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Full Length Research Paper

The *in vitro* effect of vancomycin on multidrug resistant *Staphylococcus aureus* from hospital currency notes

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We determined the *in vitro* effect of vancomycin on multidrug resistant *Staphylococcus aureus* isolates and presumptive roles of plasmid DNA using standard microbiological procedures. A total of 32 paper naira notes from seven different hospital sections (mostly from the patients) were analyzed. Thirteen notes (40.6%) gave isolates of *S. aureus* and subsequent resistant range of between 62.5 and 100% to frequently prescribed antibiotics. The same set of isolates showed three (23.1%) to be susceptible and ten (76.9%) to be resistant to vancomycin. Five of the latter ten isolates produced beta lactamase and became susceptible to some of the multidisc antibiotics and vancomycin, after the plasmid curing. This presumptively reflects important role of plasmid DNA in conferring resistance to microorganisms as a means of their adaptation especially in the vancomycin resistant *S. aureus* (VRSA). The increasing resistance to vancomycin could cause major problems to public health as it was often regarded as the last line of defense against nosocomial and community based *S. aureus* infection.

Key words: Vancomycin, multidrug, plasmid, beta lactamase, antibiotic resistance.

INTRODUCTION

The role of currency notes in the transmission of infection is not a new concept. It dates back from 1665 in England when coin was generally believed to be one of the vehicles for the transmission of plague that killed over 60,000 (Smith, 2011). The role of currency notes compares to any other fomite (Jalgaonkar et al., 2007) but with greater impact as it circulates continuously from the infected to uninfected; asymptomatic carriers to convalescence; immunocompetent to immunocompromised and/or immunosuppressed. There exist a potential for fresh infection due to mishandling of contaminated currency notes, just as it is possible for re-infection of the convalescent by pathogenic organisms it conveys (Singh et al., 2002).

Quite a number of organisms bear the potentials for survival on dry fomites like currency notes. They have evolved complicated separate physiologic resting stages that give them the advantage for surviving or hibernating due to low water activity (Grant, 2004). Highly virulent spore forming pathogenic bacteria in the genus Bacillus have been isolated in currency note by some studies (Singh et al., 2002; Toba et al., 2007). For *Staphylococcus aureus*, many studies have reported on the contamination of various items of hospital clinical equipment and air in ward with patients having Staphylococcal infection (Griffiths et al., 2002; Sexton et al., 2006) but there is less attention on the likelihood of transmitting Staphylococcal infection through the currency note especially within the hospital in the study area.

S. aureus is a notable nosocomial pathogen (Adegoke and Komolafe, 2009) to watch out for as it is found as skin or nasal flora (Adegoke and Komolafe, 2008), So, when this bacterium gets transferred to an immunocompromised individual with breached skin, any superficial and even systemic infection may result. The question now is what happens if the *S. aureus* in question is resistant to the antibiotic meant for the therapy. With the high profile of resistance of many isolates of

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S. aureus to cloxacillin (Frank et al., 2009), and many other antibiotics, public health surveillance on the pathogens they harbor is a proper step. Such resistance may be plasmid based or chromosomal. This research was aimed at studying the effect of vancomycin to plasmid mediated multidrug resistant *S. aureus* from currency notes obtained within the hospital environment.

MATERIALS AND METHODS

Source of samples

The rationale for chosen University of Uyo Teaching Hospitals (UUTHC) as the study site informed by the location of the hospital on the outskirt of Uyo Metropolis, as currency holding within the hospital will be higher than when it is on town. The naira notes of all denominations ranging from the smallest to other denominations were collected from seven different sections of hospitals around Uyo. The seven sections included: Hospital theatre ward (HTW), Hospital pharmacy (HPH), Hospital provision shop (HPS), Hospital female ward (HFW), Hospital male orthopedic ward (HMAW), Hospital female orthopedic ward (HFAW) and Sick children's ward (SCW). In these wards, thirty-two samples were collected.

Sampling period

The period of sample collection lasted for one week. Each of the sterile sample bottles used were properly labeled according to the ward where samples were collected. The samples were all transported to the microbiology laboratory for immediate analysis.

