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Plasmid DNA mediated vancomycin-resistant *Staphylococcus aureus* (VRSA) from cases of urinary tract infection

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Abstract
Vancomycin resistance commonly occurs with *Staphylococcus aureus*, a pathogen that exhibits intrinsic virulence. Sixty isolates of *Staphylococcus aureus* tested positive as vancomycin possessive isolates on Brain Heart Infusion Agar medium fortified with 6 µg/mL vancomycin from 100 clinical samples of urine from patients with cases of UTIs via agar diffusion method. Standard 30 µg vancomycin disc served as control. Increase in zones of growth inhibition in relation to vancomycin concentrations was noticed in some of the isolates while it was reversed in others, despite the increase in concentration. Typed strain was susceptible to six concentrations of vancomycin exposed and to the control. Thirty one of the sixty isolates were resistant to vancomycin control. Resistant isolates from standard vancomycin discs were then subjected to molecular investigation. Of the 31 isolates exposed to gel electrophoresis, 14 (47%)elicited plasmids of varied molecular weights ranging from 0.79-23.13 kb. The magnitudes of vancomycin-resistant isolates from the clinical samples studied, coupled with some incoherent zones of inhibition and the plasmid DNA obtained from the resistant isolates, suggest the need for infection control practitioners and epidemiologist to devise strategies to curtail the spread of this pathogen both in hospital and community settings.

Keywords: Urinary tract infection, Vancomycin-resistant *Staphylococcus aureus* (VRSA)*;* Plasmid DNA

INTRODUCTION

Urinary tract infections (UTIs) are infection that happen when microbes, often from the skin or rectum, enter the urethra, and infect the urinary tract. UTIs are characterized by frequent urination, pain with urination, feeling the need to urinate despite having empty bladder and discomfort. This type of infection involves urethra; the lower urinary tract (a condition called urethritis), kidneys;

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the upper urinary tract (a condition called pyelonephritis or bladder, (a condition called cystitis [1]. This disease has a threatening impact on people's lives and economic values, and it has been recognized to be a global problem. UTIs are the second most common cause of human illness and are responsible for more than 1 million emergency room visits and frequent hospitalizations in the United States each year. UTIs are predicted to affect 150 million individuals worldwide each year and the cost of healthcare in the United States is estimated to be $6 billion [2].

Gram-negative bacteria, accounts for 90% of UTIs, and predominantly *Escherichia coli* is the most prevalent cause of UTIs. *Pseudomonas aeruginosa*, *Klebsiella* species, *Citrobacter* species and Coagulase-negative *Staphylococci* (CoNS) are among the other uropathogens that causes UTIs [3]. Urinary tract infections caused by *Staphylococcus aureus* are rather rare. However, in certain individuals, *Staphylococcus aureus* produces ascending urinary tract colonization and infection, which is commonly subsequent to staphylococcal bacteremia originating from (e.g. instances of endocarditis), and indwelling catheters and other urinary tract instruments that increase the risk of *Staphylococcus aureus* transmission [4].

UTIs can be community or nosocomially acquired. A community-acquired urinary tract infection (CA-UTI), occurs in the community after less than 48 hours of hospitalization while nosocomially acquired urinary tract infections (N-UTI) emerge 48 hours after hospital admission or three days after release [5]. UTI prevalence is influenced by age, gender, catheterization, inpatient treatment, and long-term use of antimicrobials. *Staphylococcus aureus* the most common species of Staphylococci to cause staphylococcal infections and is a successful pathogen with regard to a combination of nasal carriage and immuno-evasive strategies. It has been increasingly recognized that different determinants have a unique role in the predilection of *Staphylococcus aureus* for establishing infection at particular sites [6].

The greatest challenge to the treatment of *Staphylococcus aureus* infection is in the selection of the appropriate therapeutic agent. This is because the pathogens have the potentials of developing resistance to almost all classes of antibiotics. Due to *Staphylococcus aureus* aggressive nature and the limited treatment options for MRSA infections, the development of VRSA and ability of these microorganisms to spread from one patient to another with ease, this poses a substantial threat to public health [7].

