ERRATUM

Molecular Investigation of Clinical Isolates of *Staphylococcus aureus* from Cases of Boil Infection

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ABSTRACT

Background: Boil, a pyodermal infection caused by *Staphylococcus aureus* is a very common skin disease. It is characterized by pus filled lump at specific anatomical loci.
Objectives: This study evaluated the antimicrobial activity of vancomycin powder in varied concentrations alongside with 30µg vancomycin standard discs, determined the minimum inhibitory concentration and plasmid profiles of the resistant isolates of *Staphylococcus aureus* from boil infection.
Methods: A total of one hundred (100) exudates from boils were collected with a sterile swab for bacteriological examination; the samples were culture on mannitol salt agar, followed by Gram staining and other conventional biochemical characterization. The isolates were tested by agar diffusion method against varied concentrations of vancomycin and a standard 30 µg vancomycin disc as a control. The resistant isolates from standard vancomycin disc were subjected to molecular investigation to determine the source of their vancomycin resistance.
Results: Remarkable zones of growth inhibitions to varied concentrations of vancomycin powder that ranged from 32µg/mL-1.0 µg/mL were recorded, although, vancomycin antibiotics are not concentration dependent. The minimum inhibitory concentration for the 60 isolates examined elicited varied values. Of the 60 isolates exposed to plasmid investigation, fourteen(14) elicited resistance that were plasmid mediated which molecular weight ranged from 0.82 kb - 27.22kb.
Conclusion: The resistance of the isolates observed from plasmid patterns with varied molecular weights could aid the transferability of that factor to other related bacteria which could be a threat to therapeutic management of boil infection.

INTRODUCTION

Boils are bumpy, red, pus-filled lumps around a hair follicle that are tender, warm, and very painful. It could appear in various forms that ranges from pea-size to golf ball-sizes.1 A yellow or white point at the center of the lump can be seen when the boil is ready to drain or discharge pus and in a severe infection, an individual may experience fever, lymph node and fatigue [2] Skin infections tend to be recurrent in many patients and often spread to other family members. Systemic factors that lower resistance commonly are detectable, including: diabetes, obesity, and haematologic disorder. Boils can be caused by other skin conditions that cause the person to scratch and damage the skin. A recurring boil is called chronic furunculosis[3]

Boils may appear on the buttocks or near the anus, the back, the neck, the stomach, the chest, the arms or legs, or even in the ear canal. Boils may also appear around the eye, where they are called stye. A boil on the gum is called intra-oral dental sinus or more commonly, a gumboil. The most common complications of boils are scarring or abscesses of the skin,, spinal cord, brain, kidneys, or other organs. Infections may spread to the bloodstream (bacteraemia) and become life-threatening[4]. Boils in the nose or ear can be particularly uncomfortable. As the lump continues to grow the entire abscess eventually softens and becomes filled with pus It may then burst through the surface of the skin, releasing the pus, or disappear gradually without bursting[5].

Staphylococcus aureus is a well known aetiologic agents that causes Staphylococcal infections and is a successful pathogen due to a combination of nasal carriage and bacterial immune-evasive strategies. It can cause a range of illnesses, from minor skin infections such as pimples, impetigo, boils (furuncles), cellulitis, folliculitis, carbuncles, scalded

skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteraemia, and sepsis[6]

Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It has been increasingly recognized that different determinants have a unique role in the predilection of *Staphylococcus aureus* for establishing infection at particular sites. 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[7]. Vancomycin is an antibacterial medication in the glycopeptide 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

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Plasmid can be transferred from antibiotic-resistant strains to antibiotic sensitive strains, thus making a sensitive strain resistant to the antibiotic. Plasmid usually occurs in bacteria and its molecular size varies from 1 to over 200 kilobase pairs. The number of identical plasmid within a single cell can be zero, one, or even thousands under some circumstances.[9] Plasmid may contain resistant genes for single or multiple antimicrobial agents and these resistant plasmid genes are transferable from one bacterium to another. And in many cases, resistant to several therapeutically useful antibiotics[10]. This study was carried out to evaluate the antimicrobial activity of vancomycin on isolates of *Staphylococcus aureus* from cases of boil infection and relates the resistance to plasmid, if present to the DNA observed.

MATERIALS AND METHODS

Collection of samples.