Processing of samples

Each of Naira notes was individually soaked in about 5 ml sterile distilled water and then swabbed with sterile cotton tipped applicator. After thorough swabbing to ensure that the microbial content was dislodged into the water, the already swabbed notes were kept separately and the water used for swabbing the naira notes was inoculated into already prepared double strength nutrient broth in McCartney bottles using sterile Pasteur pipette and incubated for 18 h at 36°C to keep the organisms at log phase. After overnight incubation, the broth tubes were examined and all turbid tubes showing microbial growth, and others with powdery deposits and uniform turbidity indicating microbial growth were subcultured to freshly prepared mannitol salt agar plate using the streak plate method, incubated at 37°C for 24 h. A golden opaque yellow, with mannitol fermentation, Gram positive cocci in clusters, catalase positive and coagulase positive isolates were identified as *S. aureus*, just as other organisms were also characterized following the schemes of Cowan (1985), Holt et al. (1994) and Cheesbrough (2006). The identified isolates were used for the next stage of analysis.

Antibiotics sensitivity testing

This test is done to note the pattern of sensitivity or the antibiotics an organism is sensitive or resistant to in order to know the treatment to administer (Talaro and Talaro, 2002). The degree of resistance or susceptibility is indicated by clear zones around the sensitivity disc and is measured to the nearest millimeter. The antibiotics disc used were Gram positive multi disc (Abtek Biological Ltd. Liverpool, England) and vancomycin single disc (Oxoid).

Standardization of inoculum

Four pure colonies of each isolate on a 24 h plate culture were randomly selected and inoculated into 2 ml of sterile peptone water broth in bijou bottles. This was incubated at 37°C for 6 h and the turbidity was adjusted by serial dilution in phosphate buffer saline (pH 7. 2) to match an opacity tube containing 0. 5 ml of 1% barium chloride in 1% suplhuric acid (a Mc Farlands 0.5 barium sulphate standard containing 10^5 cfu/ml of the inoculums). One milliliter of the culture dilution (bacteria suspension) was transferred into a well dried surface of diagnostic sensitivity test agar (DST) medium and titled to spread evenly over the entire surface of the agar plate. The excess fluid was drained off and dried within 5 mins; multi-antibiotic discs were then placed on the surface of the inoculated plate and incubated aerobically at 37°C for 18 to 24 h (over-night). *S. aureus* NCTC 6751 was used as control organisms for the sensitivity test. The diameter of the zone of inhibition was measured in millimeter. The result of each antimicrobial agent tested was reported as susceptible or resistant when the test organism was compared with the control. Zone diameter equal to wider than or not more than 6 mm smaller than the control was regarded as sensitive. No zone of inhibition or a zone diameter of 12 mm or less was regarded as resistant (Komolafe and Adegoke, 2008).

Beta-lactamase test

Based on the resistance of the *S. aureus* isolate to penicillin and other beta- lactam antibiotics used, beta-lactamase test was carried out using the method of Odugbemi et al. (1977). Strips of starch paper about 4 to 7 cm were cut and sterilized using 70% ethanol, the strips were soaked for 10 min in benzyl penicillin dissolved in phosphate buffer with 100,000 units. The cut strips were then spread evenly on Petri dishes and about 18 to 24 h old cultures grown on Nutrient Agar were inoculated on the surface of the test paper and spread over an area of 3 mm. The Petri dishes were incubated at 37°C for 30 min, Gram's iodine solution was used to flood the plate and drained off immediately. The starch paper turns uniformly black within 30 s of application: Colonies with decolourized zones are positive for beta-lactamase but colonies with black background are beta-lactamase negative. The result was read 5 mins to avoid false positive result.

Plasmid curing

Generally, it has been known that extra chromosomal DNA called plasmids is capable of conferring resistance of microorganisms to drugs (Talaro and Talaro, 2002). Based on this: plasmid curing to presumptively link the observed resistance to vancomycin and other beta-lactam antibiotics on the multi discs used was carried out (Silhavy et al., 1984). A measure of 0.1 mg/ml of acridine orange was supplemented in freshly prepared nutrient broth. The isolates from the stock were inoculated into the broth and incubated for 24 h. Using the overnight culture, antibiotic sensitivity tests were carried out and allowed for 18 to 24 h after which observation of clear zones (indicating susceptible) was made in a formerly resistant strain which was indicative of plasmid mediated resistance.