Vancomycin is an antibacterial medication in the glycopeptides class. Vancomycin binds to the acyl-D-ala-D-ala portion of the growing peptidoglycan cell wall, which is a group of amino acids. By binding, multiple mechanisms of action begin to take place resulting in bacterial inhibition. The first Vancomycin-Resistant *Staphylococcus aureus* (VRSA) strain was isolated in a Michigan hospital in 2002. After discovering this upsurge, infectious disease researchers began to explore the substance that could combat the new trend of resistance phenomenon. Vancomycin can be used for therapeutic management of bone and joint infection, skin and soft tissue infection, infective endocarditis, community and hospital acquired pneumonia including ventilator associated pneumonia. Contraindication of this antibiotics includes, hearing loss, reduced kidney function, systemic mastocytosis and low level of neutrophils [8].

Plasmids are extra chromosomal strands of DNA that are capable of self-replication, and may code for antibiotic resistance. Plasmid can be transferred from an antibiotic-resistant strain to antibiotic sensitive strain, thus making a sensitive strain resistant to the antibiotic. Plasmids usually occur in bacteria and vary in molecular sizes. The

number of identical plasmids within a single cell can be zero, one, or even thousands under some circumstances. Plasmids may contain resistant genes for single or multiple antimicrobial agents and these resistant plasmid genes are transferable from one bacterium to another [9]. And in many cases, resistance to several therapeutically useful antibiotics, host specificity, and pathogenicity in *Staphylococcus aureus* has also been due to plasmids that carry genetic determinants of resistance [10].

This study was carried out to examine the antimicrobial activity of vancomycin on isolates of *Staphylococcus aureus* from cases of urinary tract infections and the relation between resistance and plasmid DNA observed.

EXPERIMENTAL METHODS

Collection of samples. Mid-stream urine samples were collected from a total of one hundred patients visiting Saint Mary Catholic Hospital, Eleta Ibadan for treatment. The samples were collected with sterile universal bottles and processed for *Staphylococcus aureus.*

Bacteriological analysis. The samples of urine collected were cultured on MacConkey agar and Blood agar media separately to multiply the chances of getting *Staphylococcus aureus* isolates. The colonies observed after 24 hours of incubation at 37^{o}C were morphologically selected and further subculture to mannitol salt agar, followed by biochemical tested that are conventional to the identification of *Staphylococcus aureus* which includes; catalase test, coagulase test, DNase on DNase agar medium, gelatin hydrolysis and oxidase test. The isolates of *Staphylococcus aureus* identified were subculture on to commercially prepared Brain Heart Infusion Agar fortified with 6 µg/ml vancomycin and thereafter incubated at 37^{o}C for 24 hours, colonies obtained were used for the determination of the antibiogram [11].

Determination of antibiogram.

Disc diffusion method. Antimicrobial susceptibility profiles of the isolates of *S. aureus* were determined using the agar diffusion method. Of 0.0016 g from 500 mg vancomycin powder vial was weighed into 5 mL of sterile distilled water to obtain a stock concentration of 320 µg/mL, the stock was serially diluted into 160 µg/mL, 80 µg/mL, 40 µg/mL, 20 µg/mL, and 10 µg/mL in Bijou bottles. A volume of 0.1 mL(100 µL) from the prepared concentrations was pipetted into the surface of antibiotic-free paper discs (6 mm) to obtain different concentrations of vancomycin range from 32 µg/L, 16 µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, and 1 µg/mL and were allowed to dry for 24 hours in a receptacle void of contamination. Each impregnated disc was carefully impressed at equidistant on sterile set Mueller Hinton agar medium already inoculated with the isolates of *S. aureus*, followed by incubation at 37^{o}C for 24 hours. The zones of growth inhibition were measured and interpreted according to CLSI (2006).

