Molecular Identification and Antimicrobial Studies of Fluoroquinolone-Resistant *Staphylococcus aureus* and *Escherichia coli* from Humans and Farm Animals in Enugu State

BY

ADONU, CYRIL CHEKWUBE
PhD/2012667003P

DEPARTMENT OF PHARMACEUTICAL MICROBIOLOGY AND BIOTECHNOLOGY

FACULTY OF PHARMACEUTICAL SCIENCES
NNAMDI AZIKIWE UNIVERSITY AWKA

SUPERVISOR: PROF. C. O.ESIMONE

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Molecular Identification and Antimicrobial Studies of Fluoroquinolone-Resistant *Staphylococcus aureus* and *Escherichia coli* from Humans and Farm Animals in Enugu State

Being a dissertation submitted to the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka in Partial fulfillment of the requirements for the award of Doctor of Philosophy Degree in Pharmaceutical Microbiology and Biotechnolgy.

BY

ADONU CYRIL CHEKWUBE
PhD/2012667003P

SUPERVISOR: PROF. C. O. ESIMONE

JULY 2017

CERTIFICATION PAGE

I, ADONU, CYRIL CHEKWUBE, a doctoral student in the Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, with registration number **PG/PhD/2012667003P,** do hereby certify that the work embodied in this project is original and has not been submitted in part or full to this or any other University or College

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ADONU, CYRIL CHEKWUBE Date

APPROVAL PAGE

This is to certify that the PG. student, ADONU CYRIL CHEKWUBE, with the registration number **PG/Ph.D./2012667003P** has satisfactorily completed all the requirements for the award of Doctor of Philosophy (Ph.D) degree in Pharmaceutical Microbiology and Biotechnology of the Nnamdi Azikwie University. This work is original; it has not been submitted in any form for award of any degree, diploma or certificate in any University.

_____ ----------

Prof C.O Esimone (Supervisor) Date

_____ ----------

Dr .A.N. Oli (Head of Department) Date

_____ ----------

Prof I. C. Uzochukwu (Dean of Faculty) Date

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Prof. J. A. Onaolapo Date
(External Examiner)

---------- ----------

Prof . Ike Odimegwu Date
(Dean, School of Postgraduate Studies)

DEDICATION

I dedicate this work to the Almighty God , the maker of all things, visible and invisible.

Aknowledgement

First and foremost, I thank the Almighty God who made it possible for me to complete this work. I am very grateful to my supervisor Prof. C.O Esimone who was always ready and willing to help. My humble appreciation goes to the Dean of the Faculty of Pharmaceutical Sciences Nnamdi Azikiwe University Awka –Prof. Uzochukwu. – and the head of Department of Pharm. Microbiology and biotechnology–Dr Oli Angus - as well as other staff of the Department .

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List of Abbreviations

ATCC:	American typed culture collection
BDCP:	Bioresource Development and Conservation programme
CLSI:	Clinical Laboratory Standard Institute
DMSO :	Dimethylsulphoxide
DNA :	Deoxyribonucleic acid
EO :	Essential oil
FIC :	Fractional inhibitory concentrations
FQ:	Fluoroquinolone
FQREC:	Fluoroquinolone-resistant *Escherichia coli*
FQRSA :	Fluoroquinolone-resistant *Staphylococcus aureus*
GCMS :	Gas chromatography-mass spectrometry
HV:	Healthy volunteer
IMVIC :	Indole production, methyl red, voges proskae and citrate utilization test
IZD:	Inhibition zone diammeter
MARI :	multiple antibiotic resistance index
MBC:	Minimum bacteriocidal concentration
MIC :	Minimum inhibitory concentration
NARICT;	National Research Institute for Chemical Technology
NIMR;	Nigerian Institute of Medical Research
PCR ;	Polymerase chain reaction

PT :	Patient
QRQR;	Quinolone resistance determining region
ESBLs	Extended spectrum beta-lactamases
PBPs	Penicillin-binding proteins
UTIs	Urinary tract infections
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
EAEC	Enteroaggregative *E. coli*
HUS	Haemolytic-uremic syndrome
CF	Cystic fibrosis
HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
EDTA	Ethylene diamine tetraacetic acid
SPM	Sao Paulo metallo-beta-lactamase
MH	Mueller Hinton
EMB	Eosin methylene blue
MR	Methyl red test
VP	Voges proskauer
NCCLS	National Committee for Clinical Laboratory Standard
PCR	Polymerase chain reaction
SPSS	Statistical Package for Social Sciences

ORGANISMS

E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*

UNITS AND SYMBOLS

α:	Alpha
β	Beta
Zn^{2+}	Zinc ions
mg/ml	Milligram per mill
µg	Microgram
%	Percentage
CI	Confidence interval
n	Number of isolates/sample size
^{o}C	Degrees Celsius
ml	Mill
mm	Millimeter
≤	Less or equal to
≥	Greater or equal to
rpm	Revolutions per minute
TE	Tris-EDTA
µl	Micro liter
$MgCl_2$	Magnesium chloride
pM	pica mole

Taq	*Thermus aquaticus*
Mm	molar mole
bp	Base pair
min	Minutes
EtBr	Ethidium bromide
TBE	Tris boric acid EDTA
nm	Nanometer
UV	Ultraviolet
M	Mole
v	Voltage
NaCl	Sodium chloride

ABSTRACT

The increased emergence of fluoroquinolone-resistant *Escherichia. coli* and *Staphylococcus aureus* isolates of human and animal origin is a global public health problem. This is because infections caused by these isolates are presently associated with high mortality, morbidity and high drug treatment costs as there are little or no treatment options available. The aim of the study was to isolate, characterize, investigate on the epidemiology and to carry out antimicrobial studies on fluoroquinolone-resistant *E. coli* (FQREC) and *Staph. aureus* (FQRSA) isolates from farm animals and human in seven health districts in Enugu State. Specific objectives include: determination of the prevalence and distribution of FQRSA and FQREC as well as some of their resistant genes –*Qnr A, gyr A* and *Nor A* genes in the study area; evaluating the contribution of efflux pump inhibitor on fluoroquinolone resistance and evaluation of the antibacterial potentials of *Cymbopogon citratus* oil and *Cocos nucifera* essential oils alone and in combination with ciprofloxacin against the test isolates. A total of 7980 specimens of urine, faecal matter, and nasal, wound and skin swabs were collected using sterile containers and swab-sticks by random sampling techniques from humans and farm animals in Enugu State. Eight hundred and forty samples each of urine, faecal matter and nasal swabs were collected from both healthy carriers and patients, and 420 wound swabs were collected from patients alone. Futher, 1680 samples of nasal, vendors table, skin and anal swabs were collected from cattles, pigs and chickens. Isolation of *E. coli* and *Staph. aureus* were carried out using MacConkey, mannitol salt and blood agar. Identification was by Gram staining, catalase reaction, coagulase test and polymerase chain reaction. Antimicrobial 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 and resistant genes by conventional polymerase chain reaction. *In vitro* interactions of essential oils of *Cymbopogon citratus* and *Cocos nucifera* with ciprofloxacin were done by Checkerboard and thin overlay innoculum susceptibility disc methods respectively. One way ANOVA was used for data analysis using SPSS version 16. A total of 3407 *E. coli* and *Staph. aureus* comprising 920 animal isolates and 2487 human isolates were recovered from urine, faecal matter, nasal, wound and skin swabs. The prevalence of ciprofloxacin, ofloxacin, levofloxacin and pefloxacin resistance among *E.coli* isolates from the subjects were: human (12.5, 12.2, 12.6 and 13.2%), pig (5.7, 6.1, 5.7 and 7.8%), cattle (0, 0, 0 and 0%) and chicken (13.6, 14.3, 11.6 and 17.7%) respectively. The prevalence of ciprofloxacin, ofloxacin, levofloxacin and pefloxacin resistance among *Staph. aureus* isolates from the subjects were: human (21.1, 21.6, 19.4 and 22.5 %), pig (4.1, 3.4, 2.7 and 4.8 %), cattle (7.5, 7.5, 5.8 and 13.3%) and chicken (13.3 ,13.3, 13.3, and 13.3%) respectively. The results showed the range of prevalence of genes in both humans and animals to be *gyrA*, 21.4-63.4% and *qnrA*, 7.1-22.6% in FQREC and *NorA*, 0-71.4% in FQRSA. A total of 223 plasmids were detected and cured to the range of 36.4-100%, confirming the contribution of plasmid in mediating fluoroquinolone resistance in these isolates. The presence of efflux pump inhibitor (omeprazole) at 128 µg/ml resulted in a reduction in the ciprofloxacin MIC (2- to 16-fold) for FQRSA, and for FQREC (omeprazole at 64 µg/ml), the MIC values were increased for most of the isolates. Essential oil of *Cymbopogon citratus* inhibits the growth of FQRSA and FQREC and also exhibit synergism with ciprofloxacin on many of the isolates. This study showed that the sensitivity of FQREC and FQRSA increases in the presence of ciprofloxacin and *Cymbopogon citratus* essential oil combination. The prevalence of FQRSA and FQREC and their resistant genes are high in Enugu State.

