were screened for *S. aureus* and MRSA using standard cultural methods, and they were further screened for PBP2a protein and *PVL* gene using a rapid PBP2a kit and polymerase chain reaction techniques respectively. **Results:** Findings showed a 21.9% recovery of *S. aureus* from the samples and a 70.5% prevalence rate of MRSA among the *S. aureus* isolates. *S. aureus* and MRSA were more prevalent in the wound swabs. The PBP2a protein was detected in 27.3% of the *S. aureus* isolated. It was interesting to note that 61.3% of the MRSA isolates lacked the PBP2a which is known to be an integral part of the *mecA* gene. The PBP2a was mostly detected in the isolates that came from the wound swabs. The *PVL* gene was detected in 32.3% of the *S. aureus* isolates and the *PVL*-positive isolates were all from wound swabs. **Conclusions**: The significant linkage of *S. aureus* isolates under study to methicillin resistance and the *PVL* gene is a call for caution. Therefore, the trends of MRSA in hospital-related infections should be recurrently investigated to avoid indiscriminate spread.

Introduction

Staphylococcus aureus (*S.aureus)* colonization is a prominent risk factor in *S. aureus*-related infections [1]. Globally, *S. aureus* opportunistically triggers several infections which could adversely influence human morbidity and mortality [2]. Genetic and phenotypic heterogeneity

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among *S. aureus* has contributed to its resistance to diverse classes of antibiotic via the acquisition of mobile genetic elements that encodes resistance determinants or mutations in loci that influence antibiotic sensitivity [3]. This makes multi-drug resistant *S. aureus* a notable etiological agent among healthcare-related infections (HAIs) [4]. Antibiotic resistance in *S. aureus* could be extremely virulent, and most importantly the methicillin-resistant *S. aureus* (MRSA) strains are of great concern. This genetic trait may prompt the organisms to demonstrate resistance to several other antibiotics including tetracycline, penicillin, and carbapenems. Many of the difficult-to-treat bacterial infections in humans have been associated with MRSA [5].

Notably, MRSA exhibits resistance to β-lactam antibiotics, antibiotics in the class penicillin (methicillin, dicloxacillin, nafcillin, oxacillin, etc.,) and other classes of antibiotics [6]. The resistance of *S. aureus* to methicillin is resultant to the presence of the *mecA* gene which encodes the penicillin-binding proteins (PBPs) [7]. The *mecA* gene is embodied in a mobile genetic element called the staphylococcal cassette chromosome mec (SCCmec), which differs in size and genetic composition [8]. Many MRSA isolates are multi-drug resistant and are only sensitive to the glycopeptides such as vancomycin [6]. Thus, the discovery of MRSA isolates with dwindled sensitivity to glycopeptides (intermediate susceptibility to glycopeptide) is of monumental public health concern. Pathogenicity and antibiotic resistance in *S. aureus* could be attributed to many players which include virulence factors that compromise host immunity and other adverse effects [9]. This increased prevalence of MRSA and its potential to spread in hospitals and communities have been a complicated challenge in infection control [10]. Community-acquired MRSA (CA-MRSA) strains are major causes of skin and soft tissue infections (SSTIs) given its association with several virulence factors [11]. The rapid spread of CA-MRSA and its capability to thrive in hospital settings thereby displacing the conventional hospital-associated MRSA (HA-MRSA) strains, further complicates the epidemiological understanding of CA-MRSA [12]. An important virulence factor and a known marker of CA-MRSA is the cytotoxin called Panton-Valentine Leukocidin (*PVL*) which enhances its ability to cause acute infections in animal and human hosts [13]. The *PVL* is encoded by *lukS-PV* and *lukF-PV* genes [14], which are widely known to promote pathogenicity in *S. aureus* [15]. The *PVL* toxin, which is a prophage-encoded bicomponent pore-form protein, has a profound epidemiological link to prevalent CA-MRSA strains. However, its contributions to pathogenicity remain contentious, as several studies reported its relatedness to primary skin infections and necrotizing pneumonia, while others disputed its pertinence as a virulence factor [12,16].

This study aimed at detection of PBP2a protein and characterization of the *PVL* gene in *S. aureus* and their methicillin-resistant strains isolated from in-patients and patients' caregivers in a Hospital in Sokoto Town, Nigeria.