Plasmid DNA extraction. The pure isolates of *Staphylococcus aureus* obtained from the stock were subculture into nutrient broth and incubated for 24 hours, the culture of the bacteria cells was thereafter transfer into Eppendorf tubes centrifuged at 13,000rpm for 2 minutes after which supernatants were discarded. The pellet was suspended in the remaining broth by vortexing at high speed. The suspended pellet was treated as follows; 300µL of TENS (Tris25mM, EDTA 10mM, NaOH 0.1N and SDS 0.5 %) solution was added and mixed gently by inverting tubes until the solution becomes slimy. A volume of 150 µL of 3.0 M sodium acetate (pH 5.2) was vortexed for about 10 seconds and the mixture was centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred into another 1.5 mL Eppendorf tube and 900 µL of ice-cold absolute ethanol was added. This was vortexed and centrifuged at 13,000 rpm for 10 minutes after vortexing and centrifugation, the

supernatant was discarded and a white pellet was observed. 1000 µL (1 mL) of ice-cold 70% ethanol was added to the observed white pellet and centrifuge at 13,000 rpm for 5 minutes without vortexing. The supernatant was discarded and the pellets were totally air dried. The dried pellet was suspended in 40 µL of TE buffer (Tris 10 mM, 1 mM Na_2EDTA).

Gel electrophoresis of the suspended pellet

The agarose powder (0.8%) in x0.5 TBE buffer (Tris-borate, Na_2EDTA) was dissolved by boiling. This was allowed to cool to about 60^{o}C before adding 10 µL of ethidium bromide (1 mg/mL). After gentle swirling, it was poured into electrophoresis tank and comb inserted. After solidification (gelling) the comb was removed and the gel totally submerged in x 0.5 TBE buffer. The sample (suspended pellet, 15 µl) was mixed with 2 µL of loading dye and carefully loaded into the wells created by the combs alongside 100 bp DNA ladder. This set up was connected to power pack and run at 100 V for 45 minutes. The gel was thereafter observed in gel photo documentation systems [12] to read the plasmid bands waves.

Ethical consideration. Ethical approval with reference number AD 13/479/ 44755^B was obtained from the Department of Planning Research and Statistic Division, Oyo State Ministry of Health before embarking on this study. The purpose of the study was disclosed to concerned office ahead of sample collection.

RESULTS

Table 1 shows the distribution patterns of clinical sample in respect to age and gender. Table 2 shows the zones of growth inhibition of the isolates of *Staphylococcus aureus* to varied concentration of vancomycin and 30µg vancomycin disc control. The zones of growth inhibition observed varied according to concentrations of vancomycin prepared and in comparison with standard vancomycin disc.(CLSI Breakpoint for standard disk ≥16 (susceptible). Thirty(30) of the 60 isolates *Sa* (1, 2, 3, 7, 8, 10, 14, 15, 16, 17, 18, 19, 20, 24, 25, 28, 29, 30, 31, 32, 35, 39, 40, 41, 43, 44, 46, 53, 57 and 59) of *Staphylococcus aureus* examined exhibited resistance to standard 30 µg vancomycin disc exposed.

Figure 1 shows elicited plasmid DNA that ranged from, 0.25kb and 23.13 kb molecular weights from 14 of the isolates of *Staphylococcus aureus*(1, 2, 3, 8, 10, 14, 15, 16, 17, 18, 19, 25) exposed based on the resistance to vancomycin disc as shown in Figure 1.

Of the 15 isolated exposed to plasmid gel electrophoresis, only two (2) isolates, (Sa *(28* and *29*) elicited plasmid bands of low molecular weight of 0.24kb and 0.25kb as shown Figure 2.