CHAPTER ONE

1.0 Introduction

Escherichia coli is a Gram negative rod-shaped bacterium that is commonly found in the intestines of humans and animals. It was discovered by German pediatrician and bacteriologist Theodor Escherichia in 1885, and is now classified as part of the Enterobacteriaceae family of gamma-proteobacteria. It colonizes human and animal gut within hours of birth. However, *E. coli* has a dichotomous existence; while the majority of *E. coli* strains exist within the mammalian intestinal tract as harmless commensals, paradoxically several evolutionary lineages have deviated from this harmless lifestyle to become pathogens (Khachatryan *et al*., 2008).

Current dogma suggests that such latter strains of *E. coli* have acquired additional genetic elements, encoding specific virulence factors, which enable the organism to cause disease when infecting an otherwise healthy individual (Rosongren *et al*., 2009). The resulting clinical syndromes include extraintestinal infections such as urinary tract infections, septicaemia and meningitis, and intestinal infections manifesting as diarrhea. Those strains causing intestinal infections can be divided into six pathotypes viz. Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohaemorrhagic *E. coli* (EHEC) and Diffusely Adhering *E. coli* (DAEC) (Kaper *et al*., 2004). The pathotype to which particular strain belongs is defined by the clinical manifestations of disease, the repertoire of virulence factors, epidemiology and phylogenetic profiles (Johnson *et al*., 2009). Those causing extraintestinal infections include Uropathogenic *E. coli* (UPEC) and Meningitis-associated *E. coli* (MNEC). These pathotypes are called Extraintestinal Pathogenic *E. coli*, ExPEC (Bhavsar *et al*., 2007).

Staphylococcus aureus. S*taphylococcus aureus* is a facultative anaerobic, Gram-positive bacterium discovered by Dr. Alexander Ogston in 1880. Literature reports suggest that about 30 to 50% of the population has been carriers of *S. aureus* at one time in their lives (Lowy, 1998). Asymptomatic carriage of *Staphylococcus aureus* is especially common, particularly in the anterior part of the nasal cavity, where a prevalence rate of around 20% has been reported (Abudu *et al*., 2001). Apart from nasal and nasopharyngeal carriage, another common site for *S aureus* colonization is the skin, particularly the inguinal fold, rectum and axilla. (Horner *et al*., 2013). The bacterial strains can display an altered antibiotic sensitivity profile, particularly in elderly patients, with comorbid diseases, causes of immune suppression, previous antibiotherapy or history of prior hospital admission during the pasts months (Fritz *et al.*, 2012). In children, data on nasal carriage appears to be somewhat similar to the prevalence reported in adults, but there are certain peculiarities. Generally, *S. aureus* produces three broad disease types: a variety of superficial infections such as pimples, boils, abscesses and toxic epidermal necrolysis (characterized by outer layer of skin separating from deeper layers); systemic infections such as pneumonia, meningitis endocarditis (inflammation of heart valves), osteomyelitis (inflammation of bone or bone marrow), and septicemia and toxinoses such as food poisoning or toxic shock syndrome (Tong *et al*.,2015; Fowler *et al*., 2006). According to reports of the National Institutes of Health and Centers for Disease Control and Prevention, *S. aureus* infects 500,000 people yearly in America, more than 94,000 of which are cases of life-threatening, antibiotic-resistant *S. aureus* infections.(Klein *et al*., 2007). Both community-associated and hospital-acquired infections with *S. aureus* have increased in the past 20 years, and the rise in incidence has been accompanied by a rise in antibiotic-resistant strains—in particular, methicillin-resistant *S aureus* (MRSA).

MRSA is difficult to treat and has very limited treatment options. Vancomycin is the only drug of choice but there have been many reports of development of low grade to absolute resistance even to vancomycin from many parts of the globe (Robert *et al*., 2006, Bal *et al*., 2008).

Fluoroquinolone compounds such as ciprofloxacin and norfloxacin, first synthesized in the 1980s, were found to have extended antimicrobial spectra that included both Gram-positive and Gram-negative bacteria, and were hoped to be useful in eradicating multi-drug resistant bacteria such as MRSA (Gilbert *et al*., 1986). The introduction of fluoroquinolones more than 10 years ago offered clinicians orally and parenterally administrable compounds with a broad spectrum of activity and therapeutic results not seen before for a wide range of infections. Extensive use and misuse of these compounds led to the emergence and spread of resistant strains. Consequently, the use of these agents for treatment of infections have been affected due to the rapid emergence of resistance both *in vitro* and in the clinical setting (Kaartz and Seo, 1997). 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" (Acar and Goldstein, 1997).

1.1 Statement of problems.

The increasing occurrence of fluoroquinolone-resistant organisms especially fluoroquinolone-resistant *E. coli* and *S. aureus* isolates of human and animal origin is a global public health problem. Infections caused by fluoroquinolone-resistant *E. coli* and *S. aureus* are presently associated with higher morbidity, mortality, and invariably higher expenditure in treatment compared with infections caused by strains

susceptible to the drugs. Bacterial resistance to antimicrobials was first discovered in the 1940s, following the introduction of penicillin. The emergence of resistance to fluoroquinolones in virtually all species of bacteria was recognized soon after the introduction of these compounds for clinical use (McDonald *et al.*, 2001). The emergence and dissemination of resistant bacteria is an inevitable side effect of uncritical use of antimicrobials (Van den Bogaard and Stobberingh, 2000). When antimicrobials are constantly added to feeds at a subtherapeutic or therapeutic level, microorganisms develop resistance. Furthermore, the use of antimicrobials speeds up the spread of genes that encode resistance to them. (Taylor, 2001) After the introduction of a new antimicrobial not only the resistance rate of pathogenic bacteria, but also of commensal bacteria increases.