Pus samples from boil infection were collected from a total of one hundred individuals. The samples were collected with sterile cotton swab moistened with 0.1% bacteriological peptone water and processed immediately for the isolation of *Staphylococcus aureus.*

Bacteriological isolation

Each swab specimen was culture on mannitol salt agar, and incubated at 37^{0}C for 24 – 48 hours under aerobic condition. The colonies obtained were Gram stained and other conventional biochemical tests; catalase, coagulase, DNAse, gelatin hydrolysis and haemolysis on blood agar was carried out on the isolates. The isolates of *Staphylococcus aureus* identified were subculture on commercially prepared Brain Heart Infusion Agar fortified with 6µg/ml vancomycin and thereafter incubated at 37^{o}C for 24 hours, colonies obtained were used for the determination of the antibiogram.

Determination of Antibiogram

Disc diffusion technique

Antibiogram of the isolates of *S. aureus* were determined using the agar diffusion technique. 0.0016 g from 500mg vancomycin powder vial was weighed into 5mL 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 sterile universal bottles. A volume of 0.1(100µL) from the prepared concentrations was pipetted into the surface of antibiotic-free paper discs(6mm) to obtain varied concentrations of vancomycin that range from 32µg/mL, 16µg/mL, 8µg/mL,4µg/mL, 2 µg/mL, and 1 µg/mL and were thereafter allowed to dry for 24 hrs in a sterile dessicators. Each impregnated disc was carefully impressed at equidistant on sterile molten and cooled Mueller Hinton agar medium already inoculated with the isolates of *S. aureus,* followed by incubation at 37^{0}C for 24 hrs. The zones of inhibition were measured and interpreted according CLSI (2013)

Determination of Minimum Inhibitory concentration

Agar Dilution methods

The MIC of the vancomycin on the isolates was determined using micro broth dilution method with the aid of 96 well plates. The antibiotic powder were dissolved in double strength Mueller Hinton Broth to obtain a stock solution of 32µg/mL. This was serially diluted in Bijou bottles to obtain a concentrations range of 16, 8, 4, 2, 1µg/mL. Each of the concentration (100µL) was dispensed into the respective well from well 1 to well 9 leaving the last three wells for controls(MHB, MHB plus Vancomycin and MHB with culture). Each of the well with exception of well 10 and 11 were inoculated with 10 µL of the aliquot of microorganisms and incubated at 37^{0}C for 24hrs. After 24 hrs, 10µg of 0.2mg/mL of piodonitrotetrazolium violet was added to all the well. The microtitre plate was incubated for 30 minutes. Well with color change to pink indicative of microbial growth were observed. The least concentration that showed no color change was taken as the minimum

inhibitory concentration.

Plasmid DNA Extraction

The overnight (growing) nutrient broth culture of the bacteria cells (*Staphylococcus aureus*) in Eppendorf tubes were centrifuge 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 150µL of 3.0M sodium acetate (pH 5.2) was vortexed for about 10 seconds the mixture was centrifuged at 13,000rpm for 5 minutes. The supernatant was transferred into another 1.5mL Eppendorf tube and 900µL of ice-cold absolute ethanol was added. This was vortexed and centrifuged at 13,000rpm for 10 minutes thereafter followed by centrifugation, the supernatant was discarded and a white pellet was observed. 1000µL (1mL) of ice-cold 70% ethanol was added to the observed white pellet and centrifuge at 13,000rpm for 5 minutes without vortexing. The supernatant was discarded and the pellets were totally air dried. The dried pellet was suspended in 40µL of TE buffer (Tris 10mM, 1mM Na2 EDTA).

Gel electrophoresis of the suspended pellet

The agarose powder (0.8%) in x0.5 TBE buffer (Tris-borate, Na2 EDTA) was dissolved by boiling. This was allowed to cool to about 60^{0}C before adding 10 µL of ethidium bromide (1mg/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 x0.5 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 100bp 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.

RESULTS

The distribution of clinical samples of boil varied in number. Of the five anatomical loci sampled, nasal passage were recorded to have the highest(15) in female while buttock recorded in males had the lowest(6) as shown in Table 1. The zones of growth inhibition of the isolates of *Staphylococcus aureus* to varied concentration of vancomycin and 30µg vancomycin disc varied in diameter, the higher the concentration the wider the zone of inhibition recorded as shown in Table 2. Minimum inhibitory concentration of vancomycin on an every isolates of *Staphylococcus aureus* evaluated elicited varied susceptibility pattern. The resistant isolates showed varied plasmid DNA bands as elicited in Figure 1.