Table 1: Age range and distribution of samples collected among the gender studied

Age(range)	Total nos of sample	Male	Female
1-10	8	5	3
11-20	24	10	14
21-30	30	12	18
31-40	12	4	8
41-50	14	6	8
51-60	8	4	4
61 and above	4	2	2
TOTAL	100	43	57

Table 2: Susceptibility of *Staphylococcus aureus* to various concentrations of vancomycin

Isolate number	1 µg	2 µg	4µg	8µg	16µg	32µg	+C (Vancomycin Disc 30µg)	
Sa 1	12	16	20	22	24	30	14	R
Sa 2	16	22	25	28	30	33	14	R
Sa 3	10	14	16	19	22	26	12	R
Sa 4	8	15	20	23	27	30	16	S
Sa 5	11	17	22	27	30	32	16	S
Sa 6	14	17	19	24	30	34	16	S
Sa 7	10	16	20	22	26	30	14	R
Sa 8	22	15	10	24	12	20	12	R
Sa9	10	0	0	0	0	20	16	S
Sa 10	16	24	28	30	0	20	12	R
Sa 11	0	0	0	0	0	20	18	S
Sa 12	0	0	0	0	14	20	20	S
Sa 13	0	0	0	0	0	20	16	S
Sa 14	12	16	20	20	24	30	14	R
Sa 15	16	20	15	18	20	18	15	R
Sa 16	10	14	16	19	22	26	12	R
Sa 17	16	12	24	10	24	36	14	R
Sa 18	12	15	18	23	26	30	14	R
Sa 19	10	14	16	19	22	26	14	R
Sa 20	8	15	20	23	27	30	12	R
Sa 21	11	17	22	27	30	32	16	S
Sa 22	10	14	12	12	10	18	16	S
Sa 23	14	16	24	27	32	35	20	S
Sa24	16	21	16	16	14	20	14	R
Sa25	18	23	25	28	31	20	12	R
Sa 26	10	12	0	0	0	20	16	S
Sa 27	14	16	20	24	29	32	16	S
Sa 28	16	24	27	32	34	20	12	R
Sa 29	13	16	24	28	31	20	14	R
Sa 30	10	16	12	10	20	14	14	R
Sa 31	10	16	20	22	26	20	12	R
Sa 32	12	15	20	24	26	20	12	R
Sa 33	10	0	0	0	0	20	16	S
Sa 34	16	24	28	30	0	20	16	S
Sa 35	10	0	0	0	0	20	10	R
Sa36	0	0	0	0	14	24	22	S
Sa 37	0	0	0	0	0	20	20	S
Sa 38	12	16	20	20	24	30	20	S
Sa 39	16	22	25	28	30	33	14	R
Sa 40	10	14	16	19	22	26	12	R
Sa 41	16	20	24	28	32	36	14	R
Sa42	12	15	18	23	26	30	22	S
Sa 43	12	18	24	26	32	20	14	R
Sa 44	14	18	18	12	18	24	12	R
Sa 45	12	15	20	24	26	30	20	S
Sa 46	10	14	12	12	10	22	14	R
Sa 47	15	20	25	28	32	34	16	S
Sa 48	16	18	14	17	12	25	20	S
Sa 49	10	18	26	31	34	20	16	S
Sa 50	0	0	0	0	0	20	16	S
Sa 51	14	21	26	30	34	36	16	S
Sa 52	10	0	0	0	0	20	16	S
Sa 53	12	18	24	28	32	34	14	R
Sa 54	12	16	20	20	24	30	20	S
Sa 55	16	22	20	18	23	26	16	S
Sa 56	10	14	16	19	22	26	16	S
Sa 57	8	15	20	23	27	30	12	R
Sa 58	11	17	22	27	30	32	16	S
Sa 59	14	17	19	24	30	34	14	R
Sa60	10	16	20	22	26	30	20	S
ATCC Sa 129213	12	15	20	24	26	28	24	S

