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Molecular Characterization and Efficacy of Antibiotic Combinations on Multiple Antibiotic-Resistant *Staphylococcus aureus* Isolated from Nostrils of Healthy Human Volunteers

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Abstract

Background: The increased use of antibiotics in recent times has resulted in the development of resistance to antibiotics. The significant clinical implication of resistance has led to heightened interest in the study of bacterial resistance.

Purpose: This study was carried out to evaluate the effects of antibiotic-combinations against some community isolates of multiple antibiotic-resistant *Staphylococcus aureus* and also to determine if the observed resistance is plasmid or chromosomally-mediated .

Methods: Swabs were collected from nostrils of one hundred healthy human subjects, streaked in mannitol-salt agar, and incubated. The isolates were characterized to species level and fifty three (53) of these isolates were identified as *S. aureus*.

Antibiotic sensitivity of the isolates was determined using agar diffusion method. The combined in-vitro antimicrobial activities of gentamicin and other antibiotics (flucloxaccillin, cephalexin, and clindamycin) against the resistant strains (isolates 03, 13,31,40, and 70) of *S. aureus* were investigated using the checkerboard technique. The preliminary characterization of the plasmid DNA was also carried out through agarose gel electrophoresis. To determine if resistance was plasmid –mediated , the resistant isolates were cured with acridine orange and their antibiotic susceptibility pattern evaluated by agar diffusion method.

Results: The drug interaction results showed that the combination of gentamicin and the β-lactam antibiotics were synergistic. The preliminary characterization of eight (8) resistant bacteria isolates showed plasmid whose sizes ranged from 10-13kb. The plasmid curing studies revealed that the observed multi-antibiotic resistance (MAR) was both plasmid and chromosomally –mediated.

Conclusion: The observed multiple-antibiotic resistance was both plasmid and chromosomally- mediated and can be reversed using antibiotic –combinations of different mechanisms of action. This surveillance exercise may be used to devise mechanisms to stem the emergence and subsequent spread of drug resistance by the organism.

Keywords: *Staphylococcus aureus* , multi-drug resistance , antibiotic combinations, plasmids, Agarose gel electrophoresis, curing.

INTRODUCTION

Staphylococcus aureus is a Gram-positive eubacterium that is found on the surfaces of the human skin and mucous membranes. It is also found in other areas of human contact, including air, soil, dust, water and food products. *Staph. aureus* is an opportunistic pathogen in humans and animals and is one of the most frequent sources of hospital –and community-acquired infections.[1] . Many isolates of *Staph. aureus* have evolved resistance to both synthetic and traditional antimicrobial agents and their prevalence outside the hospital is of potential epidemiological threat.[2-3]. In several studies , *Staph aureus* from normal flora seem to constitute an important reservoir of antimicrobial resistance genes. These genes can be transferred to other potential pathogens[4]. The accumulation of resistance factors has rendered the bacteria immune to a variety of commonly used antibiotics.[5] thus increasing the ability of the bacteria to survive in hostile environments. The prevalence of bacterial pathogens resistant to available antibiotics has been increasing over the past several decades . This situation constitutes a major challenge for clinicians and microbiologists and is particularly acute for the treatment of infections caused by gram-positive organisms. Multi-drug resistant (MDR) *Staph aureus* is an increasing common cause of nosocomial infections . While most MDR strains are hospital acquired there has been a worrisome increase during the last few years in the incidence of community-acquired strains. Such strains are typically more virulent than hospital strains[6,7]. Antimicrobial combination therapy may be used to provide broad-spectrum coverage and, prevent the emergence of resistance mutants. Thus surveillance study in *Staph.aureus* isolated from the nostrils of human subjects is important as data obtained from this exercise may be used to stem the emergence and subsequent spread of drug-resistance among bacteria population.

This study was therefore conducted to determine the possibility of reversing the resistance patterns via antibiotic combination and equally determine if the resistance is plasmid or chromosomally mediated.

MATERIALS AND METHODS

Microorganisms

Community strains of *Staphylococcus aureus* were isolated from nostrils of 100 healthy human volunteers within Nsukka metropolis, Enugu State (after obtaining their informed consent) using sterile swab sticks. Samples were collected between July and August 2008 while isolation and identification of the bacterial isolates were performed according to standard bacteriological techniques previously established [8,9]. Thereafter all *Staph. aureus* isolates were stored in agar slants at 4^0C until used for further studies.

