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Plasmid Curing Analysis of Antibiotic Resistance in β-lactamase Producing *Staphylococci* from Wounds and Burns Patients

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Abstract: Hospitals worldwide are facing unprecedented crisis due to increasingly rapid emergence and dissemination of antimicrobial resistant staphylococci in wounds and burns and its environs via plasmid mediation. This study was conducted to evaluate the plasmid-mediated or chromosomal-mediated resistance in staphylococci. One hundred clinical swabs from wounds and burns patients were demonstrated for presence of staphylococci using mannitol salt agar. Various biochemical, DNase and β-lactamase test was carried out and the plasmid curing assay was demonstrated using 0.1 mg mL^{-1} acridine orange on antibiotic resistant isolates. The results revealed *S. aureus* (47) and coagulase negative staphylococci (CoNS) (6). β-lactamase producing species of *S. aureus* were 14 and CoNS was 1. Most isolates showed high resistance pattern to gentamicin, ciprofloxacin, norfloxacin, rifampicin, chloramphenicol, ampiclox and others. The antibiotic resistance isolates were highly indicative of plasmid-borne and few are chromosomal-borne after the plasmid curing analysis. The plasmid-mediated resistance observed among various antibiotics poses difficulty in treatment for clinicians. This high plasmid-mediated resistance among the isolates and from other studies calls for an urgent surveillance and epidemiological studies to infection control.

Key words: Antibiotic resistance, β-lactamase, multidrug, wound infection

INTRODUCTION

Staphylococcus aureus is an important pathogen that causes skin, wound and burn infections, septicaemia and endocarditis, such that infections involving antibiotic resistant strains may impact on human health (Ombui *et al*., 2000; Adegoke and Okoh, 2011; Yameen *et al*., 2010; Bashir *et al*., 2007). Coagulase-negative *Staphylococcus* (CoNS), a component of the normal skin flora has become an important opportunistic pathogen in foreign body sites causing both nosocomial and community acquired infections. Most developed countries have reported an increase in colonization and infection in hospitalized patients by CoNS while there are scanty data in developing countries (Akinjogunla and Enabulele, 2010).

Since the introduction of antimicrobials, bacteria have developed mechanisms for resisting the effects of antibiotics. The emergence of multidrug resistance in Gram positive bacteria (*Pneumococci*, *Enterococci* and *Staphylococci*) is a particularly important development (Bashir *et al*., 2007). The levels of antibiotic resistant infections in the developing world have increased steadily in the last few decades as a result of combination of microbial characteristics and the selective pressure of antimicrobial use (Blondeau and Tillotson, 2002; Ombui *et al*., 2000).

Microorganisms mechanisms of overcoming the activities of antimicrobial agents include the production of structure-altering or inactivating enzymes (β-lactamase or aminoglycoside-modifying enzymes), alteration of penicillin-binding proteins or other cell-wall target sites, altered DNA gyrase targets, permeability mutations, active efflux and ribosomal modification (Akinjogunla and Enabulele, 2010; Gad *et al*., 2010).

Plasmids, which are extra-chromosomal materials, allow the movement of genetic material, including antimicrobial resistance genes between bacterial species and genera. It may contain resistance genes for single or multiple antimicrobial agents, which have been reported among staphylococci to several therapeutically useful antibiotics including penicillin family, streptomycin, rifampicin, fusidic acid and novobiocin and fluoroquinolones (Akinjogunla and Enabulele, 2010; Ombui *et al*., 2000).

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The clinical significance of CoNS strains has emerged by their resistance characteristics to methicillin and almost all classes of antimicrobial agents, therefore limited treatment options and prolonged course of infection due to these CoNS species could therefore have severe consequences (Akinkunmi and Lamikanra, 2010; Ako-Nai *et al*., 2005). However, plasmid profiles have been found useful in epidemiological surveillance of disease outbreaks and in tracing antibiotic resistance patterns. Thus this study was designed to determine plasmid-mediated resistance isolates on staphylococci and the curing rate on each antibiotic tested.

MATERIALS AND METHODS

Collection samples: One hundred clinical wounds and burns samples were obtained from different sites in patients undergoing dressing of wounds and burns at General Hospital, Ekpan, Delta State, Nigeria. Exudates were obtained from the infected sites of each patient with sterile cotton wool swabs and applied to freshly prepared Mannitol Salt Agar (MSA) and Nutrient agar (Oxoids). The cultures were then transferred to the laboratory where they were incubated at 37°C for 24 h.

