

## Prevalence and Plasmid Profile of Clinical and Carrier Strains of Fluoroquinolone-Resistant *Staphylococcus aureus* Isolates from Humans in Enugu State, Eastern Nigeria

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**Abstract:** Currently, fluoroquinolone antibiotics are commonly used drugs for the treatment of infections in Enugu State. Consequently, the rapid emergence and spread of antibiotic resistance especially of fluoroquinolones have caused serious public health problems. This study was conducted to determine the prevalence and plasmid profile of clinical and carrier strains of fluoroquinolone - resistant *Staphylococcus aureus* (FQRSA) isolates from humans in Enugu State. A total of 1680 non-duplicate specimens comprising 420 samples each of urine and nasal swab were collected from patients and healthy carriers using sterile container and cotton-wool swab by random sampling techniques from seven health districts in Enugu State. Isolation and characterization of *S. aureus* were carried out using standard microbiological techniques. Antibacterial susceptibility studies were carried out using the Kirby-Bauer disk diffusion technique. All the fluoroquinolone-resistant isolates were evaluated for the presence of plasmid DNA by conventional polymerase chain reaction. A total of 589 *S. aureus* strains were isolated for the study. The prevalence of urinary FQRSA isolates in healthy carriers and patients were 19.2 and 25.0 % for subjects within the age 6-18 years and 27.6 and 41.1% for subjects within the age of 19-60 years respectively. For nasal isolates, the prevalence of FQRSA isolates in healthy carriers and patients were 15.0 and 22.2 % respectively. Eighty eight (94.6 %) of all the FQRSA isolates were found harbouring a total of 121 plasmids with molecular sizes ranging from 0.5 to 23.1KB. Of all the plasmids detected, the 23.2KB was the modal plasmid found in our study with the frequency of 66.7%. In general, eight (8) different plasmid profiles were observed with 6.6, 9.2 and 23.1KB occurring in FQRSA isolates from almost all the health districts. This study has shown that, though, some of the clinical and carrier strains of *S. aureus* were resistant to fluoroquinolones, the prevalence of FQRSA isolates from specimen gotten from patients was higher than that from healthy carrier. Again, both the clinical and carrier strains of FQRSA isolates were harbouring resistant plasmids in the study area.

**Key words:** Prevalence • Plasmid profile • Fluoroquinolone-resistant • *S. aureus*. Enugu State

### INTRODUCTION

*Staphylococcus aureus* is a prominent cause of human infections worldwide [1-3]. It is present in about

25-30% of humans especially adults where they always exist as normal flora of the skin [4-6]. However, if there is a wound, a break in the skin from surgical procedure or suppression of a person's immune system, then the

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presence of *S. aureus* in the skin can cause an infection [7]. The infections caused by these bacteria include toxic shock syndrome [8], food poisoning, meningitis, pneumonia and endocarditis [8, 9] as well as dermatological disorders ranging from minor infections and eczema to blisters and scalded skin syndrome [10]. These organisms have the remarkable ability to acquire antibiotic resistance determinants and currently with the increased emergence of fluoroquinolone-resistant *S. aureus* (FQRSA) isolates; it has become a warning sign for public health [11]. The genes for antibiotic resistance in *S. aureus* are located on the plasmid, these plasmids contain resistant genes against a number of antimicrobial agents [12]. With the problems of healthcare funding in the developing world such as Nigeria, most health care centers still prescribe antibiotics without necessary clinical investigations. Prolonged and inappropriate use, abuse and misuse of antibiotics in both animals and humans could lead to the spread of fluoroquinolone resistant- *S. aureus* bacteria and consequently a change in antibacterial resistance pattern [13].

## MATERIALS AND METHODS

**Materials:** Culture media and antibiotic discs were purchased from Oxoid (Cambridge, UK) and they include mannitol salt agar, Mueller-Hinton agar, blood agar, ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg), pefloxacin (5 µg) gentamicin (30 µg), ceftriaxone (30 µg), amoxicillin (25 µg), erythromycin (15 µg) and tetracycline (30 µg).