Table 1: Anatomical loci and Gender Distribution of the clinical sample

Anatomical locales	Female	Male	Total
Elbow	13	10	23
Nasal passage	15	7	22
Armpit	14	5	19
Outer ear	12	8	20
Buttock	10	6	16
Total	64	36	100

Table 2: Susceptibility of *Staphylococcus aureus from boil* to Vancomycin

	32µg1	**6 µg**	**8 µg**	**4 µg**	**2 µg**	**1µg**	**+C (Vancomycin Disc 30µg)**	
Isolates number								
Sa 1	30	26	22	20	16	12	18	S
Sa 2	34	26	22	20	18	16	24	S
Sa 3	20	18	16	15	12	10	20	S
Sa 4	28	26	25	23	20	16	19	S
Sa 5	18	16	15	15	15	12	17	S
Sa 6	18	17	14	12	10	0	19	S
Sa 7	30	26	20	16	14	0	18	S
Sa 8	32	30	21	20	18	0	22	S
Sa9	0	0	24	22	16	10	14	R
Sa 10	26	24	20	18	17	10	18	S
Sa 11	0	0	18	16	14	10	12	R
Sa 12	0	16	10	0	0	0	10	R
Sa 13	20	16	14	12	10	0	14	R
Sa 14	30	18	17	17	16	0	18	S
Sa 15	30	28	22	20	18	0	18	S
Sa 16	38	32	30	22	18	12	22	S
Sa 17	36	30	24	22	20	12	18	S
Sa 18	22	20	18	17	16	10	18	S
Sa 19	28	26	24	20	14	0	22	S
Sa 20	20	14	12	10	10	0	20	S
Sa 21	30	26	24	22	20	16	20	S
Sa 22	17	14	13	12	10	10	18	S
Sa 23	19	16	14	13	12	10	19	S
Sa24	36	31	28	26	22	0	18	S
Sa25	28	26	20	18	17	0	22	S
Sa 26	0	0	16	14	12	10	10	R
Sa 27	14	12	10	10	10	0	20	S
Sa 28	26	24	21	18	15	0	18	S
Sa 29	33	26	24	21	18	0	14	S
Sa 30	30	26	24	21	20	10	18	S
Sa 31	34	32	24	18	10	0	22	S
Sa 32	29	26	24	20	14	0	18	S
Sa 33	0	0	22	20	16	12	16	R
Sa 34	0	20	16	14	12	10	16	R
Sa 35	28	24	20	18	17	12	18	S
Sa36	19	17	16	14	12	0	19	S
Sa 37	24	22	20	16	14	12	10	R
Sa 38	17	16	14	12	10	0	22	S
Sa 39	28	24	18	14	10	0	18	S
Sa 40	38	30	26	18	21	14	20	S
Sa 41	34	30	22	20	18	10	20	S

Sa42	16	14	12	10	0	0	12	R
Sa 43	22	18	18	16	14	0	18	S
Sa 44	34	28	25	22	20	0	24	S
Sa 45	32	28	20	18	16	0	22	S
Sa 46	30	20	18	14	10	10	18	S
Sa 47	35	30	26	24	20	14	20	S
Sa 48	26	18	14	12	10	0	20	S
Sa 49	20	18	16	10	10	0	20	S
Sa 50	0	0	20	16	14	12	14	R
Sa 51	18	17	16	14	14	0	21	S
Sa 52	10	0	0	16	13	101	0	R
Sa 53	21	81	41	31	20	0	18	S
Sa 54	28	25	20	17	16	10	14	R
Sa 55	28	18	14	12	12	0	22	S
Sa 56	32	26	23	20	18	10	18	S
Sa 57	36	30	24	18	14	0	18	S
Sa 58	0	16	10	0	12	10	10	R
Sa 59	36	31	24	20	16	0	18	S
Sa60	24	18	14	10	0	0	10	R
ATCC Sa 129213	28	26	24	20	18	0	22	S

Table 3: Determination of Minimum Inhibitory Concentration of Vancomycin on test isolates.

