Salt Tolerance, Haemolysin and β-Lactamase Production, and Susceptibility Profiles of Methicillin-Resistant Staphylococci from Clinical Specimens in Benin City, Nigeria.

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ABSTRACT

Against the background that different strains of methicillin-resistant staphylococci possess different properties, the salt tolerance, haemolysin production, β-lactamase production and susceptibility profiles of methicillin-resistant and methicillin-sensitive staphylococci were determined. A total of 335 staphylococci isolates recovered from clinical specimens and consisting of 313 isolates of Staphylococcus aureus and 22 isolates of coagulase negative staphylococci were used for this study. Methicillin resistance was determined with cefoxitin, while salt tolerance, haemolysin production, β-lactamase production and antimicrobial susceptibility profiles of the isolates were evaluated using standard techniques. Methicillin-resistant Staphylococcus aureus (MRSA) tolerated higher salt concentrations (ranging from 8% - 25%) than their methicillin-sensitive (MSSA) counterparts ($P \leq 0.0001$). A similar observation between methicillin-resistant and methicillin-sensitive coagulase negative staphylococci (MRCONS and MSCONS) was observed, albeit, it was only statistically significant at 15% and 20% ($P=0.0089$ and 0.0124 respectively). The prevalence of haemolysin production did not differ significantly between MRSA and MSSA as well as between MRCONS and MSCONS ($P>0.05$). The prevalence of β-lactamase production was only significantly higher in MRSA compared with MSSA ($P=0.0022$). The susceptibility profiles did not differ significantly between methicillin-resistant and methicillin-sensitive staphylococci ($P>0.05$). MRSA and MRCONS tolerated higher levels salts than their sensitive counterparts.

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INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen causing a wide range of diseases in both immunologically normal and compromised hosts (1). Humans are a natural reservoir for *S. aureus*, and asymptomatic colonization is far more common than infection (2). The pathogen is responsible for broad spectrum of human and animal diseases ranging from skin infections to such severe diseases as pneumonia, endocarditis, osteomyelitis, septicaemia and enterocolitis (3 – 10). Infections with *S. aureus* are especially difficult to treat due to the evolution of resistance genes to antimicrobial drugs (11). Importantly, the methicillin-resistant strains (MRSA) are now the most common cause of nosocomial *S. aureus* infections and are spreading throughout communities (12). Risk factors for the development of MRSA

include previous antibiotic use, increased age, and severity of underlying illness, duration of hospitalization and multiple invasive procedures (13, 14). Patients infected with MRSA have a longer length of stay in the hospital and a higher rate of mortality than patients infected with methicillin-susceptible strain of *S. aureus* (MSSA) (13, 15). Infections with MRSA are difficult to treat (16).

Different strains of MRSA possess different qualities. In New Zealand, strains of MRSA from the community termed Western Samoa phage pattern differ from other MRSA in been more salt tolerant, adhere better to Hep2 epithelial cells, were consistently egg-yolk opacity factor negative and produced higher levels of haemolytic toxins (17). There have been conflicting reports in terms of degree of of salt tolerance of MRSA isolates, while some studies reported that the isolates do not grow in media with above 2.5% NaCl concentration (18), others reported survival at 14 % and 35% NaCl concentration (17, 19). This implies that MRSA strains may vary in their properties in Nigeria in terms ability to survive in salt environment. To our knowledge, no study has looked at the properties of MRSA and MSSA vis-s-vis degree of salt tolerance in Nigeria. Against this background, this study aims at determining the salt tolerance, haemolysin and β-lactamase production, and susceptibility profiles of methicillin-resistant staphylococci recovered from clinical specimens.

MATERIALS AND METHODS

Bacterial isolates

A total of 335 consecutive non-repetitive clinical isolates of staphylococci consisting of 313 S. aureus and 22 coagulase negative staphylococci (CONS) were used for this study. All isolates were identified using standard techniques (20). An isolate was identified as S. aureus if it was Gram positive *coccci*, catalase positive and coagulase positive. Similar criteria were used for CONS except that they were coagulase negative.

Detection of methicillin resistance

Methicillin resistance in both S. aureus and CONS was indicated by resistance to cefoxitin using the British Society for Antimicrobial Chemotherapy (BSAC) method (21). Briefly, test organisms were emulsified in sterile water and the turbidity matched with 0.5 McFarland standards. Once matched, a sterile cotton wool swab was dipped in the organism suspension and excess liquid was removed by turning the swab on side of the test tube. The entire surface of Mueller–Hinton agar plate was seeded by swabbing in three directions with the swab. A 10µg cefoxitin disc was place at the centre of the plates and the plates were incubated at 35^0C overnight. An isolate was deemed methicillin resistant if the inhibition zone diameter is ≤21mm for both S. aureus and CONS.