Culture Media and Reagents

The culture media used in the study include, Nutrient broth (Oxoid, England), Mannitol salt agar (Oxoid, England) Nutrient agar (Fluka Spain) and Peptone water. Gram Staining reagents, buffer solution, Tris-ethylenediamine tetra-acetic acid sodium sulfate (TENS), sodium acetate, Ethidium bromide and Bromo – phenol blue were all analar grade reagents.

Antibiotic Sensitivity Discs

The following antibiotics used were obtained from ABTEK, India: Amoxicillin – Clavulanic acid(AUG) 30 µg, Amoxicillin (AMX) 25µg, Erythromycin (ERY) 5µg, Gentamicin (GEN) 10µg, Cotrimoxazole (COT) 25µg, chloramphenicol (CHL) 30µg, Cephalexin (CLX) 30µg,, Clindamycin (DAL) 2µg, Flucloxacillin (FLX) 5µg, and Minocycline 30µg,. The following drugs were also used: Gentamicin (80 mg/ml) (Gentalek) Yugoslavia, Clindamycin (150 mg) (Dalacin CTM) Pfizer USA, Flucloxacillin (Floxapen 250 mg) Beecham England, Tetracycline (Tetraclin 250 mg) Greenfield Pharm. India.

Antibiotic Sensitivity Test

Antibiotic sensitivity of the isolates was determined using agar diffusion method(10). The isolates were cultured in nutrient broth at 37^0C for 24 h. Two (2) loopfuls of the suspension of each isolate were inoculated into 20 ml of sterile molten agar in 10 cm diameter Petri dishes and mixed. The plates were allowed to set and the antibiotic Sensitivity discs were aseptically placed on their surfaces. The plates were incubated at 37^0C for 24 h and the resultant inhibition zone diameters (IZDs) were measured and recorded.

Evaluation of antibiotic interaction using checkerboard technique

The stock solution of gentamicin (100 µg /ml) and flucloxacillin (100 µg /ml) were prepared using sterile water, for the evaluation of the combined activity of both drugs against some antibiotic-resistant *Staph. aureus*. Varying proportion of the drug combinations ranging from 10:0 to 0:10 of gentamicin and flucloxacillin respectively were mixed according to the continuous variation checkerboard method. Each proportion of the antibiotic combination was serially diluted (two- fold) with sterile water (2 ml) into four test tubes, after which 1 ml of the mixture was added into a clean sterilized plate. Thereafter 19 ml of molten nutrient agar was added to the drug in the plate, mixed well and allowed to set. The plates were each divided into five sectors according to the isolates. A loopful of the test organisms (0.04 ml) was collected and streaked on the surface and incubated for 24 h at 37^0C .The same procedure was repeated using a stock of gentamicin (500 µg / ml) and cephalexin (500 µg / ml), and a stock of gentamicin (300 µg/ml) and clindamycin (300 µg/ml). The combined activities of antibiotics against each of the microorganisms were determined on the basis of the fractional inhibitory concentration (FIC) index expressed by the following relationship:

$$\text{FIC INDEX} = \frac{A1}{A} + \frac{B1}{B}$$

Where A1 and B1 are the respective minimal concentration of gentamicin and either flucloxacillin,cephalexin or clindamycin producing the combined MICs, while A and B are the MICs of the single agents.

Plasmid Profile Studies Using Agarose gel Electrophoresis Extraction of Plasmid DNA.

Selected resistant isolates were grown in a 5 ml doublestrength Mueller Hinton broth for 72 h at 37^0C. The 72h grown cultures were centrifuged in a micro centrifuge for 10 min at 10,000 rpm to obtain pellets. The supernatant was gently decanted and the cell pellets were vortexed for 5 min. Thereafter, 300µg of Tris EDTA (TE) buffer and 150 µL of 3.0 M sodium aqueous acetate was added at pH 5.2 and was vortexed for 3mins to lyse the bacteria cell pellet. The samples were centrifuged again for 2 min in a microcentrifuge (Biofuge,Biotra Bio-trade Hecrus Sepatech Co. Ltd USA) and the supernatant was transferred to a fresh tube, mixed well with 0.9 ml of 100 % ethanol which had been precooled to – 20 ^{0}C (in a refrigerator) to precipitate the bacteria DNA. It was centrifuged again for 2 min and the supernatant was discarded. The pellet was rinsed twice with 1 ml of 70 % ethanol and was dried under vacuum for 2 – 3 min, after which it was resuspended in 20 - 40 µL of TE buffer for further use [11-13].

Preparation of Gel.