Isolates	**Concentration in g/Ml**			
	MIC	**-C1**	**-C2**	**-C3**
Sa 1	2	-	-	+
Sa 2	1	-	-	+
Sa 3	8	-	-	+
Sa 4	1	-	-	+
Sa 5	16	-	-	+
Sa 6	8	-	-	+
Sa 7	2	-	-	+
Sa 8	4	-	-	+
Sa9	>16	-	-	+
Sa 10	8	-	-	+
Sa 11	16	-	-	+
Sa 12	>16	-		+
Sa 13	> 16	-	-	+
Sa 14	8	-	-	+
Sa 15	4	-	-	+
Sa 16	4	-	-	+
Sa 17	2	-	-	+
Sa 18	4	-	-	+
Sa 19	2	-	-	+
Sa 20	16	-	-	+
Sa 21	4	-	-	+
Sa 22	4	-	-	+

Sa 23	4	-		+
Sa24	2	-	-	+
Sa25	2	-	-	+
Sa 26	> 16	-	-	+
Sa 27	> 16	-	-	+
Sa 28	8	-	-	+
Sa 29	4	-	-	+
Sa 30	2	-	-	+
Sa 31	4	-	-	+
Sa 32	2	-	-	+
Sa 33	4	-	-	+
Sa 34	16	-	-	+
Sa 35	4	-	-	+
Sa36	2	-	-	+
Sa 37	16	-	-	+
Sa 38	> 16	-	-	+
Sa 39	4	-	-	+
Sa 40	1	-	-	+
Sa 41	2	-		+
Sa42	>16	-	-	+
Sa 43	4	-	-	+
Sa 44	4	-	-	+
Sa 45	2	-	-	+
Sa 46	2	-	-	+
Sa 47	4	-	-	+
Sa 48	16	-	-	+
Sa 49	16	-	-	+
Sa 50	> 16	-	-	+
Sa 51	8	-	-	+
Sa 52	> 16	-		+
Sa 53	16	-	-	+
Sa 54	4	-	-	+
Sa 55	8	-	-	+
Sa 56	2	-	-	+
Sa 57	4	-	-	+
Sa 58	> 16	-	-	+
Sa 59	4	-	-	+
Sa60	>16	-	-	+
ATCC Sa 29213	>16	-	-	+

Key: C1: Mueller Hinton Broth, C2:Mueller Hinton Broth plus Vancomycin, C3: Mueller Hinton Broth with culture

L-R: BP LADDER (M), LANES (9-60)