Salt tolerance test

Salt tolerance was evaluated by determining growth of the staphylococci isolates at different concentrations of sodium chloride (NaCl) ranging from 8% to 25% in nutrient agar. The different concentrations of NaCl were incorporated into nutrient agar and the isolates streaked on these plates from an overnight culture of the staphylococci isolates on cysteine lactose electrolyte deficient (CLED) medium. The plates were incubated at 37^0C overnight.

Haemolysin production

Haemolysin production was detected using the method of Drew *et al* (22). Each bacterial isolate was inoculated unto 5% sheep blood agar and was incubated at 37^0C for 24hrs. The presence of clear zones around the colonies indicates haemolysin production.

β-lactamase production

β-lactamase was detected by the iodometric method (23). The staphylococci from an overnight culture on nutrient agar were suspended in 0.1cm^3 0.1M phosphate buffer containing 6mg/ cm^3 benzylpenicillin until it was heavily turbid. The tubes containing these were left at room temperature for one hour. After one hour, 20µL of 1% starch solution was added and followed by 20µL of 2% iodine in 53% aqueous potassium iodide. β-lactamase activity was inferred if there was decolourization of the iodine-starch complex within 5mins.

Disc susceptibility testing

Disc susceptibility tests were performed using the British Society for Antimicrobial Chemotherapy (BSAC) method (21).

Data analysis

The data obtained were analyzed with Chi square (X^2) test or Fisher's exact test as appropriate and odd ratio analysis using the statistical software INSTAT® (Graph Pad Software Inc, La Jolla, CA, USA).

RESULTS

A total of 180 (57.51%) out of 313 isolates of S. aureus were methicillin resistant and the prevalence of MRSA did not differ between isolates from in-patient and out-patient (P= 0.9369). No methicillin-resistant CONS were observed among in-patients while 64.71% of CONS from out-patients were methicillin resistant, and this was statistically significant (P= 0.0351) (Table 1).

Table 1: Distribution of methicillin-resistant *staphylococci* in relation to source of isolates

Organisms/source	No. tested	No. positive for MR (%)	OR	95%CI	P value
Staphylococcus aureus					
Out patient	229	132(57.64)	1.021	0.616,1.692	0.9369
In-patient	84	48(57.14)			

MR = Methicillin–resistant; OR Odd ration; CI = Confidence interval

Table 2: Salt tolerance profile of methicilin–resistant and methicillin–sensitive staphylococci

NaCl concentration (%)	Organisms (%)		P vale
	MR	MS	
Staphylococcus aureus	**(n = 180)**	**(n = 133)**	
8	180(100.00)	121(90.98)	0.0001
10	174(96.67)	104(78.20)	<0.0001
15	141(78.33)	30(22.56)	<0.0001
20	80(44.44)	4(3.01)	<0.0001
25	24(13.33)	0(0.00)	<0.0001

MR = Methicillin–resistant = Methicillin–sensitive; NaCl = Sodium chloride; n = number tested

MRSA were significantly more tolerant to various concentrations of NaCl used than their MSSA counterparts (P<0.001) while for CONS, the difference became significant from 15% NaCl concentration. Although, no methicillin-sensitive CONS grew at 25% NaCl as against 13% of methicillin-resistant CONS that survived 25% NaCl concentration (Table 2).

Haemolysin production did not differ significantly between MRSA and MSSA as well as between MRCONS and MSCONS (P>0.05). B-lactamase production was only significantly higher among MSRA compared with MSSA (P= 0.0022) (Table 3).

Generally, ofloxacin was the active antibacterial agent against methicillin-resistant and methicillin-sensitive S. aureus and CONS. With the exception ceftriaxone that was significantly (P= 0.0351) more active against MSCONS than MRCONS, there was no significant difference (P>0.05) in the antibacterial activity of the tested antibiotics between MRSA and MSSA as well as MRCONS and MSCONS (Table 4).

Table 3: β-lactamase and haemolysin production of methicillin – resistant and methicillin-sensitive staphylococci

Characteristics	MR	MS	P vale
Haemolysin production ***Staphylococcus aureus***			
No. tested	180	133	0.1275
No. positive (%)	163 (90.56)	112(84.21)	
No. positive (%)	10(90.91)	8(72.73)	
β-lactamase production ***Staphylococcus aureus***			
No. tested	180	133	0.0022
No. positive (%)	154(85.56)	94(70.68)	

MR = Methicilin – resitant; MS = Methicillin-sensitive

Table 4: Susceptibility profiles of methicillin – resistant and methicillin – sensitive staphylococci.