RESULTS

Proximate microbial composition of samples

Microbial analysis of sample from the seven hospital sections (Hospital theatre ward, hospital pharmacy,

Table 1. Proximate microbial composition of samples.

Hospital sections	Number of samples from hospital sections	Number of samples with *S. aureus*	Percentage *S. aureus* (%)	Other suspected organism
HTW	10	5	15.6	*E*
HPH	2	1	3.1	*E c*
HFAW	2	2	6.25	-
HMAW	7	2	6.25	*Pa*
SCW	9	1	3.1	*S e*
HPS	1	1	3.1	-
HFW	1	1	3.1	-
Total	32	13	40.6	

% Occurrence of *Staphylococcus aureus* = 40.6%, E=*Enterococccus* spp., Ec=*Escherichia coli*, Pa=*Pseudomonas aeruginosa*, Se=*Staphylococcus* epidermidis, HTW–Hospital Theatre Ward, HPH- Hospital Pharmacy, HFAW -Hospital Female Orthopaedic Ward, HMAW- Hospital Male Orthopaedic Ward, SCW - Sick Children's Ward, HPS- Hospital Provision Shop, HFW- Hospital Female Ward.

Table 2. Physical Characteristics of *S. aureus*.

Properties	Appearance	Identification
Growth on nutrient agar	Creamy glistering colonies	*Staphylococcus aureus*
Colour	Golden yellow	*Staphylococcus aureus*
Turbidity	Opaque	*Staphylococcus aureus*
Diameter/size	1-3 mm	*Staphylococcus aureus*
Surface	Smooth	*Staphylococcus aureus*
Edge	Raised	*Staphylococcus aureus*
Emulsifiability	Easily emulsifiable	*Staphylococcus aureus*
Growth on mannitol salt agar	Golden yellow	*Staphylococcus aureus*
Growth on blood agar	Shiny milky colonies with β haemolysis	*Staphylococcus aureus*
Shape	Spherical grapelike clusters	*Staphylococcus aureus*
Growth on nutrient broth	Uniformly turbid with powdery deposit	*Staphylococcus aureus*

hospital female orthopaedic ward, hospital male orthopaedic ward, sick children's ward, hospital provision shop and hospital female ward) in Naira notes revealed a variety of organisms which includes *Enterococcus* spp, *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus epidermidis* and *S. aureu*s. The *S. aureu*s were present in about 40% of the total sample (Table 1).

Characteristics of the *S. aureus*

The physical, morphological and biochemical characteristics of *S. aureus* are shown in Table 2 and sample of the characterization detail in Table 3.

In vitro antibiotic sensitivity test

The test analysis showed that the nosocomial strains of *S. aureus* were resistant to most conventional multi and single disc antibiotics on Gram positive Abtec multidisc antibiotics containing eight antibiotics – erythromycin, tetracycline, chloramphenicol, penicillin, ampicillin, gentamycin, cloxacillin and streptomycin and single disc of vancomycin antibiotics used. The resistance, susceptibility and intermediate pattern of response of the organism were noted and is hereby reported (Table 4) where the organisms were resistant to a large number of drugs about 81.7% and had intermediate of about 7.7% and few susceptible about 10.6%. The ratio of resistant vancomycin to susceptible ones were 79.9:23.1 where 76.9 and 23.1% were the percentage of resistance and susceptible isolates to vancomycin respectively (Table 5).

Beta-lactamase test

The test for the production of beta-lactamase in the bacterial population was positive due to the decolourised zones on the starch iodide paper. The positive beta lactamase production was discovered from isolates

Table 3. Morphological and biochemical characterization of *S. aureus*.

Hospital sections	Shape	Gram reaction	Motility	Growth in air	Anaerobic growth	Catalase test	Coagulase test	Glucose	Lactose	Maltose	Mannitol	Fructose	Sucrose	Xylose	Rafinose	Mannose	Phosphatase	Cellulose	Arginine	Protease	Oxidase	Growth in NaCl	Growth at 37°C	Indole	α Toxin causing Beta haemolysis	Pigmentation	Beta-lactamase test	Identification
HTW1	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HTW2	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HTW3	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HTW4	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HTW5	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HPH	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HFAW1	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HFAW2	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
SCW	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HFW	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HMAW1	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HMAW2	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*
HPS	S	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	+	+	-	β	+	+	*Staphylococcus aureus*

HTW-Hospital Theatre ward, HPH-Hospital Pharmacy, HFAW -Hospital Female orthopaedic ward, SCW-Sick Children's Ward, HFW-Hospital Female Ward, HMAW-Hospital male orthopaedic ward, HPS-Hospital Provision Shop, β-Beta-haemolysis, S-Spherical / Cocci, + means positive, - means negative.

showing resistance to vancomycin, erythromycin, tetracycline, chloramphenicol, penicillin, ampicillin, gentamycin, cloxacillin and streptomycin (Table 7).