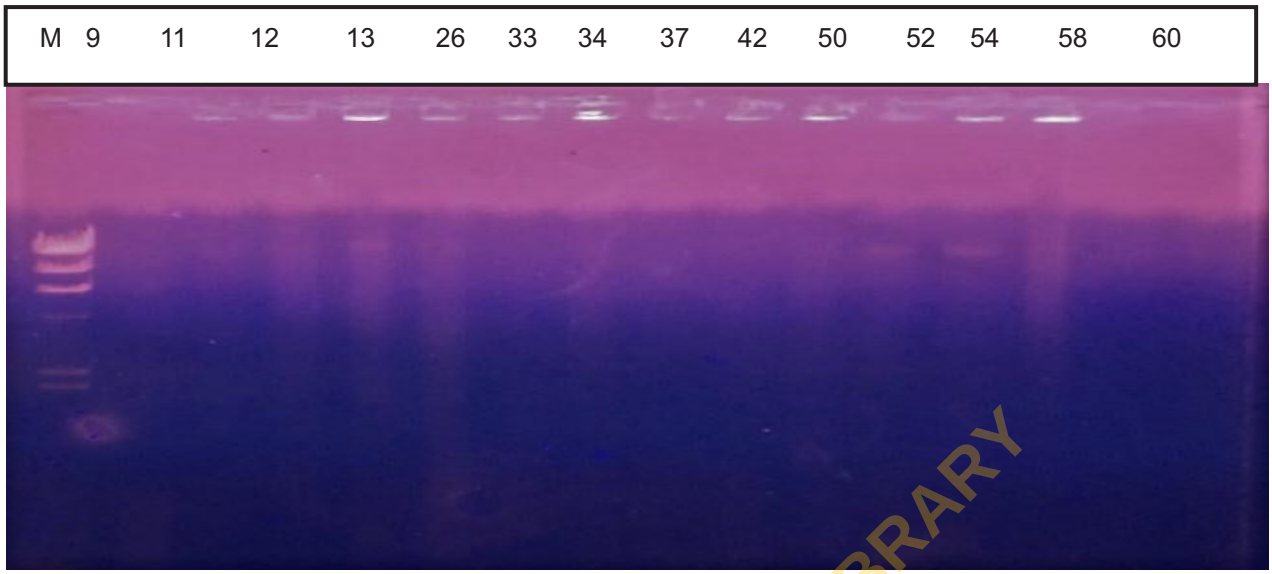

Figure 1: Plasmid DNA profiles from *Staphylococcus aureus*

DISCUSSION

A total of 100 boil samples were collected, the distribution ratio 64 females to 36 males were recorded, this variation in gender distribution observed could be attributed to the feminine care attitudes to their skin and cultural orientation. It has been recognized also, that people with hyperacious sebaceous gland secretion harbor more lipid materials on their body than those without due to their genetic make-up. Hence, the predisposition of the former to attract microbes-laden particles into their body that could give rise to development of boil[11]
In the pathological distribution of the isolates, *Staphylococcus aureus* were recorded to have the highest number from the nasal passage, followed by armpits and every other anatomical site in varied numbers - an indication of the ability of *Staphylococcus aureus* to cause infection in almost every part of the body, thus agreeing with the widely acclaimed status of *Staphylococcus aureus* as a bug of medical importance[11]
Biochemical characterization performed on the isolates yielded expected results, though with a little deviations which could be due to strain variation. All the isolates exhibited growth on mannitol salt agar within 18 to 24 hours of incubation. The isolates were all catalase positive, an indication that they are aerobic. This is in line with the fact that catalase neutralizes the toxic effect of hydrogen peroxide, the by-product of oxidative metabolism[12]
Ninety-eight (98 %) of the isolates produced coagulase enzyme including the control strain, *ATCC 29213* while others were coagulase negative. However, all the isolates were oxidase negative. The detection of the enzyme gelatinase in most of the isolates studied, serves an additional evidence of pathogenicity of isolates of *Staphylococcus aureus* while Ninety-five (95%) of the isolates were found to be DNAse positive with pronounced wide zone of clear(alpha-type) haemolysis on blood agar, thus confirming the human origin of the *Staphylococcus aureus* isolates which corroborated the report of Mathew et.al., on Staphylococci detected with haemolysins from human origin[13]

In this study, varied concentrations of vancomycin antibiotic were tested against the 60 isolates of *Staphylococcus aureus.* The zones of growth inhibition obtained were concentration related, though vancomycin activity is not concentration dependent and 30 microgram of vancomycin disc were used as a control disc. Fourteen(14) of the 60 isolates were resistant to vancomycin disc *Sa (9, 11,12, 13, 20, 26, 27, 34, 37, 38, 42, 50, 52, 58, 60)* and There are remarkable zones of growth inhibition at every concentrations. The variation in zones of growth inhibition observed in this study and varied MIC results recorded could be due to intrinsic or extrinsic factors, inherent properties within the isolates, while the resistant pattern obtained as reflected in zones of growth inhibition of *Staphylococcus aureus* to vancomycin powder at different concentration and vancomycin disc could be attributed to over-exposure or unguided use of this antibiotic[14] Plasmid DNA ranged from 0.82kb, 1.12kb, 14.45kb, 27.22kb molecular weight were obtained from the resistant isolates of

Staphylococcus aureus exposed to gel-electrophoresis. The variations in plasmids in terms of molecular weight and number were observed which agreed with the study of Kemper *et.al.,* 2011 that plasmid of *Staphylococcus aureus* were diverse in nature and its diversity could be traced to clinical source of the isolates[15]. The plasmid isolated were independent of the level of vancomycin resistance in the isolates of *Staphylococcus aureus* studied.

CONCLUSION

In this study, an account on the spread of boil infection with respect to pathogens and gender, along with resistance to vancomycin have been identified. The plasmid borne resistance obtained could be transfer to another bacteria hence aiding the spread of antibiotic resistance that can cause therapeutic failures and economic loss. Sequel to this, there is a need for public enlightenment on the therapeutic management of boil infection.

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