**Study Area:** The study was conducted in Enugu State, which is one of the 36 States in Nigeria and is located in the Eastern part of the country. The State operates district health system which is made up of seven District Hospitals at Enugu Urban, Udi, Agbani, Awgu, Ikem, Enugu-Ezike and Nsukka. Specimens of urine and nasal swabs were collected from both patients and healthy carriers residing in one of these districts.

**Ethical Approval:** Ethical approval was obtained from the ethical committee of the State Ministry of Health and the clearance reference number was MH/MSD/EC/O218. Besides, signed informed consent of all the participant subjects were also obtained.

### Inclusion Criteria:

- Age 6- 60 years.

- Apparently healthy volunteers not on any form of antimicrobial therapy at least 3 months preceding the sample collection.
- In or Out patient who has been on antibiotics at least 4 days preceding the sample collection.

### Exclusion Criteria:

- 5 years < Age > 60 years.
- Apparently healthy volunteers on any form of antimicrobial therapy at least 3 months preceding the sample collection.
- In or Out patient not on antibiotics at all or have just commenced antimicrobial therapy at most 4 days preceding the time of sample collection.

**Sample Collection and Storage:** At the respective health care facility, arrangement was made with a physician, matron and laboratory scientist/ technician who assisted in the sample collection. Prior to specimen collection, each subject was interviewed using structured questionnaire designed to obtain basic demographic data, history of illness, antibiotic intake or intake of herbal medicines and clinical information concerning the human subject. Non-duplicate specimens of urine and nasal swabs were collected per test subject. Clean-catch midstream urine and nasal swab specimens were collected from both patients and healthy volunteers and were transported to the Microbiology Laboratory of the Department of Pharmaceutical Microbiology, University of Nigeria, Nsukka for immediate culture and sensitivity tests.

### Isolation and Identification of the Test *S. aureus*:

The specimens were inoculated on blood agar and mannitol salt agar for the isolation of *S. aureus*. These isolates were identified using conventional methods as described previously [14, 15]. Yellow colonies with yellow zones on the Mannitol salt agar culture were subjected to Gram staining, catalase production and coagulase positivity tests. Positive isolates were confirmed *Staphylococcus. aureus* by conventional PCR.

**Antimicrobial Susceptibility Test:** Susceptibility was carried out by disk diffusion method according to Clinical Laboratory Standard Institute (CLSI) recommendations [16] on Mueller-Hinton agar plates with the following discs; ciprofloxacin (5 µg), ofloxacin (5 µg), levofloxacin (5 µg), pefloxacin (5 µg) gentamicin (30 µg), ceftriaxone (30 µg), amoxicillin (25 µg), erythromycin (15 µg) and tetracycline (30 µg). All culture media and antibiotics

were obtained from Oxoid, Cambridge, UK. *S. aureus* ATCC 25923 was used as the reference strain for the susceptibility tests.

**Plasmid Profiling:** The genetically confirmed fluoroquinolone resistant *S. aureus* were evaluated for the presence of plasmid DNA as described elsewhere [17]. One ml of 24 h cultures of test *S. aureus* in Tryptic Soy Broth (TSB) medium (Merck, Germany) was transferred into 1.5ml sterile Eppendorf micro-fuge tubes and centrifuged at 10,000g for 10min. The resultant pellets were dissolved in 600µl of lysis buffer (NaCl 1M, Tris-HCl 1M, EDTA 0.5M), 20µl SDS (25%), 3 µl of proteinase-K (20mg/ml) and incubated at 60°C for 1h. After the lysis, 620 µl of phenol/chloroform/isoamylalcohol (25:24:1 volume/volume) was added to the above solutions, vortexed and centrifuged at 12,00g for 10min. The supernatants were aseptically transferred to sterile micro-fuge tubes to which 1ml of 95% cold ethanol was added. The micro-fuge tubes were allowed to stand for 1h in refrigeration condition (4°C). Plasmid DNA were precipitated in each tube by centrifugation at 12,00g for 10 mins. The precipitated DNA was dissolved in 50 µl of 10mM Tris EDTA-buffer (TE) containing 10 µl of RNASE. The plasmids were run on 1.5% agarose gel electrophoresis and visualized under UV light transilluminator and photographed as described by other authors [18].