Plasmid curing

The results of plasmid curing that were carried out to verify the relationship between plasmids of *S. aureus* and its resistance to multiple antibiotics (along with beta lactamase production) revealed that a considerable number of originally resistant strain became susceptible, after curing, to both the multi disc antibiotics and single disc (Table 6 and 7).

Plasmid curing and vancomycin resistance in *S. aureus*

The results of the plasmid curing of the ten VRSA revealed 50% curing rate and the 5 previously resistant isolates of *S. aureus* became susceptible. Therefore, the ratio of resistance to susceptible was equal 50:50 (Table 7).

DISCUSSION

The proximate bacterial composition of the sample revealed that the currency notes had the following organisms: *Enterococcus* spp. (50.0%), *E. coli* (50.0%), *P. aeruginosa* (28.6%), *S. epidermidis* (11.1%) from HTW, HPH, HMAW and SCW location within the hospital respectively while 40.6% in all had *S. aureus*. Various bacteria from the notes might be as a result of shedding from various infections being suffered by the patients from which the money samples were collected; that is, the patients might have contaminated the samples with the isolates. This observation was in line with the observation of Lamichhane et al. (2009). The bacterial species isolated here have been implicated in many life-threatening infections. *E. coli* is often associated with gastroenteritis (2010), *P. aeruginosa* and *S. epidermidis* have been implicated in wound

Table 4. Antibiotic sensitivity testing using Gram-positive multi disc.

Hospital sections	CXC (5 µg)	ERY (5 µg)	GEN (10 µg)	PEN (11 µg)	STR (10 µg)	TET (10 µg)	AMP (10 µg)	CHL (10 µg)	No of drug resist	No of drug intermediate	No of drug susceptible
HTW1	R	R	R	R	R	R	R	R	8	0	0
HTW2	R	R	S	R	I	R	R	R	6	1	1
HTW3	R	S	S	R	R	I	R	I	4	2	2
HTW4	R	R	I	R	R	R	R	R	7	1	0
HTW5	R	R	R	R	R	R	R	R	8	0	0
HPH	R	R	R	R	R	R	R	R	8	0	0
HFAW1	R	R	R	R	I	R	R	R	7	1	0
HFAW2	R	R	S	R	R	R	R	R	7	0	1
SCW	R	R	I	R	R	R	R	R	7	1	0
HFW	R	R	S	R	R	R	R	R	7	0	1
HMAW1	R	R	I	R	S	S	R	R	5	1	2
HMAW2	R	R	S	R	S	R	R	R	6	0	2
HPS	R	S	I	R	S	R	R	R	5	1	2
									85	8	11

HTW-Hospital Theatre Ward, HPH-Hospital Pharmacy, HFAW- Hospital Female orthopaedic ward, SCW-Sick Children's ward, HFW-Hospital female ward, HMAW- Hospital male orthopaedic ward, HPS -Hospital Provision Shop, S-Susceptible, R-Resistant, I-Intermediate.

sepsis by a number of researchers (Bahar et al., 2010; Bowler et al., 2001; Lilani et al., 2005; Reichman and Greenberg, 2009). *S. aureus* which had highest occurrence has been recognized for cross implication in various types of infections. High level of antibiotic resistance was observed by the *S. aureus* isolates. The organisms were resistant to 81.7% of the antibiotics, had intermediate profile to 7.7% and few susceptibles to about 10.6% antibiotics. The intermediate ones depend on the concentration of the drug to specify if the organisms will be susceptible or resistant. A little increase in the concentration might make the organisms sensitive. The *S. aureus* obviously exhibited resistance to multiple antibiotics and 76.9% resistance to vancomycin that was often considered as last line of defense was isolated. Infections from this kind of organisms might be difficult to treat (el-Baz et al., 1987; William, 2005). The growth of *S. aureus* at about 4 mg/ml vancomycin shows that it could be classified as VRSA under the guidelines set out by the national committee for clinical laboratory standard (Cui and Hiramatsu, 2003). Ten isolates from different hospital sections could be placed in this category.

