Screening of Clinically Isolated *Staphylococcus aureus* for Methicillin Resistance and Enterotoxin Production

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Abstract: Biochemically identified *S. aureus* isolates from the various clinical specimens from Lagos Central District healthcare centres were screened for methicillin using MRSA agar (CHROMagar). Production of enterotoxins were detected using the RIDASCREEN® SET Total kit (r-biopharm) while filtrates of the positive samples were screened for specific enterotoxins using RIDASCREEN® SET A, B, C, D, E (r-biopharm) according to manufacturer instructions. Of the 43 coagulase positive *S. aureus* isolates, 22(51.0%) were identified as methicillin resistant *S. aureus* while 21 (49.0%) were methicillin sensitive *S. aureus*. Of the 43 *S. aureus* tested for enterotoxin, six (13.9%) possessed single enterotoxins while 4 (9.3%) possessed more than one type of enterotoxin. It also showed that coagulase-positive *S. aureus* from wound swabs had highest number of positive enterotoxins antibodies. Therefore, all *S. aureus* isolated from the laboratory specimen especially from wound specimen should be tested for enterotoxin.

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1. INTRODUCTION

Carriage of *Staphylococcus aureus (S. aureus)* appears to play a key role in the epidemiology and pathogenesis of infection (Kluytmans et al., 1997; Odu and Okonko, 2012). The ecological niches of *S. aureus* are the anterior nares (Odu and Okonko, 2012). In healthy subjects, over time, three patterns of carriage can be distinguished: about 20.0% of people are persistent carriers, 60% are intermittent carriers, and approximately 20% almost never carry *S. aureus* (Kluytmans et al., 1997; Odu and Okonko, 2012).

There are thirty two staphylococci species of which *Staphylococcus aureus* is the most important member due to its association with infection and intoxication (Bergdoll, 1989). *S. aureus* also known as "golden staph" or Oro staphira is facultative anaerobic, Gram-positive cocci in cluster bacteria. It is a pathogenic organism because it possesses variety of virulence factors that allow it to attach to host cells, invade tissues and evade the host's immune system (Freeman-Cook, 2006). *S. aureus* causes the following infections in human: superficial infection (skin abscess, boils, and impetigo) and also invasive infection causing osteomyelitis, arthritis, endocarditis, pneumonia or septicemia.

S. aureus acts as endogenous reservoir for clinical infections in the colonized individual but also as a source of cross-colonization for community spread (Pathak et al., 2010; Odu and Okonko, 2012). Healthy individuals have a small risk of contracting an invasive infection caused by *S. aureus,* but they can be carriers of the organism (Foster, 2004; Odu and Okonko, 2012). The spread of colonization occur especially in close contact areas like schools, pre-schools or households (Peacock et al., 2003), probably by the contaminated hands and surfaces (Pathak et al., 2010; Odu and Okonko, 2012).

Among its mechanism of infection is the production of enterotoxins. Staphylococcal enterotoxins are group of proteins produced by *S. aureus* that induce an emetic response in humans along with other gastrointestinal symptoms (Freeman-Cook, 2006). They are capable of producing serologically different enterotoxins as well as variety of enterotoxin-like substances that are capable of causing severe illness in humans (Thomas *et al.*, 2007).

Many of the thermal processes used in foods are severe enough to kill any vegetative cells of *S. aureus*, but their enterotoxin have been shown to maintain their activity even after these processes e.g. milk

pasteurization. Illness due to staphylococcal intoxication is usually acquired through ingestion of contaminated foods that contain preformed toxins and is the third most common cause of food poisoning in the world (Atanassova *et al.*, 2001).

A variety of studies have examined community prevalence of carriage of *S. aureus* in diverse sub-populations, such as adult outpatients, health care workers, college students, and injection drug users (Wertheim et al., 2004; Bischoff et al., 2004; Bassetti et al., 2004; Eveillard et al., 2004; Mainous et al., 2006; Odu and Okonko, 2012). The prevalence of *S. aureus* ranges from 20% to 45%, with an estimate of methicillin-resistant *Staphylococcus aureus* (MRSA) colonization from 10 community surveillance studies of 1.3% (Salgado et al., 2003; Mainous et al., 2006; Odu and Okonko, 2012).