**Plasmid Curing:** Plasmid curing was conducted by treating fluoroquinolone resistant *S. aureus* (FQRSA) isolates with sub-inhibitory concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5mg/ml of acridine orange in Mueller-Hinton broth according to a previously described method [19]. The tubes were incubated at 37°C for 24 h. After incubation, the broth was shaken

properly for homogenization of the culture and loopful of the broth culture taken and inoculated onto drug-free Mueller-Hinton agar plates and incubated for 24 h at 37°C. Susceptibility tests were carried out on the FQRSA isolates using some selected antibiotic disks and then, the diameter of zones of inhibition were taken after incubation. FQRSA isolates in which the inhibitory zone diameter against the test antibiotics increased were considered as plasmid-cured (i.e. plasmid-mediated resistance) while those in which the inhibitory zone diameter against the test antibiotics remained unchanged were considered as not cured (i.e. chromosomal-mediated resistance).

## RESULTS

### Prevalence of Fluoroquinolone Resistant *S. aureus*:

The antibiotics resistance profile of *S. aureus* isolates from both human patients and asymptomatic carriers against some fluoroquinolones and other commonly used antibiotics is shown in Table 1. A total of 589 *S. aureus* isolates were got from non-duplicate samples of urine and nasal swab of both asymptomatic healthy volunteers and patients previously or currently on antibiotics in the study area. Out of these (589) isolates, the average percentage prevalence of FQ-resistant *S. aureus* are as follows; 21.1% were resistant to ciprofloxacin, 21.6% were resistant to ofloxacin, 19.4% were resistant to Levofloxacin and 22.5% were resistant to pefloxacin respectively. The prevalence of levofloxacin resistant *S. aureus* was the least resistant rate among all the fluoroquinolones tested while that of pefloxacin resistant *S. aureus* was highest. Based on the age and sex of the test subject, the prevalence of FQRSA are shown in Table 2. The prevalence of urinary FQRSA isolates in healthy carriers and patients were 19.2 and 25.0 %

Table 1: The antibiotics resistance profile of *S. aureus* isolates from human subject.

Specimen Source	Specimen	Number of <i>S. aureus</i> isolates (% prevalence)								
		CPX	Ofx	Lev	Pef	Gn	Cef	Amx	Ery	Doxy
Healthy Volunteer	Nasal swab	36 (11.3)	33 (10.3)	36 (11.3)	35 (10.9)	25 (7.8)	30 (9.4)	279 (87.2)	64 (20.0)	257 (80.3)
	Urine specimen	17 (19.8)	17 (19.8)	19 (22.1)	20 (23.3)	21 (24.4)	23 (26.7)	77 (89.5)	22 (25.6)	76 (88.4)
	Patient	16 (22.2)	17 (23.6)	16 (22.2)	21 (29.1)	26 (36.1)	18 (25.0)	72 (100)	25 (34.7)	72 (100)
	Urine	33 (31.7)	33 (31.7)	28 (26.9)	33 (31.7)	32 (30.7)	15 (14.4)	81 (77.8)	31 (29.8)	78 (74.9)

NB: figures in parenthesis represent the prevalence(%) of antibiotics resistant isolates

KEY: Cpx = ciprofloxacin, Ofx = ofloxacin, Lev = levofloxacin, Pef = pefloxacin, Gn = gentamicin, Cef = ceftriaxone, Amx = amoxicillin, Ery = erythromycin, Doxy = doxycycline.

Table 2: Prevalence of FQRSA according to specimen source, subject age and sex.

Specimen Source	Age Range (yr)	Total no <i>S. aureus</i>	No of FQRSA(%)	Sex M (%)	F (%)
HVU	6-18	26	5(19.2)	1(20)	4(80)
	19-60	29	8(27.6)	6(75)	2(25)
PSU	6-18	40	10(25.0)	6(60)	4(40)
	19-60	56	23(41.1)	13(57)	10(43)
Hvns	6-18	116	11(9.5)	6(55)	5(45)
	19-60	133	20(15.0)	13(65)	7(35)
Pns	19-60	72	16(22.2)	4(25)	12(75)

Key: HVU = healthy volunteer urine, PSU = patient specimen of urine.  
 HVns = healthy voluneer nasal swab., Pns = patients nasal swab.