Commensal bacteria constitute a reservoir of resistance genes for (potential) pathogenic bacteria. Resistant commensal bacteria of food animals, including zoonotic bacteria, might contaminate meat and its products and thereby enter the intestinal tracts of human beings (Gorbach, 2001). Animal wastes including manures may contain high levels of bacteria with antimicrobial resistance, and are dispersed into the soil and water where we grow our crops and can eventually leach into our groundwater, lakes and rivers, and cause further contamination of our drinking water, fish and environment. (Taylor, 2001). When the resistant bacteria cause illness in a person, the medical therapy may be compromised due to difficulties in treatment (Heymann, 2000). Knowing full well that the increasing prevalence of FQ resistance is of significant public health concerns, given the association between the FQ-resistance and poor clinical outcomes, including increased mortality (Camins *et al.*, 2011; Lautenbach *et al.*, 2005), it is imperative that a detailed epidemiological and antimicrobial studies be conducted on the fluoroquinolone resistant *E. coli* and *S.*

aureus with emphasis on prevalence of these resistant isolates as well as fostering solutions through drug combinations involving alternative medicines. This is urgently needed because *E. coli* and *S. aureus* are the most common bacteria isolated from microbiology culture and /or the most common cause of morbidity and mortality in both hospital and community setting (Gaynes and Edwards, 2005; Sahm *et al*., 2001), within and outside Enugu State.

1.2 Justification

The menace posed by fluoroquinolone-resistant *E. coli,* and *S. aureus* necessitates the need to detect by phenotypic and molecular techniques the prevalence of these organisms and their resistant genes in both the hospitals and community. No information is available on the prevalence of FQREC and FQRSA isolates and fluroquinolone resistance genes with respect to specific specimen source in Enugu State, South Eastern Nigeria. This is because other studies (Van den Bogaard and Stobberingh, 1999; Nsofor and Iroegbu, 2013a) were not specific with respect to human specimen source (i.e whether it was specimen of urine, nasal swab, stool from healthy volunteers or patients previously or currently on antibiotics), animal specimen source (whether from faecal swab, skin or meat itself in term of animal) (Nsofor and Iroegbu, 2013b), subject age and sex. Moreso, documented information on the antimicrobial studies involving orthodox antibiotics and essential oil from lemongrass and coconut oil together with their interaction are lacking in the region. This project helped to detect by phenotypic and molecular detection methods the prevalence of fluoroquinolone-resistant *E. coli,* and *S. aureus* and their resistant genes from farm animals and humans in Enugu State , Nigeria.

1.3 Hypothesis

The1 hypothesis of this research work is as follows:

- Prevalences of fluoroquinolone-resistant *S. aureus* and *E. coli* are high in Enugu State
- *S. aureus* and *E. coli* are habouring plasmids and fluoroquinolone resistant genes
- Both plasmids and fluoroquinolone-resistant genes are responsible for the antimicrobial resistance nature of some *S. aureus* and *E. coli*.
- *S. aureus* and *E. coli* habouring plasmids and fluoroquinolone-resistant genes can be found in both farm animals and humans

1.4 THE AIM AND OBJECTIVES OF THE STUDY

The aim of the study was to isolate, characterize, investigate the epidemiology and to carry out antimicrobial studies on fluoroquinolone-resistant *E. coli* (FQREC) and *S. aureus* (FQRSA) in Enugu State

The specific objectives of the research are as follows

1. To determine the antibiotics resistance pattern of all the *S. aureus and E. coli* isolates to commonly used antibiotics in the study area.
2. To determine the prevalence and distribution of fluoroquinolone-resistant *S. aureus and E. coli* in the study area.
3. To determine the prevalence and distribution of fluoroquinolone-resistant genes– *Qnr A* and *gyr A* gene in *E .coli, Nor* A gene in *S .aureus* isolates.
4. To evaluate the contribution of efflux pump inhibitor in the susceptibility of the test isolates to fluoroquinolones.
5. To study the distribution of resistance plasmid in all the fluoroquinolone resistant isolates
6. To evaluate the antibacterial potentials of lemon grass essential oil and coconut oil singly and in combination with ciprofloxacin against the test isolates

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Staphylococcus aureus (S. aureus)*

Staphylococcus aureus is a gram-positive, round-shaped, bacterium that is a member of the Firmicutes. It is a ubiquitous bacterium and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase, coagulase and nitrite and is a aerobic as well as facultative anaerobe that can grow with or without the need for oxygen (Masalha, 2001).

2.1.1 Microbiology of *S. aureus*

S. aureus, a Gram positive coccus,. An estimated 30–50% of healthy subjects are intermittently or chronically colonized with *S. aureus*, with chronic nasal carriage being a risk factor for *S. aureus* bacteraemia (Wertheim *et al*., 2004). *S. aureus* grows typically aerobically but also as facultative anaerobe and is capable of biofilm formation. For epidemiological purposes multiple typing systems have been developed for methicillin sensitive *S. aureus* (MSSA) and MRSA. These include pulse-filed gel electrophoresis (PFGE), multilocus sequence typing (MLST), and typing of the variable tandem repeat region of staphylococcal protein A (*spa* typing). Additionally MRSA isolates are distinguished by the Staphylococcal Casette Chromosome (SCC) *mec* types, which carries the gene for methicillin resistance. To date, at least eight SCC*mec* types have been distinguished, however new types are being described (Li *et al*., 2011). Whereas *SCCmec* types I, IV, and V encode exclusively for beta-lactam resistance, the larger *SCCmec* types II and III carry non-beta-lactam antibiotic resistance genes. Since 1960 ~80% of all *S. aureus* isolates have been resistant to penicillin and within 2 years of introduction of methicillin in 1959, *S.*

aureus strains developed resistance to methicillin through the acquisition of the *mec*A gene (MRSA). Early MRSA isolates were only associated with hospital acquisition (HA), however since approximately 1990s, community associated (CA) MRSA emerged. CA-MRSA generally differs in genetic background from HA-MRSA, is associated with SCC*mec* IV, V or VII, and tends to be resistant to fewer antibiotic classes. Given outbreaks of CA-MRSA in the hospital or HA-MRSA spreading into the community (Seybold *et al*., 2006), the distinction between HA- and CA-MRSA is increasingly difficult. Genetic interchange between MRSA strains also make molecular classification complicated (Lindsay, 2010). Classification as CA- versus HA-MRSA in fact may vary depending which approach is employed: epidemiologic, SCC*mec* type, PVL status, or sensitivity to clindamycin (David *et al*., 2008). Worldwide, CA-MRSA strains differ in their *SCCmec* type, PFGE pattern, and MLST and *spa* profiles; in the US for instance the most frequent strain is USA-300, which is increasingly being reported in European countries in cystic fibrosis (CF) or non CF subjects (Deurenberg and Stobberingh, 2009). *S. aureus* isolates harbor a multitude of virulence factors, which overlap to a large degree in MSSA and MRSA. The leukocytolytic toxin Panton-Valentine leukocidin (PVL) is more frequently expressed in MRSA than MSSA strains. PVL has been epidemiologically associated with severe cutaneous infections and has initially been attributed as the main cause for severe, necrotizing lung infections based on clinical observations and experiments in animals using isolated PVL (Labandeira-Rey *et al*., 2007). More recently the role of PVL as the main virulence factor for necrotizing lung infections has been questioned (Voyich *et al*., 2006).