The prevalence of *S. aureus* in healthy populations are 20.0% in nasal cavity of students in Kano state, 36.0% and 40.0% in women's urine in two centres in Nigeria, 17.3% in nasal cavity of Turkish children and 32.4% in nasal cavity of adults in USA (Onanuga et al., 2008). The prevalence of *S. aureus* in infection are 43.7% in conjunctivitis among children in University College Hospital (UCH), Ibadan (Adeyeba *et al*., 2010), 67.9% in UTI of children with nephritic syndrome in Kano (Adeleke and Asani, 2009) and 22.8% in Urinary tract infection at University of Benin Teaching Hospital (UBTH), Benin City (Akortha and Ibadin, 2008).

The incidence of community-acquired and hospital-acquired *S. aureus* infections has been rising with increasing emergence of drug-resistant strains called methicillin-resistant *S. aureus* (Fluit et al., 2001; Deresinski et al., 2005; Mainous et al., 2006; Odu and Okonko, 2012). MRSA is an established pathogen in most health care facilities. Previously limited to hospitals, MRSA infections have been increasingly reported in the community (Naimi et al., 2003; Nguyen et al., 2005; Harbarth et al., 2005; Ma et al., 2005; Ochoa et al., 2005; Mainous et al., 2006; Odu and Okonko, 2012). Because many clinical infections arise from spread from a healthy carrier, an understanding of the risk factors for carriage of *S. aureus* is crucial to understanding the potential for invasive infections and transmission of MRSA; however, most surveillance of *S. aureus* and MRSA has focused on individuals with invasive infections rather than on an entire population (Harbarth et al., 2005; Ma et al., 2005; Ochoa et al., 2005; Mainous et al., 2006; Odu and Okonko, 2012).

Few studies, however, have focused on which individuals are most likely to be colonized with *S. aureus* and which are most likely to specifically have MRSA (Mainous et al., 2006; Odu and Okonko, 2012). The aim of this study therefore, was to screen isolates of *Staphylococcus aureus* for methicillin-resistant and enterotoxin production.

2. MATERIALS AND METHODS

2.1. Study area and Sample collection

Clinical specimens of blood, ear, eye, finger, vaginal, nail, palm, throat, urethral, urine, semen, and wound swabs were collected from Government Healthcare centres in Lagos Central District, Lagos State, Nigeria and transported to the Immunology unit, Microbiology Division, Nigerian Institute of Medical Research, Yaba, Lagos for microbiological analysis.

2.2. Isolation of *S. aureus* strains

Samples of blood, urine, semen, ear, eye, finger, vaginal, nail, palm, throat, urethral and wound swabs were inoculated onto Mannitol Salt agar (MSA) and Staphylococcus agar (SA) plates. The cultured plates were incubated aerobically at 37^0c for 24 hours. Discrete colonies of *S. aureus* isolates from these clinical specimens were stored in 30% glycerol in Tryptone Soya broth and kept at-20^0C.

2.3. Identification of *S. aureus* strains

The stored isolates in broth were sub-cultured onto and incubated aerobically at 37^0C for 24 hours. Colonies identifiable as discrete onto the Muller Hinton agar, Mannitol Salt agar and Staphylococcus agar were carefully examined macroscopically for cultural characteristics. All isolates were subjected to various morphological characterization and gram stained to determine their gram reaction. Biochemical tests were carried out as described by Jolt *et al.* (1994) to determine the identity of the *S. aureus* isolates with reference to Bergey's Manual of Determinative Bacteriology. The isolates were identified by comparing their characteristics with those of known taxa, as described by Jolt *et al.* (1994) and Cheesbrough (2006).

2.4. Detection of Methicillin

All confirmed *S. aureus* isolates were sub-cultured on Muller Hinton Agar incubate at 37^0c for 24hours. After which a discrete colony was streaked out on CHROMagar™ MRSA agar (CHROMagar) and incubate aerobically at 37^0C for 24hours. *S. aureus* isolates were screened for methicillin resistance by the disk diffusion method of the National Clinical and Laboratory Standards Institute (CLSI, 2006). Overnight cultures from *S. aureus* were subcultured on Mueller-Hinton agar, and a 1-µg oxacillin disk was placed on the inoculated plate. Zone diameters were measured and recorded after a 24-hour incubation at 37°C; the results were classified as sensitive (≥ 13 mm), intermediate (11–12 mm), or resistant (≥ 10 mm).