Isolation of Staphylococci and species identification: Colonies growing on nutrient agar slants were streaked on top of freshly prepared plates of MSA and incubated at 35°C for 24 h. Primary characterization of isolates was based on the gram stain, morphological and cultural characteristics, fermentation on MSA, catalase and coagulase (tube) test and DNase agar test. The ability of each *S. aureus* and CoNS isolates to elaborate penicillinase was evaluated using the method described by Ako-Nai *et al*. (2005). The pure colonies were further sub-cultured and stored on nutrient agar slants at -20°C for further analysis.

β-lactamase assay: This test was carried out as described by Ako-Nai *et al*. (2005). Strips of starch paper measuring 4×7 cm were cut and sterilized with 70% ethanol. These strips were then soaked for 10 min in a solution of benzyl penicillin dissolved in phosphate buffer containing 10^5 units. They were spread over an area of 2 to 3 mm. Each test paper was then used to test two organisms at time with the inocula placed at least 2 cm apart. The Petri dishes were then incubated for 30 min at 37°C after which the plate was flooded with Grams iodine solution. This was immediately drained off. This caused the starch paper to turn uniformly black within 30 sec of application. Colonies with decolourized zones thereafter were indicative of β-lactamase production. Results were read within 5 min as black background tends to decolourize, making interpretations more difficult.

Determination of antibiotic resistance profile: Wounds and burns isolates were subjected to antibiotic screening by disk diffusion method as described by CLSI (2008). Inocula were prepared by diluting overnight cultures in sterile NaCl (0.9%) suspension and then marched with the McFarland turbidity index. Bacterial suspensions were then plated on to Mueller Hinton Agar and the commercially available antibiotic discs were placed on lawn of culture and the plates incubated overnight at 37°C. Sensitivity, intermediate and resistance were determined by the zone of complete growth inhibition around each disk according to reference standards. Reference type *S. aureus* strain (ATCC 25923) was used as positive control.

Plasmid curing analysis: Plasmid curing was carried out in order to determine the location (plasmid-borne or chromosomal) of the drug resistance marker(s). The curing (elimination) of the resistant plasmids of the *S. aureus* and CoNS isolated was done using sub-inhibitory concentration of 0.10 mg mL^{-1} of acridine orange as described by Rasool *et al*. (2003), Yah *et al*. (2007) and Akortha and Filgona (2009) with slight modification. Isolates were grown for 24 h at 37°C in Mueller-Hinton broth containing 0.1 mg mL^{-1} acridine orange. The broth was agitated to homogenize the content and loopful of the broth medium were cultured on MHA plates and antibiotic sensitivity testing was carried out as previously described. Absence of zone of inhibition on Mueller Hinton agar was indicative of plasmid-mediated resistance (plasmid cured) while presence of zone of inhibition on Mueller Hinton agar was indicative of chromosome-mediated (plasmid not cured).

RESULTS

This study revealed a high isolation rate of *Staphylococcus* spp. 53(53%) with 47 isolates as *S. aureus* and 6 isolates as CoNS. Of the 47 *S. aureus* isolates, 14(30%) were β-lactamase producing species and 33(70%) were non-β-lactamase producing species. Whereas, only 1 (17%) β-lactamase producing species

was indicated of the 6 CoNS isolates. The characterization test with DNase agar showed 38(72%) isolates of staphylococci as positive and 15(28%) isolates as negative (Table 1, 2). Most of the antibiotics employed showed high resistance pattern against the tested staphylococci. 46 *S. aureus* was tested against gentamicin, 28 (60%) isolates were resistant, 15 (32%) sensitive and 3 (9%) intermediate while all CoNS (100%) assayed showed resistance. The staphylococcal isolates tested against the fluoroquinolones used in this study showed high resistance pattern in Norfloxacin (62%), Ciprofloxacin (57%) and Levofloxacin (51%) in *S. aureus* while in CoNS, the resistance pattern in norfloxacin, ciprofloxacin and levofloxacin are 67, 100 and 83%, respectively (Table 3). The plasmid curing analysis on resistant isolates as revealed in Table 4 showed that most resistant isolates were cured (plasmid-borne) and few isolates were resistant against tested antibiotics after curing (chromosomal-borne) as compared with the initial resistant pattern before curing (pre-curing).