2.1. 2. *Staphylococcus aureus,* Components and Products

S. aureus is distinguished from other Staphylococcal species on the basis of the gold pigmentation of colonies and positive results of coagulase, mannitol-fermentation, and deoxyribonuclease tests (Wilkinson, 1997). *S. aureus* consists of the following components and products; genome, cell wall, capsule, surface proteins, toxins, enzymes and other products.

The Staphylococcal genome consists of a circular chromosome (of approximately 2800 bp), with prophages, plasmids, and transposons. Genes governing virulence and resistance to antibiotics are found on the chromosome, as well as the extrachromosomal elements (Novick, 1990).

The Staphylococcal cell wall is 50 percent peptidoglycan by weight. Peptidoglycan consists of alternating polysaccharide subunits of *N*-acetylglucosamine and *N*-acetylmuramic acid with 1,4-β linkages. The peptidoglycan chains are cross-linked by tetrapeptide chains bound to *N*-acetylmuramic acid and by a pentaglycine bridge specific for *S. aureus*. Peptidoglycan may have endotoxin-like activity, stimulating the release of cytokines by macrophages, activation of complement, and aggregation of platelets. Differences in the peptidoglycan structure of staphylococcal strains may contribute to variations in their capacity to cause disseminated intravascular coagulation (Kessler, 1991). Ribitol teichoic acids, covalently bound to peptidoglycan, are major constituents of the cell wall. Lipoteichoic acid is a glycerol phosphate polymer linked to a glycolipid terminus anchored in the cytoplasmic membrane. Most Staphylococci spp produce microcapsules. Of the 11 types of microcapsular polysaccharide serotypes that have been identified, types 5 and 8 account for 75 percent of human infections. Many staphylococcal surface proteins

have certain structural features in common. These features include a secretory signal sequence at the N terminal, positively charged amino acids that extend into the cytoplasm, a hydrophobic membrane-spanning domain, and a cell-wall–anchoring region, all at the carboxyl terminal. Protein A, the prototype of these proteins, has antiphagocytic properties that are based on its ability to bind the Fc portion of immunoglobulin.Several of these related proteins bind extracellular-matrix molecules and have been designated microbial-surface components recognizing adhesive matrix molecules (MSCRAMM). Recent studies suggest that these proteins play an important part in the ability of Staphylococci spp to colonize host tissue.(Patti *et al* ., 1994)

Staphylococci produce numerous toxins that are grouped on the basis of their mechanisms of action. Cytotoxins, such as the 33-kd protein-alpha toxin, cause pore formation and induce proinflammatory changes in mammalian cells. The consequent cellular damage may contribute to manifestations of the sepsis syndrome.(Bhakdi and Tranum-Jensen, 1991, Walev *et al.*, 1995). The pyrogenic-toxin superantigens are structurally related, sharing various degrees of amino acid sequence homology. They function as superantigens by binding to major histocompatibility complex (MHC) class II proteins, causing extensive T-cell proliferation and cytokine release.(Marrack and Kappler, 1990). Different domains of the enterotoxin molecule are responsible for the two diseases caused by these proteins, the toxic shock syndrome and food poisoning (Harris *et al*., 1993). Despite little amino acid sequence homology, toxic shock syndrome toxin 1 is structurally similar to enterotoxins B and C. The gene for toxic shock syndrome toxin 1 is found in 20 percent of *S. aureus* isolates (Marrack and Kappler, 1990). The exfoliative toxins, including epidermolytic toxins A and B, cause skin erythema and separation, as seen in the staphylococcal scalded skin

syndrome. The mechanism of action of these toxins remains controversial. Panton–Valentine leukocidin is a leukocytolytic toxin that has been epidemiologically associated with severe cutaneous infections.(Cribier *et al*., 1992).

Staphylococci produce various enzymes, such as protease, lipase, and hyaluronidase, that destroy tissue. These bacterial products may facilitate the spread of infection to adjoining tissues, although their role in the pathogenesis of disease is not well defined.

2.1.3. Epidemiology of Staphylococcal Disease

2.1.3.1 Colonization and Infection

Humans are a natural reservoir of *S. aureus*. It is part of the normal microbiota present in the upper respiratory tract,(Schenck *et al,* 2016) and on skin and in the gut mucosa (Wollina, 2017). *S. aureus*, along with similar species that can colonize and act symbiotically but can cause disease if they begin to take over the tissues they have colonized or invade other tissues, have been called "pathobionts".(Schenck *et al,* 2016). While *S. aureus* usually acts as a commensal bacterium, asymptomatically colonizing about 30% of the human population, it can sometimes cause disease (Tong *et al*.,2015)

2.1.3.2 Transmission

Persons colonized with *S. aureus* strains are at increased risk of becoming infected with these strains. Most cases of nosocomial infection are acquired through exposure to the hands of health care workers after they have been transiently colonized with staphylococci from their own reservoir or from contact with an infected patient. Outbreaks may also result from exposure to a single long-term carrier or environmental sources, but these modes of transmission are less common (Casewell and Hill, 1986).

2.1.4 Molecular Pathogenesis of *Staphylococcus aureus* Infection

S. aureus has a diverse arsenal of components and products that contribute to the pathogenesis of infection. These components and products have overlapping roles and can act either in concert or alone. A great deal is known about the contribution of these bacterial factors to the development of infection (Waldvogel, 1995; Foster *et al*, 1997; Crossley and Archer, 1997). Considerably less is known about their interaction with each other and with host factors and their relative importance in infection. The virulence of *S. aureus* infection is remarkable, given that the organism is a commensal that colonizes the nares, axillae, vagina, pharynx, or damaged skin surfaces (Tong *et al*., 2016). Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. Whether an infection is contained or spreads depends on a complex interplay between *S. aureus* virulence determinants and host defense mechanisms. The biology of colonization of the nares, the primary reservoir for staphylococci, is incompletely understood. Mucin appears to be the critical host surface that is colonized in a process involving interactions between staphylococcal protein and mucin carbohydrate (Shuter *et al.*, 1996). *S. aureus* adheres and invades host epithelial cells using a variety of molecules that are collectively termed Microbial surface components recognizing adhesive matrix molecules (MSCRAMM). A number of bacterial products (including MSCRAMM) have been suggested to be important for adhesion and attachment to nasal epithelial cells, but two factors (clumping factor B and wall-associated teichoic acid) have so far proven roles in nasal colonization of humans and rats (Patti *et al*.,1994). The role of other commensals, secretory IgA, or specific staphylococcal adhesins is unknown.The risk of infection is increased by the presence of foreign material. Several factors contribute to the increased susceptibility to infection. Devices such as intravenous

catheters are rapidly coated with serum constituents, such as fibrinogen or fibronectin, which enable staphylococci to adhere through MSCRAMM-mediated mechanisms and to elaborate glycocalices that further facilitate colonization (Cheung and Fischetti, 1993, Vaudaux *et al*., 1993). Intravenous catheters are frequently implicated in the pathogenesis of nosocomial endocarditis.