2.5. Detection of staphylococcal enterotoxins

Staphylococcal enterotoxins were detected using the RIDASCREEN® SET Total kit (r-biopharm). *S. aureus* isolates from Muller Hinton Agar were introduced into 1ml Brain Heart infusion (Oxoid) and incubate at 37°C for 18-24 hours to ensure optimal formation of enterotoxins. After incubation, cultures were centrifuged at 3500g for 5minutes at room temperature and placed on ice-pack immediately after spinning. Each supernatant wasaspirated into a clean sterile tube and assayed immediately for enterotoxin antibody. The required numbers of microtiter strips were inserted into the ELISA micro-wells holder. With the first well used as blank, 100µl of positive and negative controls were added successively followed by the supernatants. The plate was covered with adhesive paper and incubated at 37^0C for 60 minutes. One in twenty dilution of the wash buffer was prepared and using ELISA washer 100µl of the wash buffer solution was used to wash the micro-wells, the procedure wasperformed four more times. The plate holder was tapped gently on paper adsorbent to remove excess fluid, 100µl of substrate were added to all micro-wells except blank well, cover with adhesive tape and incubate at 37^0C for 60 minutes thereafter the washing step was performed. 100µl of conjugate were added to all micro-wells and incubate at room temperature in the dark for 15minutes. 100µl of the Stop solution were added and read at 450/630nm using ELISA reader.

2.6. Detection of specific staphylococcal enterotoxins

Filtrates of the RIDASCREEN® SET Total (r-biopharm) positive samples were screened for specific enterotoxins using RIDASCREEN® SET A, B, C, D, E (r-biopharm), following manufacturer instructions. The supernatants were used and possible because the tests were between 24 hours of each other.The required numbers of microtiter strips were inserted into the ELISA micro-wells holder. With the first well used as blank, 100µl of positive and negative controls were added successively followed by the supernatants. The plate is covered with adhesive paper and incubated at 37^0c for 60minutes. One in twenty dilution of the wash buffer was prepared and using ELISA washer 100µl of the wash buffer solution was used to wash the micro-wells, the procedure was performed four more times. The plate holder was tapped gently on paper adsorbent to remove excess fluid, 100µl of Conjugate 1 were added to all micro-wells except blank well, cover with adhesive tape and incubate at 37^0c for 60minutes thereafter the washing step was performed. 100µl of Conjugate 2 were added to all micro-wells except blank well and incubate at 37^0c for 30 minutes and the plate was washed. 100µl of substrate/chromogen were added to each well, blank well inclusive and covered. The plate was incubated at 37^0c for 15minutes, 100µl Stop solution were added and read at 450/630nm using ELISA reader.

2.7. Data Analysis

The carriage rates of *Staphylococcus aureus* was calculated by using positive samples as numerator and the total numbers of samples used in this study as denominator. The data generated from this study were presented using descriptive statistics. The data was subjected to Fisher's Exact Test for comparison of proportions to determine any significant relationship between carriage rate and different clinical specimens. Confidence level was set at p= 0.05. Prevalence of *S. aureus* enterotoxins, MSSA and MRSA were estimated with 95% confidence intervals. All the variables were adjusted for clinical specimens and agar. A complete case series analysis was used. The independent variables included were: isolates (MSSA versus MRSA), enterotoxins (single versus multiple) and agar (MHA versus SA). Chi-square tests were used to test for statistical significance (5%).

3. RESULTS ANALYSIS

Eighty three (83) *S. aureus* isolates were obtained in this study, of which *S. aureus* was most predominant in semen and blood culture [19(22.9%)]. This was followed by high vaginal swab (HVS) with eleven (13.3%) isolates. Ear, eye, throat and urethral swabs had the least prevalence [2(2.4%)]. Of the 83 *S. aureus* isolates obtained in this study, 43(52.0%) were identified as coagulase-positive *S. aureus* (CPSA). The distribution pattern of *S. aureus* isolates in the various clinical samples was shown in Table 1. The highest prevalence of CPSA isolates occurred in ear and urethral swabs (100.0%). This was followed by wound swabs (89.0%), urine (75.0%), HVS (55.0%), palm, throat and finger swabs (50.0%) and semen (37.0%). Eye and nail swabs had zero prevalence of CPSA (Table 1).