A 1.0 g of agarose was dissolved in 100 ml of Tris Borate EDTA buffer (TBE), thus forming 1.0% gel. The agarose solution was allowed to cool to a temperature of about 40^0C. Thereafter ethidium bromide was added and the mixture poured into a gel tray. This was allowed for 20 min to solidify and the comb was carefully removed from the gel. The gel carrier was removed from the pouring tray and was placed in the gel electrophoresis box. A 250 ml TBE was used to fill the electrophoresis box until the gel was submerged.

Electrophoresis of the DNA Samples

Using micropipette, a 50 µL sample of DNA and 3 µL of loading dye were added together and carefully mixed by pipetting the solutions up and down[13] . Each sample was loaded carefully into the gel wells, one sample per well and this was placed on the gel box at the negative charge end of the electrophoresis machine. Buffered water was added which sealed the agarose containing the sample DNA and acts as electrolyte by moving the current as well as the sample DNA towards the positive end for 2 h with a voltage of 63 V. The

agarose containing the sample DNA was removed and allowed to drain off. With the aid of UV light, UV certified safety glasses and camera, a picture showing size and movement of the sample DNA was taken to determine the mobility in millimeter using a known sample standard [13,14].

Plasmid DNA curing

Plasmid curing was carried out using the method of Berman *et al* (1984) [15] . Inoculum cultures of selected isolates were prepared and standardized (McFarland 0.5). Nutrient broth was prepared and supplemented with acridine orange to a final concentration of 25 µg / ml. The pH was adjusted to 7.6 with 1 N NaoH and distributed into test tubes (5 ml each) and then autoclaved (121 ^{0}C for 15 min.) Then the broth tubes were separately inoculated with 0.1 ml of the standardized inoculum of the selected isolates. These were incubated for 24h at 37 ^{0}C. Following the incubation, bacterial cells were recovered by streaking on a nutrient agar plates. The plates were incubated for 24 h at 37 ^{0}C. The recovered cells were tested for antibiotic sensitivity using the agar diffusion method.[16]

RESULTS

Table 1 Shows the Multiple Antibiotic Resistance indices (MARI) of the isolates. A total of 54% of the isolates had MAR indices less than 20%. The rest of the isolates (45%) had MAR indices greater than 20%. Tables 2-4 show the FIC indices for the combined drug activity of gentamicin and some antibiotics. In table 1 the interaction of gentamicin and flucloxacillin against the resistant isolates of *Staph. aureus* were mostly synergistic. Out of the combinations, 80 % produced synergistic effects against the isolates, 4 % produced antagonism while 12 % produced indifference effects and 4 % produced additive effects.

Table 2 shows that the combination of gentamicin and cephalexin against the resistant isolates were more of additivity. On the whole, 28 % of the combinations produced synergism, 36 % produced additive effects, 20 % produced antagonism, and 16 % produced indifference effects. The combination of gentamicin and clindamycin (table 3) were mostly antagonistic. A total of72 % of the combinations produced antagonism, 20 % produced additive effects, 8 % produced indifference effects while none gave synergistic effect.

Table 5 and fig.1 show that there is presence of resistance plasmid in the isolates whose sizes ranged from 10-13 kb . Antibiotic resistance, including vancomycin resistance has been shown to be due to presence of resistance plasmid [17]. The presence of plasmid DNA cannot be correlated with resistance, so a plasmid curing procedure to eliminate the plasmid from the cells was carried out, after which resistance to the antibiotics was retested

Table 1: Multiple Antibiotic Resistance Indices (MARI) of the Isolates

ISOLATE	*MARI(%)*	*ISOLATE*	*MARI(%)*	*ISOLATE*	*MARI(%)*	*ISOLATE*	*MARI(%)*
01	25.0	34	58. 3	58	16.7	76	16.7
02	8.3	35	25.0	60	16.7	77	25.0
03	83.0 *	36	25.0	61	16.7	79	75.0
08	16.7	45	83.3	64	8.3	87	33.3
09	16.7	47	8.3	66	16.7	89	25.0
12	0.0	51	0.0	67	8.3	90	33.3
13	75.0 *	52	8.3	70	91.7 *	92	58.0
14	8.3	53	0.0	71	33.3	93	16.7
19	16.7	54	8.3	72	8.3	94	8.3
29	25.0	56	16.7	74	16.7	96	0.0
31	83.3 *	57	25.0	75	33.3	97	50.0

Table 2: FIC indices for the combinations of gentamicin and flucloxacillin against the resistant isolates