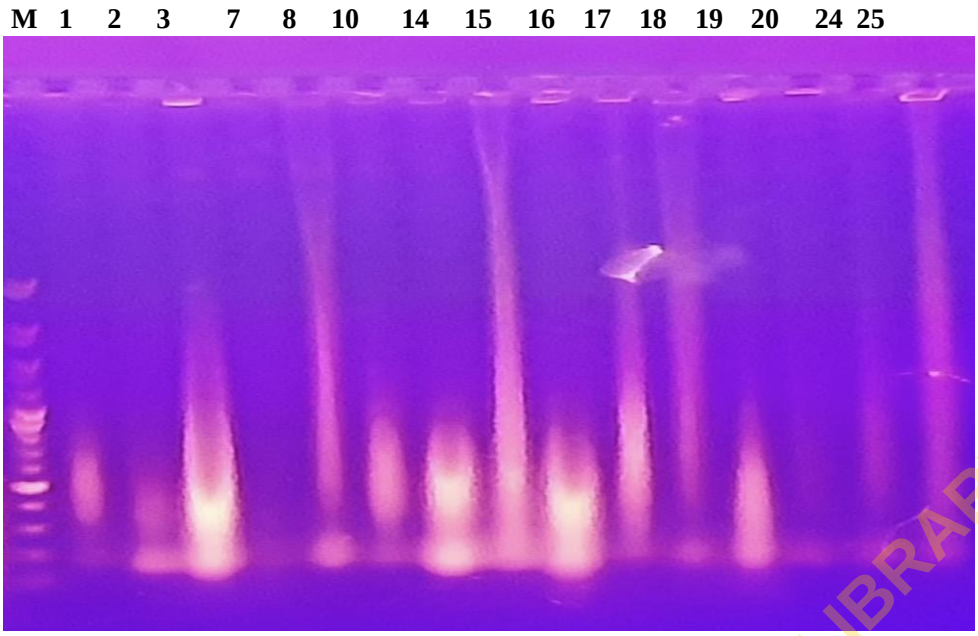

Figure 1: Plasmid DNA profiles from *Staphylococcus aureus*

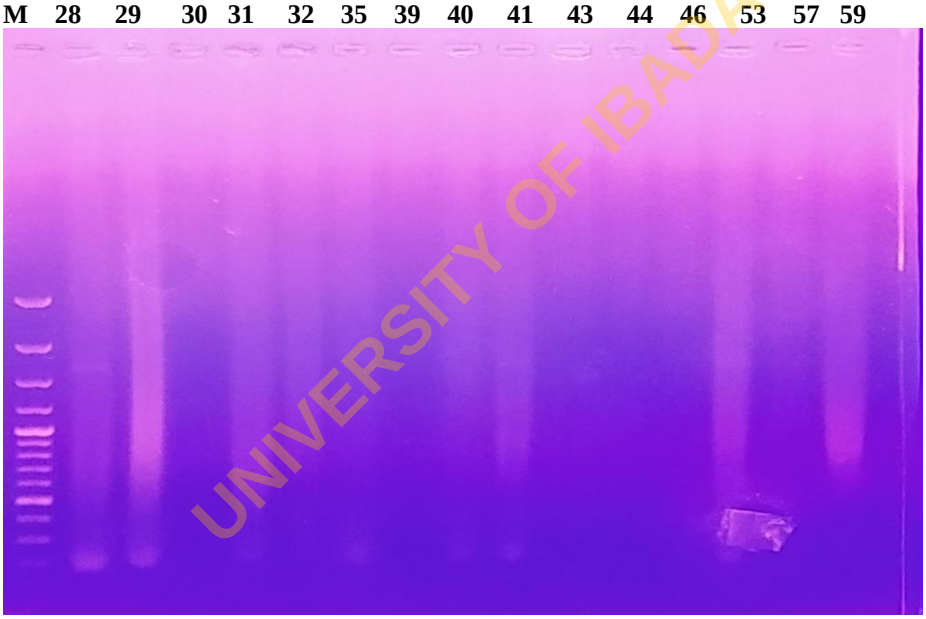

Figure 2: Plasmid DNA profiles from *Staphylococcus aureus*

DISCUSSION

Staphylococcus aureus is a successful pathogen due to potential of toxins armaments and immuno-evasive strategies. It can cause a range of illness of which urinary tract infection is inclusive [13]. One hundred (100) patients within the age of 1 -60 years and above were sampled in this study. The age and gender distribution profiles elicited higher number of female than males in this study, but the highest samples were collected within the age bracket of between 11 to 20 and 21-30 years. These age brackets fall within the sexually active age, which could maximize the chances of UTIs as a results of illicit gender trans-border relationship that makes females easily prone to

this infection due to the closeness of their urethra to the vagina. And also, the distribution patterns recorded in this study could be attributed to inherent predisposing factors among the gender which are considered to be one of the epidemiological factors aiding the spread and control of infection, this corroborate the study of Sammy Selim *et al.* [14] on antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem

During the last ten years, the prevalence of VRSA was alarming, with initial incidence and spike in critical care units, spreading to the hospital then escaping to the rest of the community. Although various genotypes and subtypes of resistance are found among *Staphylococcus aureus*, there is yet to be known uniform mechanisms for vancomycin metabolism[15].Thirty (30) of the 60 isolates *Sa* (*1, 2, 3, 7, 8, 10, 14, 15, 16, 17, 18, 19, 20, 24, 25, 28, 29, 30, 31, 32, 35, 39, 40, 41, 43, 44, 46, 53, 57* and *59*) of *Staphylococcus aureus* examined exhibited resistance to standard 30 µg vancomycin disc exposed. This could be attributed to genetic variation amongst the isolates examined or other cryptic inherent factors in the organisms tested. Increase in zone of growth inhibition in relation to increase in concentration of vancomycin prepared was observed in many of isolates in this study, isolates *Sa* (*11, 12, 13, 35, 36, 37* and *50*) were totally resistant (total growth in the plates) but susceptible to (32 µg) and (30 µg) of vancomycin disc used in this study.

The variation in zones of growth inhibition observed, could be due to strain variation among the isolates or adaptive to selective pressure in local environment. The ratios of resistant and susceptibility patterns to vancomycin disc diffusion, varied from one isolate to the others. Although, disc diffusion test is not used as a determinant or index of vancomycin efficacy in hospital practice, which corroborate the study of Tiwari *et al.* [16] on vancomycin resistant *Staphylococcus aureus* from tertiary care hospital in India.

The zones of growth inhibition observed in some isolates exposed to varied concentrations of vancomycin powder where susceptibility are not relative to increase in concentrations of vancomycin in this study, deviates from the foundational norm of the higher the concentration, the higher the zone of growth inhibition, this was an indication that vancomycin is not concentration dependent but on time, dosage and infectious agent to be treated, which corroborates the finding of a case study of Cong *et al.* [17] on VRSA infection.

Plasmid DNA ranged from, 0.25 kb and 23.13 kb molecular weights were obtained from 14 of the isolates of *Staphylococcus aureus* (*1, 2, 3, 8, 10, 14, 15, 16, 17, 18, 19, 25*) based on the resistance to vancomycin disc as showed in Figure 1, (Sa *28* and *29*) elicited bands of low molecular weight in Figure 2 in this study. The resistant isolates that have no plasmid DNA bands could be attributed to the possibility of their resistance factors not been plasmid borne. The variations in plasmids in terms of plasmid DNA copies and molecular weight observed, agreed with the findings of Faron, *et al.* [18] on resistance mechanisms, epidemiology, and approaches to screening for Vancomycin-Resistant Enterococcus (VRE) in the health care setting. The plasmids isolated in this study were independent of the level of vancomycin concentration and resistance in the isolates of *Staphylococcus aureus* investigated.

Limitation to the study. The bacterial isolates of *Staphylococcus aureus* obtained in the present study could not be compared with the types of urinary tract infection inflaming the patients, this is because patients' details that could reflect the exact clinical or diagnostic data were not provided.

Conclusion. In this study, an account of vancomycin resistant *Staphylococcus aureus*

was established amongst the isolates examined, and sequel to the plasmid DNA of varied molecular weight obtained, suggesting the molecular source of resistance capable of causing therapeutic failures when managed with the such or related antibiotics, there is therefore, need for proper monitoring of antibiotics application and if possible, species dependent therapy should be devised and implemented to block the phenomenon of antibiotic resistance spread.

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