2.1.4.1 Invasive Infections

Staphylococcal bacteremia may be complicated by endocarditis, metastatic infection, or the sepsis syndrome. The endothelial cell is central to these pathogenic processes. Not only is it a potential target for injury, but also its activation contributes to the progression of endovascular disease. Staphylococci avidly adhere to endothelial cells and bind through adhesin–receptor interactions (Vercellotti *et al*.,1984, Ogawa *et al*.,1985 and Tompkins *et al*.,1990). *In-vitro* studies demonstrated that after adherence, staphylococci are phagocytized by endothelial cells. The intracellular environment protects staphylococci from host defense mechanisms as well as the bactericidal effects of antibiotics. Vesga *et al*.,(1996) demonstrated that the intraendothelial-cell milieu fosters the formation of small-colony variants. These factors may enhance bacterial survival and contribute to the development of persistent or recurrent infections (Proctor *et al*.,1995). Staphylococcal strains that cause endocarditis are resistant to serum, adhere to both damaged and undamaged native valvular surfaces, are resistant to platelet microbicidal proteins (Wu *et al*., 1994) and elaborate proteolytic enzymes that facilitate spread to adjacent tissues. The adherence of staphylococci to the platelet–fibrin thrombus that forms on damaged valvular surfaces may involve the adherence of MSCRAMM proteins to exposed matrix molecules. Staphylococcal endocarditis also occurs on undamaged valves. The

invasion of endothelial cells by *S. aureus* may initiate the cellular alterations, including the expression of tissue factor, that promote the formation of vegetations. The potential role of MSCRAMM is best illustrated by collagen-binding protein. Its presence facilitates infection of bones and joints in animals (Patti *et al.*,1994). The cellular events leading to septic shock are similar in staphylococcal infection and infection with Gram-negative bacteria. In both cases, monocytes and macrophages have a central role, although polymorphonuclear leukocytes, endothelial cells, and platelets also play a part. The monocytes release tumor necrosis factor α and interleukin-1, interleukin-6, and interleukin-8 after contact with intact staphylococci, peptidoglycan, or lipoteichoic acid (Timmerman *et al*., 1993; Heumann *et al*.,1994). In contrast, the expression of interleukin-1 and interleukin-6 by endothelial cells requires bacterial phagocytosis (Yao *et al*., 1995) As a result of cytokine and cellular activation, the complement and coagulation pathways are activated, arachidonic acid is metabolized, and platelet-activating factor is released. These events, in turn, cause fever, hypotension, capillary leak, disseminated intravascular coagulopathy, depression of myocardial function, and multiorgan dysfunction. Several staphylococcal components appear to be capable of initiating the sepsis syndrome (Bone, 1994) Peptidoglycan, especially when combined with lipoteichoic acid, reproduces many of the physiologic responses of endotoxin in animal models of sepsis (Spike *et al*., 1982; De Kimpe *et al*.,1995). Alpha toxin alone reproduces many of the findings of sepsis, including hypotension, thrombocytopenia, and reduced oxygenation, in animal models (Bohach *et al*., 1990).

2.1.4.2. Toxin-Mediated Disease

Pyrogenic-toxin superantigens cause life-threatening disease that is characterized by the rapid onset of high fever, shock, capillary leak, and multiorgan dysfunction. Superantigens are T-cell mitogens that bind directly to invariant regions of MHC class II molecules, bypassing intracellular protein ingestion and digestion and subsequent peptide presentation by antigen-presenting cells. The MHC-bound superantigens then attach to T cells according to the composition of the variable region of the T-cell–receptor β chain. Toxic shock syndrome toxin 1 binds all variable-region β2–positive T cells, causing an expansion of clonal T cells (5 to 20 percent of resting T cells as compared with 0.01 percent of T cells for processed antigens), resulting in the massive release of cytokines by both macrophages and T cells. These cytokines mediate the toxic shock syndrome, whose pathophysiology mimics that of endotoxin shock. In both syndromes, bacterial products induce the release of excessive quantities of cytokines, which then cause tissue damage. (Bohach *et al*., 1990).

2.1.4.3. Host Response to Infection

The typical pathological finding of staphylococcal disease is abscess formation. Leukocytes are the primary host defense against *S. aureus* infection (Verdrengh and Tarkowski, 1997) .The migration of leukocytes to the site of infection results from the orchestrated expression of adhesion molecules on endothelial cells. This cytokine-mediated process is triggered by bacteria and tissue-based macrophages. After infection, cytokines are first demonstrable within vessels, extending into tissues as inflammatory cells migrate to the sites of infection (Yao *et al*., 1997). *S. aureus*–infected endothelial cells also express intercellular adhesion molecule 1 (CD54), vascular-cell adhesion molecule 1 (CD106), and MHC class I molecules and probably contribute to this process (Beekhuizen *et al.*, 1997). The presence of opsonizing

antibody directed against capsule, peptidoglycan, or complement facilitates phagocytosis *in vitro* (Karakawa *et al*.,1988). The role of antibody *in vivo* is less certain, since the titer of antistaphylococcal antibodies is not correlated with protection from infection, except in the case of toxic shock syndrome, in which the presence of anti–toxic shock syndrome toxin 1 is protective (Wergeland *et al*., 1989; Freedman and Beer, 1991). At present, it is not known which staphylococcal components are capable of inducing protection from subsequent infection.

2.1.4.4. Antibiotic Resistance in *Staphylococcus aureus*

High replication rates coupled with the great ability to perform horizontal gene transfer (especially through conjugation) allow bacteria to develop antibiotic resistance and to spread it quickly. By 1942, the first penicillin resistant strains of *S. aureus* had been isolated in hospitals (Deurenberg *et al.*, 2007). These penicillin resistant strains contained a plasmid encoding a penicillin-hydrolyzing enzyme, penicillinase. Less than 20 years after the first strains of *S. aureus* were found to be resistant to penicillin, 80% of all strains had acquired penicillin resistance. As new antibiotics such as methicillin and vancomycin were used to fight *S. aureus*, resistance to these antibiotics also began to develop. Methicillin was first used to treat *S. aureus* in 1959 and just after 2 years of use, methicillin-resistant *S. aureus* (MRSA) strains had be isolated (Deurenberg *et al*.,2007). Methicillin resistance first developed and became transferable through the mecA gene. The mecA gene encodes a protein, penicillin-binding protein PBP2a, which cannot be bound by beta-lactam antibiotics (penicillin, methicillin…) and in turn prevents the disruption of cell wall formation by these antibiotics. This gene is located on mobile genetic element called the Staphylococcal Cassette Chromosome mec (SCCmec) (Deurenberg *et al*., 2007).

2.2 *Escherichia coli*

What is *Escheichia coli?*

Escherichia coli (also known as *E. coli*) is a gram-negative, facultatively anaerobic, rod-shaped, coliform bacterium of the genus Escherichia that is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Tenaillon *et al*., 2010). It is one of the most frequently encountered bacterial species of animal and human commensal intestinal flora (McDonald *et al*., 2001). This organism is used as an indicator of recent surveillance programmes to monitor the occurrence of antimicrobial resistance in the enteric microflora of both humans and farm animals (Tuber, 1999). In addition, *E. coli* is the primary cause of urinary tract infections in humans (McDonald *et al*., 2001) and is the most frequent nosocomial and community-acquired pathogen in all regions (Diekema *et al*.,1990). Resistance to fluoroquinolones develops more rapidly in *E. coli* than in other members of the Enterobacteriaceae (Gales *et al*.,1998).