Table 1: Distribution of *Staphylococcus aureus* and coagulase-positive *Staphylococcus aureus* (CPSA) obtained from clinical samples

Samples	No. Tested (%)	No CPSA positive (%)
Semen	19(22.9)	7(37.0)
Ear Swab	2(2.4)	2(100.0)
Eye Swab	2(2.4)	0(0.0)
Throat Swab	2(2.4)	1(50.0)
Urethral Swab	2(2.4)	2(100.0)
Wound Swab	9(10.8)	8(89.0)
Urine	4(4.8)	3(75.0)
HVS	11(13.3)	6(55.0)
Finger	4(4.8)	2(50.0)
Nail	5(6.0)	0(0.0)
Palm	4(4.8)	2(50.0)
Blood	19(22.9)	10(53.0)
Total	**83(100.0)**	**43(52.0)**

Table 2 shows the distribution of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) isolates. Of the 43 CPSA isolates, 22(51.0%) were identified as MRSA while 21(49.0%) were MSSA. All the CPSA isolates from semen and urethral swabs were MSSA while all the CPSA isolates from the ear and throat swabs were MRSA. The highest number of isolates that were MRSA was obtained from wound swabs while least number of MRSA were isolated from finger, palm swabs and urine samples (Table 2).

Table 2: Distribution of methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA)

Samples	No. of CPSA (%)	MRSA (%)	MSSA (%)
Semen	7(16.3)	0(0.0)	7(100.0)
Ear Swab	2(4.6)	2(100.0)	0(0.0)
Throat Swab	1(2.3)	1(100.0)	0(0.0)
Urethral Swab	2(4.6)	0(0.0)	2(100.0)
Wound Swab	8(18.6)	7(87.5)	1(12.5)
Urine	3(6.9)	1(33.3)	2(66.7)
HVS	6(13.9)	5(83.3)	1(16.7)
Finger	2(4.6)	1(50.0)	1(50.0)
Palm	2(4.6)	1(50.0)	1(50.0)
Blood	10(23.3)	4(40.0)	6(60.0)
Total	**43(100.0)**	**22 (51.0)**	**21 (49.0)**

Key: CPSA – Coagulase positive *S. aureus*

Table 3 showed the distribution of enterotoxins in coagulase positive *S. aureus*. Six (60.0%) coagulase-positive *S. aureus* of the 43 tested for enterotoxin possessed single enterotoxins while 4 (40.0%) possessed more than one type of enterotoxin. Coagulase-positive *S. aureus* from wound swab had highest number of positive enterotoxins antibodies. One of the blood culture isolate was positive for single and multiple enterotoxins.

Table 3: Distribution of enterotoxin in CPSA

Source of *S. aureus*	CPSA isolates (%)	No. Positive (%)	Single (%)	Multiple (%)
Ear Swab	2(4.6)	1(50.0)	1(100.0)	0(0.0)
Wound Swab	8(18.6)	6(75.0)	3(50.0)	3(50.0)
HVS	6(13.9)	2(33.3)	2(100.0)	0(0.0)
Blood	10(2.3)	1(10.0)	0(0.0)	1(100.0)
Total	**43(100.0)**	**10(23.3)**	**6 (60.0)**	**4 (40.0)**

Key: CPSA – Coagulase positive *S. aureus*

None of the single enterotoxin isolates was positive for enterotoxin A and B while Enterotoxin D was the highest enterotoxin detected from Coagulase positive *S. aureus*. Enterotoxin C, D and E was detected from coagulase-positive *S. aureus* obtained from wound swabs (Table 4).

Table 4: Sample distribution of enterotoxin (single) in CPSA

Samples	No. of CPSA	Enterotoxins				
		A	B	C	D	E
Ear Swab	2				1	
Wound Swab	9			1	1	1
HVS	10				2	

4. DISCUSSION

S. aureus is a common cause of bacteraemia in the community especially as a nosocomial infection. In this study, 83 *S. aureus* isolates were obtained, of which *S. aureus* was most predominant in semen and blood culture [19(22.9%)]. This was followed by high vaginal swab (HVS) with eleven (13.3%) isolates. Ear, eye, throat and urethral swabs had the least prevalence (2.4%). Also, of the 83 *S. aureus* isolates obtained in this study, 43(52.0%) were identified as coagulase-positive *S. aureus* (CPSA). The highest prevalence of CPSA isolates occurred in ear and urethral swabs (100.0%). This was followed by wound swabs (89.0%), urine (75.0%), HVS (55.0%), palm, throat and finger swabs (50.0%) and semen (37.0%). Eye and nail swabs had zero prevalence of CPSA. Odu and Okonko (2012) showed that 32.0% of apparently healthy students of University of Port Harcourt, Nigeria below 30 years of age had *S. aureus* out of which 37.5% were MRSA. The 52.0% prevalence reported for *S. aureus* in this study is not comparable to the 32.4% reported by Mainous et al. (2006) and the 32.0% reported by Odu and Okonko (2012).