Antibacterial agents(µg/disc)	MR (%)	MS (%)	P value
Staphylococcus aureus	**n = 180**	**n = 133**	
Amoxicillin-clavulanate (30)	0(0.00)	1(0.75)	0.8191
Cloxacillin (5)	18(10.00)	15(11.28)	0.8589
Cefuroxime (30)	2(1.11)	0(0.00)	0.6156
Ceftazidime (30)	0(0.00)	0(0.00)	N.D
Ceftriaxone (30)	41(22.78)	18(13.53)	0.0547
Gentamicin (10)	96(53.33)	78(58.65)	0.4121
Ofloxacin (5)	139 (77.22)	94 (70.68)	0.2375
Erythiomycin (5)	106 (58.89)	77 (57.89)	0.519
Amoxicillin-clavulanate (30)	0(0.00)	2(18.18)	0.4762
Cloxacillin (5)	2(18.18)	1(9.09)	1.0000
Cefuroxime (30)	0(0.00)	0(0.00)	N.D
Ceftazidime (30)	0(0.00)	0(0.00)	N.D
Ceftriaxone (30)	0(0.00)	5(45.45)	0.351
Gentamicin (10)	7(63.64)	5(45.45)	0.6699
Ofloxacin (5)	10(90.91)	10(90.91)	1.0000
Erythiomycin (5)	7(63.64)	8(72.73)	1.0000

MR = Methicillin – resistant; MS = Methicillin – sensitive; n = number tested.

DISCUSSION

Methicillin–resistant staphylococci cause nosocomial and community infections which may result in high morbidity and mortality (12,13). The study was informed by the conflicting reports globally about the degree of tolerance to salt concentration of Staphylococci sps, hence the need to evaluate the pattern of salt tolerance of isolates in the communicate. This may provide an impetus in assessing the association between the degree of virulence and molartality vis-svis degree of salt tolerance of the isolates.

The prevalence of MRSA did not differ significantly (P = 0.9369) between isolates

recovered from in-patients and out patients. In the past, MRSA infections were mostly confined to patients with risk factors such as hospitalization or recent healthcare contact (24). More recently, however, there has been a dramatic increase in community-associated MRSA (24, 25). This may explain the non-significant difference in the prevalence of MRSA between isolates recovered from in-patients and out-patient. However, for CONS, MRCONS were only observed among out-patients.

It was observed in this study that strains of MRSA survived in high NaCl concentrations of up to 25% while MSSA did not grow at 25% NaCl. Survivals of MRSA at the various NaCl concentrations were significantly higher than MSSA. Multiple genes, including the branched-chain amino acid transporter genes *brnQ* and the arsenic operon regulatory gene *arsR* cooperatively participate in salt tolerance (1, 26, 27). The presence of salt (NaCl) induces methicillin resistance (28). It is possible that the genes coding for methicillin resistance and salt tolerance are close to each other. However, molecular studies are needed to verify this. It is important to note that some authors report that above NaCl concentration of 2.5% many strains of MRSA did not grow (18). Other authors have reported MRSA strains growing in 14% and 35% NaCl media (17,19). It may appear that MRSA from different locations tolerate salt differently as Bruins *et al.* (18) study was conducted in the Netherlands, Adhikari *et al.(*17) and Ganjion *et al. (*19) studies were conducted in New Zeland and Iran respectively. Our study was able to isolate MRSA strains with salt tolerance of 25% NaCl concentration

The finding that haemolysin production prevalence did differ significantly between MRSA and MSSA ($P > 0.05$) agrees with previous reports (29, 30). A similar finding for CONS (between MRCONS and MSCONS) was observed in this study. In terms of β-lactamase production, MRSA had significantly higher prevalence of than MSSA ($P = 0.0022$). However, for CONS, there was no significant difference in the prevalence of β-lactamase production between MRCONS and MSCONS ($P = 1.0000$).

Enzymes such as coagulase, β-lactamase and haemolgsin are considered indices of pathogenicity among staphylococci (31). Survival in higher salt concentrations is necessary for colonization (32). Though these pathogenic indices were observed in all *S.aureus* and CONS some were more prevalent in MRSA and MRCONS.

There was no significant difference ($P > 0.05$) in the susceptibility profiles of methicillin–resistant and methicillin-sensitive *S.aureus* and CONS, except for ceftriaxone among CONS where only MSCONS were more susceptible ($P = 0.0351$). This is surprising as methicillin-resistant staphylococci were expected to be more resistant to antibacterial agents. Antibiotics use is unregulated and over the counter sales of antibiotics without prescriptions are rife in Nigeria (33,34, 35).

Conclusively, MRSA and MRCONS are more tolerant to high salt concentrations than MSSA and MSIONS. With the exception of MRSA that produced more β-lactamase, there were no difference in haemolysin production and antibacterial susceptibility profiles of MRSA and MSSA and MRCONS and MSCONS.

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