Methods

Sample collection

To collect clinical samples, we obtained ethical clearance from the Human Research Ethics Committee of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto, with reference number: UDUTH/HREC/2016/No493. Permission was also gotten from each participant. This study employed a cross-sectional research design to collect 201 samples (from March to May 2017) based on the sample size calculated using the formula by **Kadam and Bhalerao** [17]. Using sterile swab sticks, we collected 43 surgical wound swabs, 43 nasal swabs and 43 skin swabs from in-patients with surgical wounds (i.e. 3 samples from each patient), and 36 nasal swabs, and 36 skin swabs collected from patients' caregivers (i.e. 2 samples from each caregiver) attending UDUTH. We ensured an aseptic collection of samples, and they were immediately transported to the Microbiology Laboratory of Specialist Hospital, Sokoto in a polythene Ziploc bag for analysis.

Isolation and identification of *S. aureus*

The samples were inoculated into the sterile nutrient broth (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated at 37°C for 24 hours. A loop-full suspension from the nutrient broth was streaked aseptically onto Mannitol salt agar (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated for 24 hours at 37°C [18]. Discrete and golden-yellow colonies were presumptively isolated as *S. aureus*. These isolates were preserved on nutrient agar slants after a purification process involving sub-culturing the isolates.

Gram staining and catalase tests were determined using methods previously described by **Aditi et al.** [19]. Coagulase test was carried out to distinguish *S.*

aureus (coagulase positive) from coagulase-negative *Staphylococcus* (CONS) using the Staphaurex latex slide agglutination test kit (Oxoid Ltd., England). Furthermore, commercially available Microbact Identification Kits that are composed of 12 standardized biochemical substrates in micro-wells were used to identify *S. aureus* from among the coagulase-positive isolates.

Detection of methicillin-resistant *Staphylococcus aureus*

Oxacillin-resistant screening agar (ORSA) media was prepared following the manufacturer's instruction and used for the isolation of MRSA. Confirmed *S. aureus* isolates were inoculated on ORSA media and incubated at 37°C for 24 hours. After incubation, blue colonies on ORSA media were described as MRSA isolates. The MRSA isolates were then preserved in nutrient agar slants.

Detection of penicillin-binding protein

To ascertain the presence of penicillin-binding protein (PBP2a) in the MRSA isolates, this study employed a sero-agglutination technique that involves the use of the rapid PBP2a kit (Oxoid Ltd., England).

Following the manufacturer's instruction, 4 drops of the extraction reagent were introduced into a micro-centrifuge tube which contains approximately 1.5 x 10^9 (3-5µL) cells. The tube was vortex occasionally to eliminate clumps until a turbid suspension was visible. Afterward, the tube was placed in boiling water, heated for three minutes and removed to cool at room temperature. A drop of the second extraction reagent was added into the tube and was then shaken. The tube was centrifuged at 3000 rpm at 15 cm rotation radius for 5 minutes. The test for PBP2a was carried out using the supernatant.

On the latex agglutination test card, one of the circles is used for the PBP2a test, while the other circle is for the control, and they are properly labeled as thus. The latex reagents were mixed and one drop of test latex and control latex were added to each of the labeled circles. A 50 µL volume of the supernatant was added to the test and control circle respectively. The latex and supernatant in each circle were mixed thoroughly using a mixing stick. The card was slightly agitated for about three minutes and the presence of agglutination was observed under normal lighting conditions. The presence of agglutination indicates a positive reaction.

Molecular detection of *Panton-Valentine Leukocidin* gene in the *Staphylococcus aureus* isolates

Molecular test was performed using polymerase chain reaction (PCR) to determine the presence of *Panton-Valentine Leukocidin* (*PVL*) genes in the MRSA isolates.

DNA extraction

The DNA extraction kit of the Zymo Research Protocol was used to extract the isolates' DNA according to the manufacturer's instructions. The MRSA isolate was cultured in Luria broth and incubated for 24 hours at 37°C. Subsequently, we centrifuged 3 ml of the MRSA broth culture at 10,000 xg for a minute and then discarded the supernatant into a disinfecting Jar. The harvested cell pellet was removed and 200 µL of deionized water was added and it was then vortex to ensure the proper mixture. Thereafter, lysis buffer (400 µL) was added to the mixture. At a temperature of 70°C, the mixture was then incubated for 15 minutes to achieve complete lysis of the cells and at that point, the mixture solution became viscous to prevent the Zymo-spin column from clogging. To a Zymo-spin^{TM} IV spin filter in a collection of tubes, 400 µL of the solution was transferred and then centrifuged for a minute at 7000 rpm. To the filtrate in the collection tube from the preceding step, 1200 µL of DNA binding buffer was added and mixed properly. Then in a new collection tube, 800 µL of the mixture was dispensed into a Zymo-spin IIC column and centrifuged for 1 minute at 10,000 xg. From the above step, the obtained was discarded and the step was repeated a second time. Again, to the Zymo-spin column in a new collection tube, 200 µL DNA prewash buffer was added and centrifuged at 10,000 xg for a minute. Again, 500 µL of DNA wash buffer was transferred to the Zymo-spin column and centrifuged at 10,000 xg for 1 minute. Finally, the Zymo-spin was introduced into a clean 1.5 micro-centrifuge tube and to the column matrix, 100 µL DNA elution buffer was directly added. And to elude the DNA, it was centrifuged for a minute at 10,000 x g.