DRUG RATIOS GEN: FLUC	*FIC index ISOLATE 03*	*FIC index ISOLATE 13*	*FIC index ISOLATE 31*	*FIC index ISOLATE 40*	*FIC index ISOLATE 70*
10:0					
9:1	1.10 (-)	1.90 (-)	2.00 (*)	1.10 (-)	0.50 (++)
7:3	0.65 (++)	0.43 (++)	0.76 (++)	0.65 (++)	0.50 (++)
5:5	0.38 (++)	0.75 (++)	0.33 (++)	0.19 (++)	0.17 (++)
3:7	0.43 (++)	0.65 (++)	1.00 (+)	0.43 (++)	0.50 (++)
1:9	0.24 (++)	0.55 (++)	0.50 (++)	0.24 (++)	0.50 (++)
0:10					

*= Antagonism, ++ = Synergism, - = Indifference, + = Additivity
FIC index= Fractional inhibitory concentration index.
GEN= Gentamicin FLU= Flucloxacillin.

Table 3: FIC indices for the combination of gentamicin and cephalexin against the resistant isolates.

DRUG RATIOS GEN:CLX	*FIC index ISOLATE 03*	*FIC index ISOLATE 13*	*FIC index ISOLATE 31*	*FIC index ISOLATE 40*	*FIC index ISOLATE 70*
10 : 0					
9: 1	0.65 (++)	2.20 (*)	1.00 (+)	2.00 (*)	1.82 (-)
7: 3	0.59 (++)	1.30 (-)	1.00 (+)	2.00 (*)	1.00 (+)
5: 5	0.63 (++)	3.00 (*)	1.00 (+)	1.00 (+)	2.00 (*)
3: 7	1.55 (-)	1.70 (-)	1.00 (+)	1.00 (+)	1.00 (+)
1: 9	0.93 (++)	0.95 (++)	1.00 (+)	0.5 (++)	0.50 (++)
0: 10					

*= Antagonism, ++ = Synergism, - = Indifference, + = Additivity
FIC index= Fractional inhibitory concentration index.
GEN= Gentamicin
CLX= Cephalexin.

Table 4: FIC indices for the combination of gentamicin and clindamycin against resistant isolates.

DRUG RATIOS GEN: DAL	*FIC index ISOLATE 03*	*FIC index ISOLATE 13*	*FIC index ISOLATE 31*	*FIC index ISOLATE 40*	*FIC index ISOLATE 70*
10: 0					
9: 1	1.30 (-)	2.60 (*)	2.0 (*)	2.00 (*)	1.00 (+)
7: 3	1.90 (-)	3.80 (*)	2.0 (*)	2.60 (*)	1.00 (+)
5: 5	2.50 (*)	5.80 (*)	2.0 (*)	3.00 (*)	1.00 (+)
3: 7	3.10 (*)	6.20 (*)	2.0 (*)	3.40 (*)	1.00 (+)
1: 9	3.70 (*)	7.4 (*)	2.0 (*)	3.80 (*)	1.00 (+)
0: 10					

*= Antagonism, ++ = Synergism, - = Indifference, + = Additivity
FIC index= Fractional inhibitory concentration index.
GEN= Gentamicin
DAL= Clindamycin

Table 5: Electrophoretic mobility of standard DNA and plasmid DNA of the sample isolates

Mobility (mm)of the standard DNA	*Molecular weight (Kb)*	*Sample Isolates*	*Mobility (mm)of the isolates*
4.0	23.13	03	4.0
6.0	9.42	13	4.5
7.5	6. 56	31	5.0
9.0	4. 36	34	5.0
13.0	2.32	40	3.0
15.0	2.03	45	4.0
		70	4.0
		79	4.0