Numerous studies conducted in different countries and in different populations of patients on dialysis have consistently documented that a large proportion of such patients carry *Staphylococcus aureus* in their skins, nares and that the risk of them becoming infected with their own strains is quite high (Herwaldt, 1998; Odu and Okonko, 2012). Studies have been done in adults in intensive care units (Majumder et al., 2001; Anupurba et al., 2003; Saxena et al., 2003; Rajaduraipandi et al., 2006; Pathak et al., 2010; Odu and Okonko, 2012) and among patients at high risk of *S. aureus* infection (Chacko et al., 2009; Pathak et al., 2010; Odu and Okonko, 2012) but studies on prevalence of nasal carriage and antibiotic susceptibility pattern of *S. aureus* in children are few (Ramana et al., 2009; Chatterjee et al., 2009; Pathak et al., 2010; Odu and Okonko, 2012). One possible explanation of high prevalence of *S. aureus* in resource rich countries could be low rates of exposure to antigens due to better personal hygiene leading to decreased clearing of pathogens in the tested patients (Sivaraman et al., 2009; Pathak et al., 2010). However, transmission of infections caused by these strains is readily established by close contact (Xander et al., 2006; El- Jalil et al., 2008). Furthermore, transmission from humans to animals (Seguin et al., 1999) or from animals to man (Juhász-Kaszanyitzky et al., 2007) may further complicate the epidemiology of these organisms (El- Jalil et al., 2008).

This study showed that methicillin-resistant *S. aureus* (MRSA) isolates had higher prevalence (51.0%) than and methicillin-sensitive *S. aureus* (MSSA) with prevalence of 49.0%. All the CPSA isolates from semen and urethral swabs were MSSA while all the CPSA isolates from the ear and throat swabs were MRSA. The highest number of isolates that were MRSA was obtained from wound swabs while least number of MRSA was isolated from finger, palm swabs and urine samples. The 51.0% prevalence reported for methicillin-resistant *S. aureus* in this study is higher than the 17.9% in Loeffler *et al.* (2005) study. The result indicated MRSA, as common nosocomial pathogens of human. Ramana *et al.* (2009) reported a prevalence of 16.0% for *S. aureus*, of which 19.0% were MRSA among school going children (5 to 15 years) in Narketpally, Andhra Pradesh, India. Other study by Chatterjee *et al.* (2009) using polymerase chain reaction (PCR) showed a prevalence of 52.5% for *S. aureus* of which 3.9% were MRSA. They identified living in mud-thatch housing as factor associated with nasal carriage in their study (Pathak et al., 2010; Odu and Okonko, 2012).

Carriage of MRSA or MSSA varies in different geographical areas (Madani et al., 2001; Abudu et al., 2001; Sa-Leao et al., 2001; El- Jalil et al., 2008; Odu and Okonko, 2012). The prevalence of MRSA in some countries is still low. In the Netherlands for example, it is as low as 1.0% (Lytkainen et al., 2004; El- Jalil et al., 2008; Odu and Okonko, 2012). The prevalence of

MRSA among *S. aureus* isolates in the study by Mainous et al. (2006) was 2.58%, for an estimated population carriage of MRSA of 0.84%.

Ten coagulase-positive *S. aureus* were positive for enterotoxin production, a prevalence of 23.0% which is lower than what was observed in North Palestine (41.2%). The prevalence was in the ranges of 17.8% to 86.6% recorded by Adwan et al. (2006). The differences in prevalence observed might may be due to differences in ecological origin of the strains, sensitivity of detection methods, genes being detected and number of samples. Humphreys *et al*. (1989) found that enterotoxin production was higher among blood culture isolates of *S. aureus* from septicaemic patients.

5. Conclusion

This study may be useful in that our findings point out that all enterotoxin producing *S. aureus* were found to be methicillin resistant. Majority of the methicillin-resistant *S. aureus* were not enterotoxins producer. Therefore, all *S. aureus* isolated from the Laboratory specimen especially from wound specimen should be tested for enterotoxin. Detection of methicillin-resistant should be incorporated into the routine susceptibility testing for bacterial isolation especially for *S. aureus*.

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