Conscientiously, the administration of sub-lethal concentration of acridine orange inhibits plasmid replication in the offspring without affecting the bacterial chromosome (Mesasa et al., 2004). After treatment with appropriate concentration of acridine orange (0. 1 mg/ml), it was seen that five strains from HTW1, HTW4, HPH, SCW and HMAW contained plasmid DNA and those not treated with acridine orange grew in the presence of vancomycin. The acridine orange eliminate the plasmid DNA in the bacterial cell making them unable to grow in the presence of vancomycin antibiotic. It was shown that the group whose plasmid was cured later resembled the three previous vancomycin sensitive *S. aureus* (VSSA) strain from HFAW1, HMAW1 and HPS. The quality control measures for curing experiments basically in concentration and time of observation have been recommended (Jochen, and Bernd, 1986)

Meanwhile, the acquisition of plasmid DNA by the VRSA might be due to interspecific transfer from vancomycin –resistant enterococcus (Krzysztof et al., 1999) but for the isolates from HTW2, HTW3 and HTW5, HFAW2 and HFW, their resistance might be a result of a thicked cell wall which allows the bacteria to inhibit vancomycin molecules from reaching its major target on the cytoplasmic membrane of the cell

Table 5. Antibiotic sensitivity testing using vancomycin single disc.

Hospital sections	Vancomycin
HTW1	R
HTW2	R
HTW3	R
HTW4	R
HTW5	R
HPH	R
HFAW1	S
HFAW2	R
SCW	R
HFW	R
HMAW1	S
HMAW2	R
HPS	S

HTW-Hospital theatre ward, HPH-Hospital Pharmacy, HFAW1-Hospital female orthopaedic ward, HMAW-Hospital male orthopaedic ward, SCW -Sick Children's Ward, HFW-Hospital Female ward, HPS-Hospital provision shop, R-Resistance, S-Sensitive.

Table 6. Antibiotic sensitivity test using multidisc after plasmid curing.

Hospital sections	CXC (5 µg)	ERY (5 µg)	Gen (10 µg)	Pen (11 µg)	Str (10 µg)	TET (10 µg)	Amp (10 µg)	CHL (10 µg)	No of drug resist	No of drug intermediate	No of drug susceptible
HTW1	R	R	R	R	S	R	R	R	7	0	1
HTW4	R	R	S	R	R	R	R	R	7	0	1
HTW5	R	I	S	R	S	S	R	S	3	1	4
HPH	R	S	S	R	S	S	R	S	3	0	5
HMAW	R	R	S	R	S	R	R	R	6	0	2
									26	1	13

HTW-Hospital Theatre ward, HPH-Hospital Pharmacy, HMAW2 - Hospital Male Orthopaedic ward 2, R -Resistance- (65%), S-Susceptible- (32.5%), I-Intermediate- (2.5%), Total number of drug for the five sections = 40.

Table 7. Antibiotic sensitivity testing of *S. aureus* to vancomycin after plasmid curing.

Hospital sections	β-Lactamase production	Vancomycin status
HTW1	+	VSSA
HTW2	+	VRSA
HTW3	+	VRSA
HTW4	+	VSSA
HTW5	+	VRSA
HPH	+	VSSA
HFAW2	+	VRSA
SCW	+	VSSA
HFW	+	VRSA
HMAW2	+	VSSA

HTW-Hospital Theatre ward, HPH - Hospital Pharmacy, HFAW2 - Hospital Female orthopaedic ward 2, SCW-Sick Children Ward, HFW-Hospital Female Ward, HMAW - Hospital male Orthopaedic ward 2, Percentage resistance and susceptibility = 50:50, R-Resistance, S-Susceptibles.

(Cui et al., 2006). By and large, antibiotic use in clinical, veterinary and agricultural practices has been presumed as the major selective force for the acquisition of resistance (Bonomo and Rossolini, 2008). Antibiotic abuse should be discouraged while currency notes should also be handled with care to prevent it from being the vehicle for infection transfer.

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