2.2.1 Molecular mechanism of *Escherichia coli* Pathogenicity

Escherichia coli typically colonizes the gastrointestinal tract of humans and animals within a few hours after birth. Usually, *E. coli* and its human or animal host coexist in good health and in symbiotic relationship for decades. These commensal *E. coli* strain rarely cause disease except in immunocompromised hosts or where the normal gastrointestinal barriers are breached as in peritonitis, for example. The niche of commensal *E. coli* is the mucous layer of the mammalian colon. The bacterium is a highly successful competitor at this crowded site, comprising the most abundant facultative anaerobe of the human and animal intestinal microflora. Despite the enormous body of literature on the genetics and physiology of this species, the

molecular mechanisms whereby *E. coli* assures this auspicious symbiotic relationship in the colon are poorly characterized. One interesting hypothesis suggests that *E. coli* might exploit its ability to utilize gluconate in the colon more efficiently than other resident species, thereby allowing it to occupy a highly specific metabolic niche (Croxen and Finlay, 2010). However, there are several highly adapted *E. coli* clones that have acquired specific virulence attributes, which confer an increased ability to adapt to new niches and allows them to cause a broad spectrum of diseases. These virulence attributes are frequently encoded on genetic elements that can be mobilized into different strains to create novel combinations of virulence factors, or on genetic elements that might once have been mobile, but have now evolves to become `locked` into the genome. Only the most successful combinations of virulence factors have persisted to become specific `PATHOTYPES of *E. coli* that are capable of causing disease in healthy individuals and animals. These pathotypes produce three broad clinical syndromes; enteric/diarrhea disease, urinary tract infections (UTIs) and sepsis/meningitis. Among the intestinal pathogens there are six well-described categories- *Enteroaggregative E. coli (EAEC), Enteropathogenic E. coli (EPEC), Enterohemorrhagic E. coli (EHEC), Enterotoxigenic E. coli (ETEC), Enteroinvasive (EIEC)* and *Diffusely adherent E. coli (DAEC)* (Kaper *et al*., 2004). UTIs are the most common extraintestinal *E. coli* infections and are caused by Uropathogenic *E. coli UPEC).* An increasingly common cause of extraintestinal infections is the pathotype responsible for meningitis and sepsis- *Meningitis-associated E. coli (MNEC).* The *E. coli* pathotypes implicated in extraintestinal infections are called Extraintestinal pathogenic *E. coli*; ExPEC (Bhavsar *et al*., 2007). Enteropathogenic *E. coil*, Enterohemorrhagic *E. coli*, and Enterotoxigenic *E . coli* can also cause disease in animals using many of the same virulence factors that are present in human-strains

and unique colonization factors that are not found in human. An additional animal pathotype. Known as Avian pathogenic *E. coli (APEC)*, cause extraintestinal infections primarily respiratory infections, pericarditis, and septicemia of poultry - (Croxen and Finlay, 2010). The various pathotypes of *E. coli* tend to be clonal groups that are characterized by shar ed O (lipopolysaccharide, LPS) and H (flagella) antigens that define serogroups (O antigen only) or serotypes (O and H antigens) (Shames *et al*., 2009). Pathogenic *E. coli* strains use a multi-step scheme of pathogenesis that is similar to that used by other mucosal pathogens, which consists of colonization of a mucosal site, evasion of host defenses, multiplication and host damage. Most of the pathogenic *E. coli* strains remain extracellular, but EIEC is a true intracellular pathogen that is capable of invading and replicating within epithelial cells and macrophages. Other *E. coli* strains might be internalized by epithelial cells at low levels, but do not seem to replicate intercellularly.

2.2.1.1 Adhesion/colonization.

Pathogenic *E. coli* strains posses specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra. Most frequently, these adhesions form distinct morphological structures called fimbriae (also called pili or fibrillae), which can belong to one of several different classes. Fimbriae are rod-like structures of 5-10 nm diameter that are distinct from flagella. Fibrillae are 2-4 nm in diameter, and are either long and wiry or curly and flexible (Asadulghani *et al*., 2009). The afa adhesins that are produced by many diarrhoeagenic and uropathogenic *E. coli* are described as fimbrial adhesins , but in fact seem to have a fine fibrillar structure that is difficult to visualize (Ogura, 2009). Adhesins of pathogenic *E. coli* can also include outer-membeane proteins, such as intimin of UPEC and EHEC, or other non-fimbrial proteins. Some surface structures

trigger signal transduction pathways or cytoskeletal rearrangement that can lead to disease. For example, the members of the dr family of adhesins that are expressed by DAEC and UPEC bind to the Decay-Accelerating Factor (DAF, also known as C55) which results in activation of phosphatidylinositol 3-Kinase (PI-3-Kinase) and cell-surface expression of the Major Histocompatibility Complex (MHC) class I-related molecule (Croxen and Finlay, 2010).

The IcsA protein of EIEC nucleates actin filaments at one pole of the bacterium, which allows it to move within the cytoplasm and into adjacent epithelial cells on a 'tail' of polymerized actin (Rasko *et al*., 2008). Even surface structure that are present on commensal *E. coli* strains can induce signaling cascades if the organism encounters the appropriate receptor. The LPS of *E. coli* and other Gram-negative bacteria binds to Toll-like receptor 4 (TLR4), triggering a potent cytokine cascade that can lead to septic shock and death (Rasko *et al*., 2008). Flagellin, the main component of flagella, can bind to (TLR4), thereby activating interieukin (IL-8) expression and an inflammatory response (Lloyd *et al*,. 2007).

2.2.1.2. Toxins.

More numerous than surface structures that trigger signal transduction pathways are secreted toxins and other effector proteins that affect a variety of basic eukaryotic biological processes. Concentrations of important intracellular messengers, such as cyclic AMP, cyclic GMP and Ca2+, can be increased, which leads to ion secretion by the actions of the heat-labile enterotoxin (LT), heat-stable enterotoxin a (STa) and heat-stable enterotoxin b (STb), respectively – all of which are produced by different strains of ETEC. The Shiga toxin (STx) of EHEC cleaves ribosomal RNA, thereby disrupting protein synthesis and killing the intoxicated epithelial or endothelial cells (Maurelli, 2007). The cytolethal distending toxin (CDT) has DNase activity that

ultimately blocks cell division in the G2/M phase of the cell cycle (Maurelli, 2007). Another toxin that blocks cell division in the same phase, called Cif (cycle-inhibiting factor), does not possess DNase activity, but might act by inhibition of Cdk1 kinase activity (Maurelli, 2007). The cytotoxic necrotizing factors (CNF 1 and CNF 2) deaminate a crucial glutamine residue of RhoA, Cdc42 and Rac, thereby locking these important signaling molecules in the 'on' position and leading to marked cytoskeletal alterations, multinucleation with cellular enlargement, and necrosis. The Map protein of EPEC and EHEC has at least two dependent activities – stimulating Cdc42-dependent filopodia formation and targeting mitochondria to disrupt membrane potential in these organelles (Kenny and Rasko, 2002).