Detection of *PVL* gene using polymerase chain reaction and agarose gel electrophoresis

For each MRSA isolate, a master mix of 25 µL was prepared in an eppendorf tube as follows: 12.5 µL master mix (Biolabs), 1 µL each of forward and reverse *mecA* primer (5'-ATC ATT AGG TAA AAT GTC TGG ACA TGA TCC A-3' and 3'- GCA TCA AGT GTA TTG GAT AGC AAA AGC-5')

respectively with a base pair of 433 bp (Inqaba Biotec), 5.5 nuclease-free water and 5 µL of the extracted DNA were dispensed into an eppendorf tube. To properly mix the content, the tube was briefly vortexed, and using PCR protocol, the tube was inserted into a thermal cycler (Applied Biosystem 9700) so that the DNA can be amplified. The cycling protocol involved initial denaturation for 5 minutes at 40ºC, subsequently denatured at 94ºC for 30 seconds, annealed at 56ºC, extension step for 1 minute at 72ºC with 35 cycles, and a final extension time of 7 minutes at 72ºC. The holding temperature was 10ºC for infinity (∞). The amplicon (PCR product) underwent electrophoresis on 1% agarose gel, and upon completion of the staining procedure with ethidium bromide. The PCR product was viewed under a short wave ultraviolet trans-illuminator and gel bio-imaging system (UVP imaging system, Upland, CA, USA).

Statistical analysis

Descriptive statistics was used in analyzing the data via Microsoft excel 2013.

Results

Analysis of samples collected

The 201 samples collected for this study comprise 120 males and 81 females. Among these samples, 129 (64.2%) were from admitted patients in the surgical ward of UDUTH, while 72 (35.8%) were from the caregivers (relatives) of the patients (**Table 1**). Also, 26 (12.9%) of the study participants were within the age group 1-17 years, 128 (63.7%) were within the age group 18-40 years, and 47 (23.4%) were within the age group 41 years and above.

Isolation of *Staphylococcus aureus*

Among the samples collected, *S. aureus* was isolated from 21.9% (i.e. 44 *S. aureus* isolates) from the samples. Most of the *S. aureus* isolated **(Table 2)** in this study were from the wound swabs of the patients 35 (79.5%), while the nasal swabs obtained from patients' caregivers had 9 (20.5%) prevalence.

Detection and distribution of methicillin resistance *Staphylococcus aureus*

Oxacillin-resistant agar (ORSA) was used to isolate methicillin-resistant *S. aureus* (MRSA) from the *S. aureus* isolates. With a 70.5% isolation rate of MRSA from among the *S. aureus* isolates, **Table 2** shows the distribution of the MRSA isolates according to their sample sources, and the wound samples had the highest prevalence.

Detection of Penicillin Binding Protein 2a in the *Staphylococcus aureus* isolates

The prevalence of Penicillin Binding Protein (PBP2a) was carried out on the phenotypically isolated *S. aureus*. The detected prevalence of PBP2a among the isolated *S. aureus* isolated from patients and their caregivers was 12 (27.3%), with **Table 2** showing the distribution of PBP2a across sample sources.