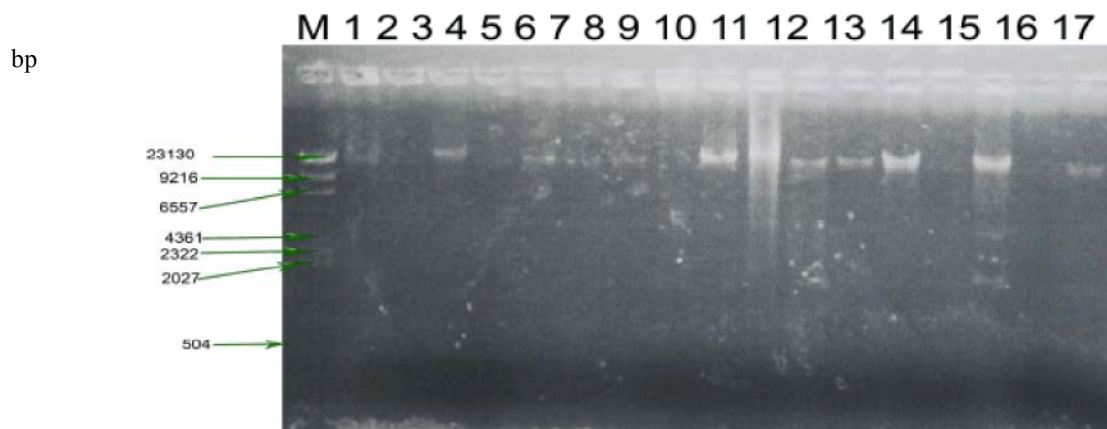


Fig. 1: The Image of gel electrophoresis of Plasmid DNA from the first group of FQRSA isolated from NASAL SWAB of healthy volunteers.

Lane M is HIND III Marker, Lane 16 has 3 positive bands of sizes 23.1 kb, 4.4kb and 2.0 kb. Lanes 1,3, 5, 9,10, 11, 12, 13, 14, 15 and 17 had a band of size 23.1kb

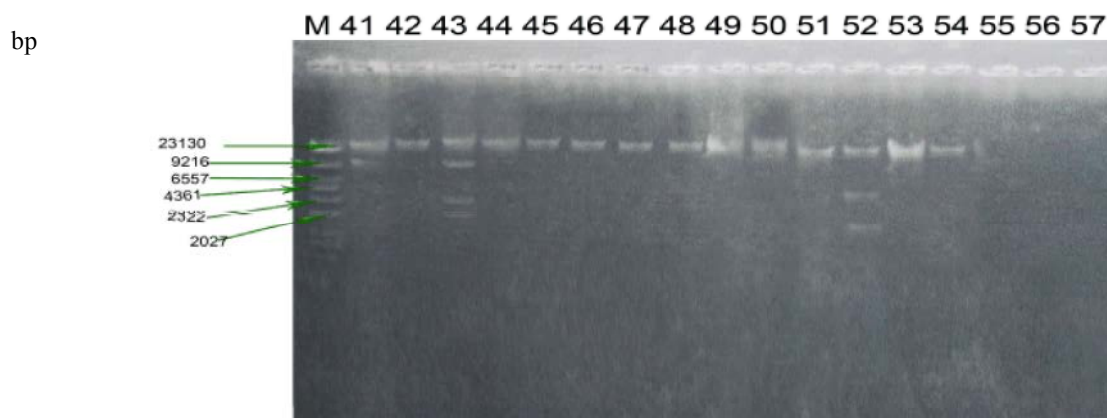


Fig. 2: The Image of gel electrophoresis of Plasmid DNA from the FQRSA isolated from nasal swab specimens of patients on antibiotics.