The various toxin are transported from the bacterial cytoplasm to the host cells by several mechanisms. Heat labile enterotoxin (LT) is a classic A-B subunit toxin that is secreted to the extracellular milieu by a type II secretion system (Tauschek *et al*., 2002). Several toxins, such as Sat, Pet and EspC, are called autotransporters because part of these proteins forms a β-barrel pore in the outer membrane that allows the other part of the protein extracellular access (Hyland *et al*., 2008). The SPATES (serine protease autotransporters kof enterobacteriaceae) are a subfamily of serine protease autotransporters that are produces by diarrhoeagenic and uropathogenic *E. coli and Shigella* strains. Enteropathogenic *Escherichia coli*, EHEC and EIEC contain type III secretion systems, which are complex structures of more than 20 proteins forming a 'needle and syring' apparatus that allows effector proteins, such as Tir and IpaB, to be injected directly into the host cell (Hyland *et al*., 2008). The UPEC haemolysin is the prototype of the type I secretion mechanism that uses ToIC for export from the cell (Swimm and Kalman, 2008). No type IV secretion systems have been described for pathogenic *E. coli*, with the exception of the type IV-like systems

that are involved in conjugal transfer of some plasmids. By one means or another, pathogenic *E. coli* have evolved several mechanisms by which they can damage host cells and cause disease.

2.2.2 Pathovars and pathogenesis

2.2.2.1. Enteropathogenic *Escherichia coli* (EPEC)

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of potentially fatal diarrhea in infants in developing countries (Croxen and Finlay, 2010). EPEC strains were first described in the 1940s for their association with infantile diarrhea during summer outbreaks in developed countries. While this phenomenon has apparently subsided in developed countries, EPEC still presents a major problem in developing countries, where frequent outbreaks can have mortality rates approaching 30% (Chen and Frankel, 2005). The EPEC pathotype was so named by Neter *et al*., in 1955 while describing primary intestinal pathogens not typically present in the feces of healthy individuals. The primary serogroups identified among EPEC isolates include O26, O55, 86, 111, O114, O119, O125, O126, O127, O128ab, O142, and O158 (Trabulsi *et al*., 2002). Signs and symptoms manifested in EPEC infections include diarrhea, vomiting, fever, and malaise (Chen and Frankel, 2005). This pathovar belongs to a family of pathogens that form attaching and effacing (A/E) lesions on intestinal epithelial cells; other members of the family include EHEC, rabbit diarrhoeagenic *E. coli* (RDEC),the murine pathogen *Citrobacter rodentium* and the recently identified *Escherichia albertii* (formerly known as *Hafnia alvei)*, a pathogen that is associated with diarrhea in humans (Croxen and Finlay, 2010). The attaching bacteria efface the microvilli and subvert host cell actin to form distinct pedestals beneath the site of attachment. This phenotype is provided for EPEC by genes encoded on a 35 kb PAI

known as the locus of enterocyte effacement (LEE) (McDaniel *et al*., 1995). The LEE is highly regulated and encodes a type III secretion system (T3SS) that translocates bacterial effector proteins into the host cell cytoplasm. Seven effectors are encoded by the LEE, but there are several non-LEE encoded (Nle) effectors in addition to these (Deng *et al*., 2004); the roles of many of these effectors are unknown.

2.2.2.2. Enterohaemorrhagic *Escherichia coli* (EHEC)

Enterohaemorrhagic *Escherichia coli* (EHEC) strains are a subset of Shiga toxin-producing *E. coli* (STEC) responsible for hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) in humans. Although most EHEC strains produce Stxs, EHEC O157:H7 are especially virulent and are responsible for the majority of HUS cases of bacterial etiology worldwide (Gyles, 2007; Serna and Boedeker, 2008). The Shiga toxins (Stxs), extremely potent cytotoxins produced by the bacteria in the intestine and act systemically on sensitive cells in the kidneys, brain, and other organs brings about this most severe sequelae, the hemolytic uremic syndrome and other devastating manifestations of EHEC (Gyles, 2007). These cytotoxins enter the host cells expressing toxin receptors and block protein synthesis by irreversibly damaging ribosomal RNA. EHEC comprised of hundreds of O:H serotypes and are commonly carried by healthy wild and domesticated ruminant animals (Beutin *et al.*, 1993; Cerqueira *et al.*, 1999; Kaddu-Mulindw *et al.*, 2001). Ruminants are not sensitive to Stxs due to an absence of vascular Stx receptors (Pruimboom-Brees *et al.*, 2000), and the widespread carriage of stx genes by *E. coli* colonizing ruminant animals has not been satisfactorily explained; hypotheses include a modulation of immune respone by Stxs (Hoffman *et al.*, 2006) and antiviral activity of STEC (Ferens *et al.*, 2006).

Apart from their ability to produce Shiga toxin, EHEC strains induce attaching and effacing (A/E) lesionas in the host's gut epithelium. While these strains are

pathogenic for humans, they can reside as reservoirs of infection in many livestocks including cattle, swine, and poultry. Disease outbreaks in humans are usually associated with the ingestion of some type of food product such as undercooked beef, fresh vegetables, and unpasteurized milk. In young children and the elderly particularly, this disease can progress to HUS, with a dramatic increase of morbidity and mortality. Enterohemorrhagic *Escherichia coli* (EHEC) cause hemorrhagic colitis and are often associated with devastating or life-threatening systemic manifestations. Almost all EHEC O157:H7 isolates harbor a 92 kb virulence plasmid called pO157, which has approximately 100 ORFs and encodes several virulence factors. However, the main virulence factor of EHEC is the phage-encoded Shiga toxin (Stx; also known as verocytotoxin). There are two subgroups of Stx, Stx1 and Stx2, which can be found in various combinations in EHEC isolates, with Stx2 being more prevalent in hemorrhagic colitis and HUS than Stx1. The initial attachment of EHEC to colonocytes is not well defined. EHEC possesses 16 potential fimbria-like operons; but, these have not been extensively studied. Research has identified a type IV pilus, called the haemorrhagic coli pilus that is involved in adherence and biofilm formation; flagella and the *E. coli* common pilus might also be involved in attachment to host cells (Erdem *et al*., 2007). As with EPEC, intimate attachment of EHEC to host cells occurs through interactions between intimin and Tir. Attachment can also be enhanced by the interation of intimin with nucleolin, a surface-localized intimin receptor, the expression of which is increased by Stx2. As Stx is released upon bacterial lysis, the increase in nucleolin expression may be important for the attachment of progeny EHEC (Robinson *et al*., 2006).

2.2.2.3 Enterotoxigenic *Escherichia coli* (ETEC).

Enterotoxigenic *Escherichia coli (*ETEC) is the most common cause of travelers' diarrhea and can have fatal consequences for children <5 years of age. ETEC is a major cause of illness and death in neonatal and recently weaned pigs (Nataro and Kaper, 1998). However, pigs older than approximately 8 weeks appear to be resistant to infection. Strains of ETEC that cause diarrhea in pigs, posses two types of virulence factors, adhesins and enterotoxins, both of which are essential for disease to occur. Human diarrhea caused by ETEC is most common disease caused by pathogenic *Escherichia coli* strains. It is estimated that there are more than 650 million cases of ETEC infection each year, resulting in nearly 800,000 deaths (Turner *et al.*, 2006). Majority of these cases occur in underdeveloped countries. Thus, ETEC strains pose a significant threat to the indigenous populations of these countries as well as travelers and military personnel visiting them. Human ETEC strains are acquired via the ingestion or handling of contaminated food and water. Infection is characterized by a rapid onset of watery diarrhea which is usually self-limiting but can cause life-threatening dehydration.