Detection of *Panton-Valentine Leukocidin* Gene

The *PVL* gene was detected in 10 of 31 MRSA isolates. The *PVL* genes detected were all from wound swap samples **(Plate 1).**

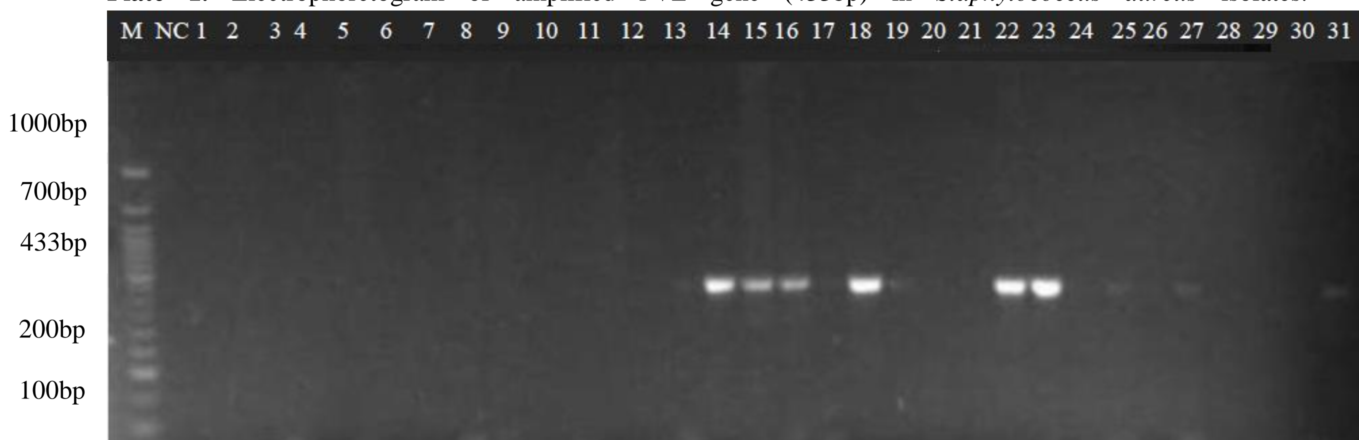
Table 1. Distribution of samples from subjects according to sources and gender.

Gender	Hospital patients			Patients' caregivers		Total
	Wound swab	Nasal Swab	Skin swab	Nasal swab	Skin swab	
Male	30 (14.9%)	30 (14.9%)	30 (14.9%)	15 (7.5%)	15 (7.5%)	**120 (59.7%)**
Female	13 (6.5%)	13 (6.5%)	13 (6.5%)	21 (10.4%)	21 (10.4%)	**81 (40.3%)**
Total	**43 (21.4%)**	**43 (21.4%)**	**43 (21.4%)**	**36 (17.9%)**	**36 (17.9%)**	**201 (100%)**

Table 2. Prevalence of *S. aureus*, MRSA, and PBP2a among the sample sources.

Sample sources	Hospital patients			Caregivers			Total		
	S. aureus	MRSA	PBP2a	*S. aureus*	MRSA	PBP2a	*S. aureus*	MRSA	PBP2a
Wound	26 (59.1%)	22 (71%)	8 (66.7%)	–	–	–	26 (59.1%)	22 (71%)	8 (66.7%)
Nasal	6 (13.6%)	3 (9.7%)	1 (8.3%)	7 (15.9%)	5 (16.1%)	3 (25%)	13 (29.5%)	8 (25.8%)	4 (33.3%)
Skin	3 (6.8%)	0 (0)	0 (0)	2 (4.5%)	1 (3.2%)	0 (0)	5 (11.3%)	1 (3.2%)	0 (0)
Total	**35 (79.5%)**	**25 (80.7%)**	**9 (75%)**	**9 (20.4%)**	**6 (19.3%)**	**3 (25%)**	**44 (100%)**	**31 (100%)**	**12 (100%)**

Plate 1. Electrophoretogram of amplified *PVL* gene (433bp) in *Staphylococcus aureus* isolates.



Keys:
Lane 14, 15, 16, 18, 19, 22, 23, 25, 27 and 31 shows amplified *PVL* gene (433bp).
Lane NC: Negative Control.
Lane M: 100bp Molecular DNA ladder.
M = Size marker, bp= Base pair, H = Hospital samples