Lane M is HIND III Marker, Apart from lanes 55, 56 and 57 without plasmid band, other lanes had at least one plasmid band of size 23.1kb. In lanes 41, 42, 43 51 and 52, each has more than one bands.

(for subjects within 6-18 years) and 27.6 and 41.1% (for subjects within 19-60 years) respectively. For nasal isolates, the prevalence of FQRSA isolates in healthy carriers and patients in antibiotics are 15.0 and 22.2% respectively. Plasmid profile of both urinary and nasal FQRSA isolates are shown on the gel images of Figures 1-4.

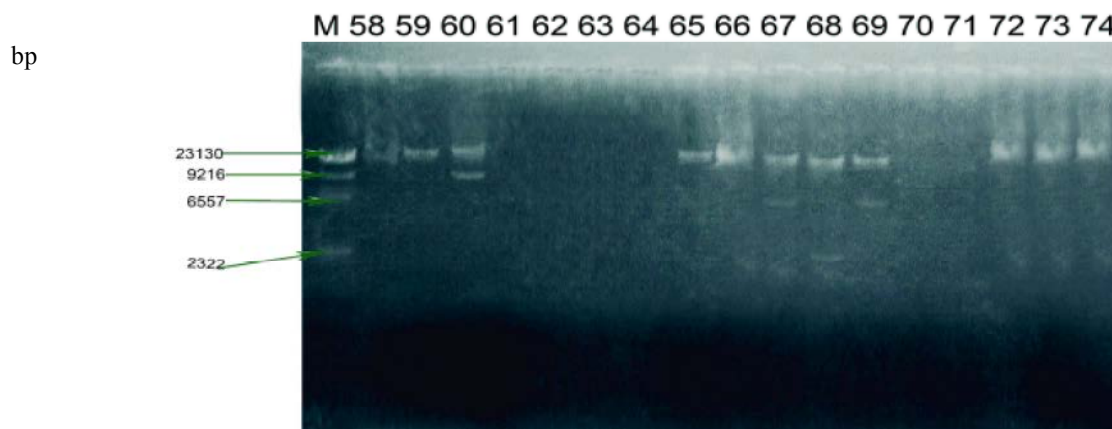


Fig. 3: The Image of gel electrophoresis of Plasmid DNA from the FQRSA isolated from urine specimen of healthy volunteers.

Lane M is HIND III Marker, Apart from lanes 61, 62, 63, 64 and 70 and 71 without plasmid band, other lanes had at least one plasmid band of size 23.1kb.

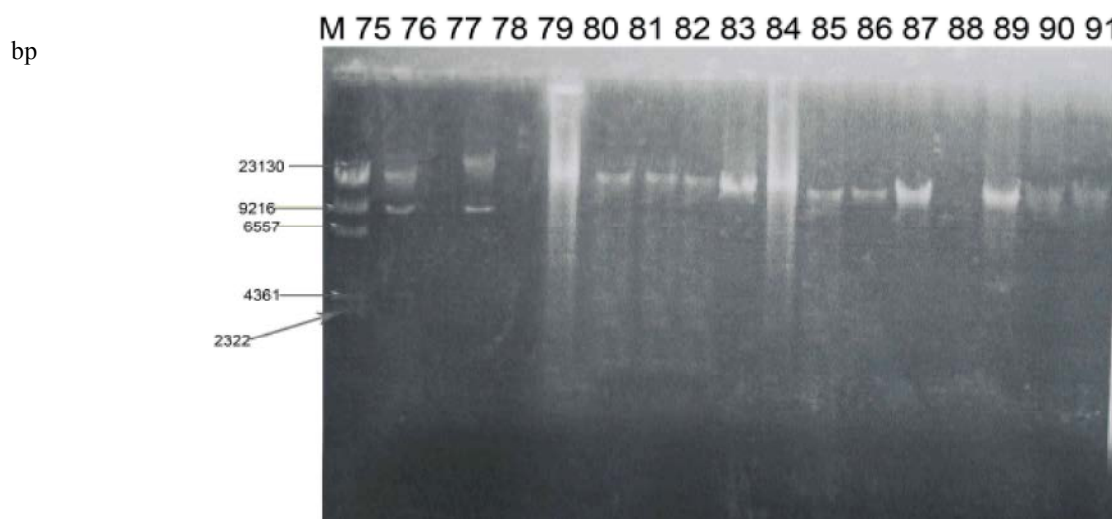


Fig. 4: The Image of gel electrophoresis of Plasmid DNA from the first group of FQRSA isolates from urine specimens of patients.