Table 1: Biochemical characterization and identification of wounds and burns isolates from hospital patients in accident and emergency unit

	No. of staphylococcal isolates (%)			
	Positive		Negative	
Biochemical test	No.	%	No.	%
Gram staining	53	53	47	47
Catalase	53	53	47	47
Coagulase	47	89	6	11
DNase	38	72	15	28
Mannitol fermentation	21	40	32	60

Table 2: Characterization and occurrence of β-lactamase (βL) and non-β-lactamase producing *S. aureus* and CoNS isolated from wounds and burns patients

		βL producer		Non-βL producer	
Isolate	No of occurrence	No.	(%)	No.	(%)
S. aureus	47	14	30	33	70
CoNS	6	1	17	5	83

DISCUSSION

Several studies have revealed an increase in antibiotic resistance pattern associated with *S. aureus* and more importantly on CoNS, which has equally being posing a great health challenge to humans and animals and it was also observed in this study (Bashir *et al*., 2007; Ombui *et al*., 2000; Yameen *et al*., 2010; Akinkunmi and Lamikanra, 2010; Akinjogunla and Enabulele, 2010). This trend of increase in antibiotic resistance was also revealed in a study on hospital currency notes by Adegoke and Okoh, 2011. In determining the mechanism of resistance to antibiotics by staphylococci, β-lactamase production and plasmid curing assay was conducted. We observed a fairly high level of β-lactamase production in staphylococcal isolates especially among the *S. aureus*. High levels of β-lactamase production in Staphylococci had also been reported (Gad *et al*., 2010; Adegoke and Okoh, 2011; Akinjogunla and Enabulele, 2010; Ako-Nai *et al*., 2005). The plasmid curing assay revealed that most of the antibiotic resistant staphylococci isolated in this study were plasmid-mediated since 56-91% of the isolates showed zones of inhibition (cured) when tested against the selected antibiotics while 2.6-31% showed zones of inhibition (plasmid not cured) indicating chromosomal-borne resistant gene. The screening of the isolates with acridine orange resultantly suggest that the resistance markers were stably lost, which is in line with that of previous studies (Akinjogunla and Enabulele, 2010; Adegoke and Okoh, 2011; Gad *et al*., 2010; Yah *et al*., 2007; Akortha and Filgona, 2009).

Table 3: Antibiotic susceptibility profile on isolated *Staphylococcus* spp. from wounds and burns hospital patients

	Staphylococcus aureus group (n = 47)						CoNS (n = 6)					
	Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant	
Antibiotics	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Levofloxacin	11	23	7	15	24	51	0	0	0	0	5	83
Erythromycin	0	0	14	30	30	64	0	0	1	17	5	83
Norfloxacin	6	13	5	11	29	62	1	17	0	0	4	67
Ceftriaxone	0	0	0	0	5	11	0	0	0	0	1	17
Cefuroxime	1	2	0	0	4	9	0	0	0	0	1	17
Ciprofloxacin	3	6	14	30	27	57	0	0	0	0	6	100
Streptomycin	8	17	6	13	39	83	0	0	0	0	6	100
Chloramphenicol	4	9	8	17	29	62	1	17	0	0	4	67
Ampiclox	12	26	3	6	38	81	0	0	1	17	5	83
Gentamicin	15	32	4	9	28	60	0	0	0	0	6	100
Co-trimoxazole	1	2	0	0	7	15	0	0	0	0	1	17
Rifampicin	4	9	2	4	36	77	0	0	0	0	5	83

Table 4: Plasmid curing analysis of resistant staphylococci isolated from wounds and burns patients with Acridine orange (0.1 mg mL^{-1})

		Cured (%)		Resistant (%) (Post-curing)	
Antibiotics used	No. resistant (Pre-curing)	No.	(%)	No.	(%)
Ciprofloxacin	33	26	78.8	6	18.2
Norfloxacin	33	27	81.8	5	15.2
Gentamicin	34	23	67.6	9	26.5
Streptomycin	39	24	61.5	8	20.5
Rifampicin	41	23	56.1	9	21.9
Erythromycin	35	27	77.1	5	14.3
Chloramphenicol	33	30	90.9	2	6.1
Ampiclox	38	31	81.6	1	2.6
Levofloxacin	32	23	71.9	9	28.1

CONCLUSION

This study had examined the incidence of staphylococci, its susceptibility patterns to different antibiotics and the drug resistance factor mediated by β-lactamases and plasmid-borne genes. Thus indicating that most resistance to antibiotics is plasmid-mediated, this can easily be transferred from one strain to another or from one organism to another within the same environment. However, this study intends to carry out the molecular study on the plasmid-borne and chromosomal-borne gene associated with antibiotic resistance. Therefore, hospital and community management of infection control and surveillance of antibiotic resistance is highly and urgently necessary.

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