Discussion

Staphylococcus aureus and its association with multidrug resistance (MDR) have drawn substantial public attention globally as a prevalent cause of clinical infections. From the 201 samples collected for this study, 21.9% occurrence of *S. aureus* was observed. Even though *S. aureus* is a normal flora of the skin, the prevalence of *S. aureus* in this study is significantly high for a hospital setting despite the aseptic measures put in place during hospital procedures. The frequency in which *S. aureus* was isolated in this study is more than 14.8% reported by **Oche et al.** [20]. Using oxacillin-resistance screening agar (ORSA), this study had an MRSA prevalence of 31 (70.5%). The use of ORSA in the detection of MRSA might have been the reason for the high prevalence of MRSA recorded in this study. Previous research has reported the possibility of a false-positive phenotypical result of MRSA due to hyper-productive penicillinase ability even in the absence of *mecA* [21]. Comparing this study to other studies, one will see 28% in Bauchi [22], 26.9% in Central Nigeria [21], 30.4% in Ibadan [23] all in Nigeria, and 31.4% in Brazil [24]. Although highly aseptic methods are applied in surgical procedures, infectious microorganisms, including MRSA still manifest in surgical wounds. By implication, the result of this study is suggesting that nasal carriage of MRSA by the patients, caregivers, and/or healthcare workers might have been the cause of MRSA infections in surgical wounds. Cross-contamination between patients and healthcare workers has been reported in hospital settings [25], and nasal carriage of MRSA which may be a marker for skin colonization may aid in

trans-positioning MRSA to the surgical wound of patients [26]. Also, poor hygienic conditions and non-adherence to relevant antibiotics policy have been suspected as possible reasons for these resistant strains [27]. In general, oxacillin-resistance screening agar (ORSA) is considered to be less sensitive in an attempt to detect methicillin-resistance that is mediated by the *mecA* gene that is known to code for producing the enzyme known as penicillin-binding protein (PBP2a) that is also known to bestow resistance to antibiotics in the β-lactam class [28]. Studies by **Nwankwo et al.** [29], and **Onemu and Ophori** [30] also recorded a high MRSA prevalence of 62% in Kano-Nigeria and 79% in Benin City-Nigeria respectively, and one thing these studies have in common with this current study is that ORSA was used for MRSA detection. In addition, when the result of this study is compared to results from other studies that detected the *mecA* gene using polymerase chain reaction (PCR), a lower prevalence of 22.2% [31] and 19.2% [32] was recorded in Southwestern Nigeria. As with other forms of resistance, abusive administration of antibiotics and guidelines, and substandard practices to control infections may be responsible for the high frequency of MRSA that was recorded [33]. In this study, the MRSA isolates were predominantly from patients wound samples, other studies have similarly reported the predominance of MRSA in the hospital environment [22, 34].

In this study, the PBP2a test results revealed that 12 (27.3%) of the *S. aureus* isolates were PBP2a producers. An interesting thing from this study was that 19 (61.3%) of the *S. aureus* isolates that were identified to be resistant to methicillin were non-PBP2a producers, but one of the susceptible *S. aureus* isolates was found to produce PBP2a. This observation suggests that other factors that aren't characterized may be involved in the transcription of the *mecA* gene, resulting in phenotypic β-lactam resistance in the MRSA isolates. As seen in this study, the low occurrence of PBP2a is in line with reports from previous studies [35]. Among MRSA isolates, there is a global variation (between geographical location and population) in the reports of Panton-Valentine Leukocidin (*PVL*), given that various reports from different countries have shown an increasing prevalence of *PVL* among MRSA isolates [36, 37]. The PCR result of this study showed that 32.3% of the 31 MRSA isolates tested had the *PVL* gene, and they were all from surgical wound samples. As such, most of the MRSA isolated from the wound samples has the *PVL* gene; an indication that the infections may be associated with CA-MRSA, especially since *PVL* is a community-acquired MRSA marker that has been implicated in causing soft-tissue and dermal infections [38]. In this study, all the isolates that harbored the *PVL* gene were from the wound samples (hospital-associated). A probable reason for the presence of the *PVL* gene in the clinical samples could be that the organism isolated from the patients may have been acquired even before the patients were hospitalized, or transmitted to the patients' wounds through physical contact between the patients and their caregivers and/or healthcare workers. This current study showed a very high prevalence of *PVL* gene than what was previously reported in comparable studies [39, 40].

Conclusion

In comparison to what is expected from a medical facility, the recovery rate of MRSA from the *S. aureus* isolates in this study is very high. And the PBP2a that is coded for by the *mecA* gene that again is responsible for methicillin and other β-lactam antibiotics resistance mediation is conspicuously absent in two-thirds of the MRSA isolates. Hence, we believe that other unidentified factors could be responsible for the methicillin resistance that was detected in the isolates, or that the use of cultural method (ORSA) in the identification of MRSA could give false positive results. In addition, the high rate of *PVL* gene among hospital-related isolates is a call to look into the trend of new MRSA that may flood the hospitals.

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Conflicts of interest: None.

Financial disclosure: None

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