Lane M is HIND III Marker, Apart from lanes 76, 78 and 88 without plasmid band, other lanes had at least one plasmid band of size 23.1kb.

**Plasmid Profile:** Tables 3 and 4 show the distribution of plasmids in the FQRSA isolates according to specimen source and location respectively. Eighty eight (94.6 %) out of 93 FQRSA isolates were found harbouring a total of 121 plasmids with molecular sizes ranging from 0.5KB to 23.1KB. Of all the plasmids detected, the 23.2KB was the modal plasmid with the frequency of 66.7%. All the isolates bearing this plasmid also harboured one or more smaller plasmids and they were resistant to six or more antibiotics including fluoroquinolones, gentamicin and ceftriaxone. In general, eight different

plasmid profiles were observed with 6.6, 9.2 and 23.1KB occurring in almost all the health districts in the test FQRSA isolates.

Based on the location (health districts), 36, 34, 18, 17, 15, 14 and 6 plasmids were detected in human FQRSA isolates from Udi, Agbani, Enugu urban, Enugu-Ezike, Nsukka, Awgu and Ikem districts respectively. The plasmid distribution in both Udi and Agbani districts followed a unique pattern, 23.1 and 9.2 KB plasmids were the most frequent (66.7 and 70.6%) respectively, the remaining plasmids harbored by

Table 3: The distribution of Plasmids in FQRSA according to specimen source in both humans

Plasmid Size KB	Frequency of Distribution according to specimen Source				
	HVnF N=38 (%)	PnF N=30 (%)	HVUF N=15 (%)	PUF N=38 (%)	PWS N=23 (%)
0.5	01(2.6)	01(3.3)	00(0)	01(2.6)	0(0)
1.0	00(0)	00(0)	00(0)	00(0)	0(0)
2.0	02(5.3)	02(6.6)	00(0)	01(2.6)	0(0)
2.3	00(0)	01(3.3)	01(6.7)	01(2.6)	0(0)
4.4	02(5.3)	02(6.6)	00(0)	01(2.6)	0(0)
6.6	02(5.3)	01(3.3)	02(13.3)	02(5.3)	5(21.7)
9.2	02(5.3)	09(30)	01(6.7)	03(7.9)	5(21.7)
23.1	29(78.3)	14(46.7)	11(73.3)	29(76)	13(56.5)

Key: HVNF = Healthy Volunteer Nasal FQRSA, PnF = Patients Nasal FQRSA.  
HVUF = Healthy Volunteer Urinary FQRSA, PUF = Patient Urinary FQRSA

Table 4: The Distribution of Plasmid in FQRSA according to health districts in the study area

Plasmid size (KB)	Frequency of Distribution according to health district						
	Agbani N=34(%)	Enugu Urban N=18(%)	Udi N=34(%)	Euugu Ezike N=17(%)	Ikem N=36(%)	Nsukka N=6(%)	Awgu N=14(%)
0.5	0	0	0	0	0	1(6.7)	1(7.1)
1.0	0	0	0	0	0	0	0
2.0	2(5.9)	1(5.6)	2(5.6)	0	0	1(6.7)	0
2.3	1(2.9)	0	1(2.7)	0	0	1(6.7)	0
4.4	1(2.9)	2(11.2)	2(5.6)	0	0	0	0
6.6	2(5.9)	2(11.2)	2(5.6)	1(5.9)	1(16.7)	0	0
9.2	4(11.8)	2(11.2)	5(13.9)	3(17.6)	0	3(20)	3(21)
23.1	24(70.6)	11(61.1)	24(66.7)	13(76.5)	5(83.3)	8(53.3)	10(71.4)

N= Number of Plasmid

these FQRSA isolates occurred at a considerable low rate 2.7 - 5.9%. The FQRSA isolates from other districts were more diverse in terms of plasmid distribution. Though six plasmid profiles were observed in the FQRSA isolate from Udi and Agbani health districts, there was an even distribution across the different plasmid size observed in the study. In Awgu districts, 1.0-6.6 KB plasmid were not detected in the isolates. 2.0KB plasmids were present in FQRSA isolates from Nsukka, Udi and Agbani health districts.