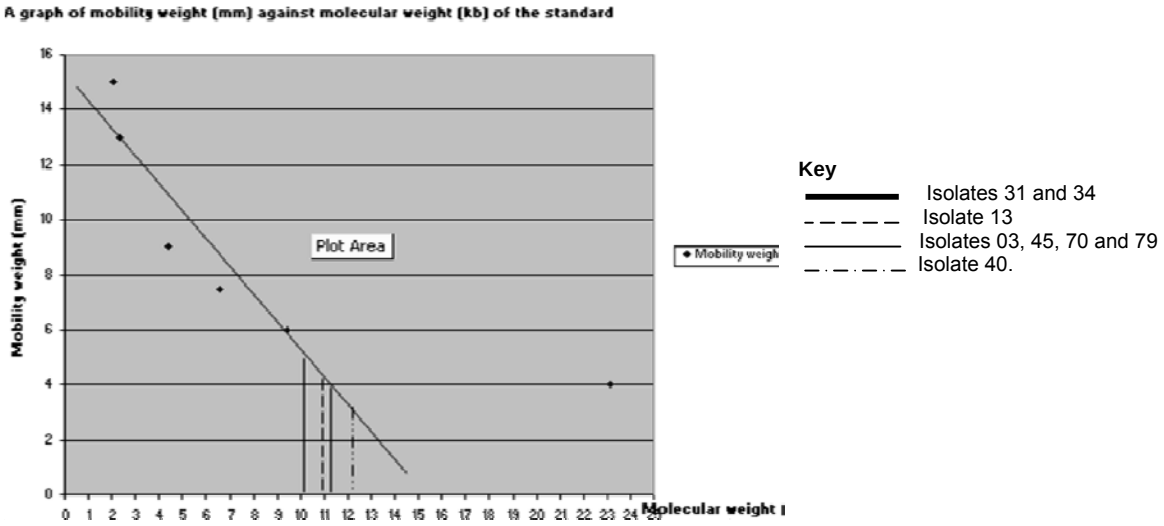

Fig 1: Mobility (mm) against molecular weight (kb) of the standard DNA

Table 6a: Result of sensitivity test before plasmid curing.

ANTIBIOTICS	*Inhibition Zone Diameter(mm) produced by the disc Antibiotics*			
	Isolate13	*Isolate40*	*Isolate45*	*Isolate79*
AUG	0	16	0	0
AMX	0	15	0	7
ERY	18	9	19	17
TET	0	9	0	O
CXC	0	O	0	0
GEN	9	0	12	12
COT	0	0	0	O
CHL	24	11	9	14
CLX	11	0	0	14
DAL	26	0	28	0
FLX	0	0	0	23
MIN	14	0	O	12

KEY: FLX=Flucloxacillin, COT=Cotrimoxazole, CXC=cloxacillin, CHL=Chloramphenicol, TET=Tetracycline,DAL=Clindamycin,MIN=Minocycline,GEN=Gentamicin, ERY=Erythromycin,CLX=Cephalexin,AUG=Amoxicillin-clavulanic acid and, AMX= Ammoxycillin

Table 6b: Result of sensitivity test after plasmid curing.

ANTIBIOTICS	*Inhibition Zone Diameter(mm) produced by the disc Antibiotics*			
	Isolate13	*Isolate40*	*Isolate45*	*Isolate79*
AUG	0	16	0	0
AMX	0	15	0	7
ERY	18	9	19	17
TET	13*	9	0	O
CXC	0	O	0	0
GEN	9	13*	12	12
COT	0	0	0	O
CHL	24	11	9	14
CLX	11	0	0	14
DAL	26	22*	28	24*
FLX	0	0	0	23
MIN	14	0	O	12

KEY:FLX=Flucloxacillin,COT=Cotimoxazole,CXC=cloxacillin,CHL=Chloramphenicol,TET=Tetracycline,DAL=Clindamycin,MIN=Minocycline,GEN=Gentamicin,ERY=Erythromycin,CLX=Cephalexin,AUG=Amoxicillin-clavulanic acid and,AMX=Ammoxycillin *= Improved inhibition zone diameter

DISCUSSION:

A total of 54 % of the isolates had MAR indices less than 20%. The rest of the isolates (45%) had MAR indices greater than 20%. When the MAR index is greater than 20%, it shows that the organisms were isolated in an environment where antibiotics are abused widely[18,19]. Research, has shown that resistance in Staphylococcus is often a consequence of wide use of the antibiotics in farming, hospitals and community [20]. The wide use of vancomycin as growth promoter in animals led to the emergence of vancomycin – resistant staphylococcus bacteria in many hospitals around the world. [21]. In addition to medical misuse, inappropriate use of antibiotics in the agricultural setting is a major contributor to the emergence of antibiotic – resistant bacteria. This situation was first documented in 1963, when increased levels of resistance in a particular strain of *Salmonella typhimurium* were observed at several British feedlots [22]. The overall results of interactions showed that the combination of gentamicin with β - lactam antibiotics can be synergistic. Penicillins are known to exert their antimicrobial effect by inhibition of the synthesis of peptidoglycan, a heteropolymeric component of the cell wall, which provides a rigid mechanical stability by virtue of its highly cross-linked lattice wall structure [23-25], and the result of this inhibition is loss of bacteria cell rigidity and subsequent rupture or lysis of the bacteria cells [23,26]. Gentamicin kills bacteria by interfering with the bacteria's ability to synthesize protein. Specifically, gentamicin binds tightly to ribosomes, which are located inside of the cell. Other antibiotics, including penicillin-like antibiotics, (beta-lactams), kill bacteria by interfering with cell wall synthesis, making the cell wall porous. When combined with gentamicin, the beta-lactam allows a lot more gentamicin to get inside the cell to attach to the ribosomes, which means that a lower concentration of gentamicin is needed to give the same bacteria-killing effect than without the beta-lactam. Thus the β-lactam antibiotics e.g. flucloxacillin **o**r cephalexin are known to enhance the permeability of gentamicin into the bacterial cells and interaction of gentamicin with carbapenen has been reported to be synergistic [27]. The combinations of gentamicin and clindamycin were predominantly antagonistic. This may be because both have similar mechanism of action (inhibition of protein synthesis). It has been reported that antimicrobial agents acting through protein synthesis show moderate susceptibility resistance profile against *s. aureus* [25,28] .Many studies have shown improved efficacy of certain antibiotics when combined with antibiotics of other classes [28]. This present study has therefore shown a possibility of reversing the multidrug resistance patterns via antibiotic combinations, (possibly of different mechanism of action) against strains of *Staph. aureus* that are resistant to the single agents. This observation could be useful in clinical practice requiring the use of antibiotics in the management of infections / diseases caused by *S. aureus*. Agarose gel electrophoresis was employed for the molecular characterization of the isolated plasmids from the bacteria strains. The relative profiles of the DNA fragments and plasmids were characterized on the basis of their comparative molecular weights and distance travelled through the electrophoretic agarose system [29]. The presence of some plasmid DNA in the isolates corresponding to the reference standard DNA fragments suggests that their antimicrobial resistance is possibly plasmid-mediated [26]. The isolated plasmids may be responsible for possibly mediating some or all of the expressed resistances of the microorganisms. Bacteria employ an extensive repertoire of plasmid, transpossons and bacteriophages to facilitate the exchange of resistance and virulence determinants among and between species. As a result, the opportunity for rapid emergence of high –level resistance even in the absence of direct selection by specific antimicrobial pressure abound [30]. Resistance gene curing was done to firmly establish the role of the isolated plasmids in the observed resistance patterns of the microorganisms. In bacteria, the acquisition of resistance may

be due to chromosomal mutations or through plasmids that are often capable of transfer from one strain of organism to another, even across the species barrier.[26]

The process of transfer and acquisition of resistance determinants among microorganisms is a natural, unstoppable phenomenon exacerbated by the abuse, overuse and misuse of antimicrobials in the treatment of human illness and in animal husbandry, aquaculture and agriculture [31-32]. Moreover, the drugs to which the isolates were resistant to, are commonly used antibiotics in the studied environment.[26].

Curing agents such as acridine orange if administered to bacterial populations in sub-lethal doses, can lead to the elimination of plasmid DNA without harming the bacterial chromosome and thus maintaining the ability to reproduce and generate offspring [33]. Population of bacteria containing plasmids that are subjected to agents such as acridine orange will become more and more dominated by plasmid free cells with time. [16,33]. Elimination of R-factors by acridines seem to be due to a selective interference in the replication of the plasmid [34,35,] and is most clearly demonstrable in exponentially growing cultures [35]. Thus, curing by acridines normally involves loss of the whole plasmids.

Table 6b reveals that sample isolate 13 had an appreciable improvement in sensitivity to tetracycline. Likewise sample isolate 40 had significant improvement in sensitivity to clindamycin and gentamicin. Sample isolate 79 also presented with improved sensitivity to clindamycin when the plasmid DNA was removed by acridine orange. Thus, the resistance to tetracycline by sample isolate 13; gentamicin and clindamycin by isolate 40 and clindamycin by isolate 79 can be postulated to be through plasmid DNA . It is possible that this plasmid DNA represents the transposon – associated van A gene cluster found in vancomycin resistant enterococci which could be horizontally transferred to other species consequently conferring resistance to the antibiotic in that species [36]. However, positive identification of the plasmid and its genetic contents was beyond the scope of this work.

In conclusion, the checkerboard results showed that the antibiotic resistance pattern can be reversed using combined antimicrobial agents of different mechanisms of action. The resistance observed was both chromosomally and plasmid-mediated. It is therefore recommended that there should be sustained surveillance and monitoring of antibiotic resistance from both human and animals. Such studies would form a basis for a sound antibiotic –use policies.

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