## DISCUSSION

Widely varying percentages of resistance to fluoroquinolones have been associated with particular bacterial species, clinical settings, origins of strains, geographic locations and local antibiotic policies [14]. Antibiotic usage is probably the most important factor that promotes the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine [20]. The World Health Organization policy perspective on medicine indicates that even when drugs are made available, more than fifty percent are

prescribed, dispensed or sold inappropriately while 50% of patients fail to take the medicines correctly resulting in harmful consequences [21]. One of the major consequences of such inappropriate use of antibiotics is the development of resistance strains of the hitherto susceptible organism. In the present study, we carried out the antibiotic sensitivity tests to select all the fluoroquinolone-resistant *S. aureus* from various sources. The prevalence of urinary FQRSA isolates in healthy carriers and patients were 19.2 and 25.0 % (for subjects within 6-18 years) and 27.6 and 41.1% (for subjects within 19-60 years) respectively. For nasal isolates, the prevalence of FQRSA isolates in healthy carriers and patients in antibiotics were 15.0 and 22.2 % respectively, hence, the prevalence of FQRSA isolates from patients urine and nasal swab is higher than that of FQRSA isolates from the specimen of healthy volunteer subjects. Our finding is the first documentation of the prevalence of clinical and carrier strains of FQRSA from urine and nasal swab specimens in Enugu State.

Based on the age of the human subject used, there was a direct relationship between the age of the subject and the percentage fluoroquinolone (FQ) resistance

for the test isolates in both patients and healthy carriers. The reason for this may be due to the limited use of fluoroquinolones in children below 18 years, for fear of tendon damage). Again, the increased consumption of fluoroquinolones for the treatment UTI, prostatitis and other diseases in old age may contribute to high FQ- resistance in older subject (19-60 years) than in younger ones (6-18 years).

Based on the sex of human subject used, the FQRSA isolates showed higher prevalent rate in male subject (55.4 %) than in female subject (44.6%). This is in agreement with the results of Ito *et al.* [22] who reported that resistance to quinolones was higher in men, perhaps because of the association between UTIs and prostatitis, where quinolones are widely used. Conversely, with isolates from patient's nasal swab specimen, the percentage FQRSA isolates from female subject is greater than FQRSA isolates from male subject. The reason for this is not clear but may be connected to the increased inappropriate use and abuse of antibiotics and herbal remedies for treatment of urogenital infections and other ailments which is more in female than in male in the study area. The difference in the prevalence rate of *S. aureus* against fluoroquinolone antibiotics tested in both the healthy carrier and the patients as observed in this study is most likely due to the difference in drug structure. This is because the fluoroquinolone MICs against organisms differ due to the effect of drug structure as previously noted [23-26].

### CONCLUSION

The prevalence of FQRSA isolates from specimen gotten from patients was higher than that from healthy carrier. Again, both the clinical and carrier strains of FQRSA isolates were harbouring resistant plasmids in the study area. Therefore, early and accurate detection of fluoroquinolone resistance in bacteria especially *S. aureus* isolates from clinical and non clinical samples is of public health importance due to the multi-antibiotic resistance nature of these organisms. The rapid spread of fluoroquinolone resistance among bacteria isolates from both clinical and apparently healthy carriers may be caused by the prolonged use and misuse of the antibiotics in various homes as well as their inappropriate use in animal husbandry.

**Recommendation:** We recommend proper detection and surveillance of fluoroquinolone resistance not only from clinical subjects but also from samples collected from the community and asymptomatic healthy carriers. It is also

important to discourage the use of fluoroquinolones without proper antibiotic sensitivity test in humans.

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