Enterotoxigenic *E. coli* attachment to the epithelial cells of the small intestine is mediated through colonization factors (CFa), which can be non-fimbrial, fimbrial, helical or fibrillar. A large number of CFs has been identified, of which CFA/I, CFA/II ands CFA/IV are the most common (Turner *et al*., 2006). The cognate receptors for the CFs are poorly defined, although researchers have found interactions between CFA/I and carbohydrate moieties of non-acid glycosphingolipid and glycoproteins; and also between CFA/IV and the acid glycosphingolipid sulphatide (Jansson *et al.*, 2006). A recent study demonstrates that flagella that are transiently bound at the tip with the secreted adhesin EtpA can be used as epithelial-cell

adherence factors. Both CFs and flagella anchor ETEC for initial attachment to host cells, but more intimate attachment may be facilitated by the outer-membrane proteins Tia and TibA (Roy *et al*., 2009).

2.2.2.4. Enteroaggregative *Escherichia coli* (EAEC).

Although it is considered to be an emerging pathogen, EAEC is the second most common cause of travelers' diarrhea after ETEC in both developed and developing countries; and is also becoming recognized as a common cause of endemic and epidemic diarrhea worldwide. Diarrhea caused by EAEC is often watery, but it can be accompanied by mucus or blood. EAEC colonization can occur in the mucosa of both the small and large bowels, which can 'lead to mild inflammation in the colon (Nataro and Kaper, 1998). Much like the details of its transmission and epidemiology, the understanding of EAEC and its pathogenesis is limited, in part owing to the paucity of suitable animal models for its study and the heterogeneity of virulence factors. The characteristic phenotype of EAEC is aggregative adhesin, which involves the formation of a stacked-brick pattern of HEp-2 cells and is mediated by the genes that are found on a family of virulence plasmids called pAA plasmid. These plasmids encode the necessary genes for the biogenesis of the aggregative adherence fimbriae (AAF), which are related to the Dr. family of adhesins and mediate the adherence of EAEC to the intestinal mucosa. AAF- and flagellin-mediated adherence induces an IL-8 response, which leads to the transmigration of neutrophils (Harrington *et al*., 2005).

2.2.2.5. Diffusely Adherent *Escherichia coli* (DAEC).

This is heterogeneous group that generates a diffuse adherence pattern on HeLa and HEp-2 cells. This pattern is mediated by proteins encoded by a family of related operons, which include both fimbrial (for example, Dr. and F1845) and afimbrial

(Afa) adhesins, collectively designated Afa-Dr. adhesins. DAEC isolates that express any of the Afa-Dr adhesins (often designated Afa-Dr DAEC) colonize the small intestine and have been implicated in diarrhea in children between the ages of 18 months and 5 years, as well as in recurring urinary tract infections (UTIs) in adults (Servin, 2005).

All Afa-Dr adhesins interact with brush border-associated complement decay-accelerating factor (DAF), which is found on the surface of intestinal and urinary epithelial cells. Binding to DAF results in the aggregation of DAF molecules underneath the adherent bacteria. It also triggers a Ca^{2+} -dependent signaling cascade, which results in the elongation and damage of brush border microvilli through the disorganization of key components of the cytoskeleton (Servin, 2005).

2.2.2.6. Enteroinvasive *Escherichia coli (*EIEC).

Enteroinvasive *Escherichia coli* (EIEC) is a highly infectious pathotype that causes bacillary dysentery and bloody diarrhea. This pathovar differs from the other *E. coli* pathovars, because it includes obligate intracellular bacteria that have neither flagella nor adherence factors (Ogawa *et al.*, 2008). Infection commences in the colon, where the bacterium passes through microfold cells (M cells) by transcytosis to reach the underlying submucosa. The disruption of tight junctions and the damage that is caused by inflammation also give EIEC access to the submucosa. EIEC are released from dead macrophages into the submucosa, from where they invade the basolateral side of colonocytes with the aid of effectors that are secreted by the T3SS.

2.2.2.7, Uropathogenic *Escherichia coli* (UPEC)

Uropathogenic *Escherichia coli* (UPEC) infections account for roughly 80% of all UTIs, causing cystitis in the bladder and acute pyelonephritis in the kidneys. UPEC has the challenge of moving from the intestinal tract to establish an infection in the urinary tract, where it uses peptides and amino acids as the primary carbon source for fitness (Alteri *et al*., 2009). The ability to ascend the urinary tract from the urethra to the bladder and kidneys reflects exceptional mechanisms for organ tropism, evading innate immunity and avoiding clearance by micturition. Several highly regulated virulence factors contribute to this complex pathogenesis, including multiple pili, secreted toxins (for example Sat and vacuolating autotransporter toxin (Vat)), multiple iron acquisition systems and a polysaccharide capsule (Wiles *et al*., 2008). Entry of UPEC into the urinary tract is followed by adhesion to the uroepithelium. This attachment is mediated by fimbrial adhesin H (FimH), which is found at the tip of the phase-variable type 1 pili. FimH binds to the glycosylated uroplakin la that coats terminally to differentiate superficial facet cells in the bladder (Wiles *et al.*, 2008). Interactions between FimH and uroplakin IIIa were found to lead to phosphorylation events that are required to stimulate unknown signaling pathways for invasion and apoptosis (Thumbikat *et al*., 2009). UPEC invasion is also mediated by FimH binding to α3 and β1 integrins that are clustered with actin at the sites of invasion, as well as by microtubule destabilization (Dhakal and Mulvey, 2009). These interactions trigger local actin rearrangement by stimulating kinases and Rho-family GTPases, which results in the envelopment and internalization of the attached bacteria. Once internalized, UPEC can rapidly replicate and form biofilm-like complexes termed intracellular bacterial communities (IBCs) or pods, which serve as

transient, protective environments. UPEC can leave the IBCs through a fluxing mechanism, motile UPEC leaves the epithelial cells and enters the lumen of the bladder.

During infection, the resulting influx of polymorphonuclear leucocytes results in tissue damage, and UPEC attachment and invasion results in apoptosis and exfoliation of bladder cells. In addition, sublytic concentrations of the pore-forming haemolysin A (HlyA) toxin can inhibit AKT activation and lead to host cell apoptosis and exfoliation. This breach of the superficial facet cells temporarily exposes the underlying transitional cells to invasion and dissemination of UPEC. Invading bacteria are trafficked in endocytic vesicles enmeshed with actin fibres, where replication is restricted. Disruption of host actin permits rapid replication, which can lead to IBC formation in the cytosol or fluxing out of the cell. This quiescent state may act as a reservoir that is protected from host immunity and may therefore permit long-term persistence in the bladder.

2.2.2.8 Neonatal Meningitis *Escherichia coli* (NMEC)

Neonatal Meningitis *Escherichia coli* (NMEC), a common inhabitant of the gastrointestinal tract, is the most frequent cause of Gram-negative-associated meningitis in newborns. Fatality rates can approach 40% (Kaper *et al*., 2004), and survivors are usually burdened with severe neurological sequelae. The pathogenesis of NMEC is complex, as the bacteria must enter the bloodstream through the intestine and ultimately cross the blood-brain barrier into the central nervous system , which leads to meningeal inflammation and pleocytosis of the cerebrospinal fluid (Croxen and Finlay, 2010). Initial colonization, after the bacteria have been acquired prenatally from the mother, is followed by transcytosis through enterocytes into the bloodstream.

The progression of disease is dependent on high bacteraemia (>10^3 colony-forming units per ml of blood), so survival in the blood is crucial. Protection from the host immune responses is provided by an antiphagocytic capsule, made up of a homopolymer of polysialic acid, and serum resistance, resulting form manipulation of the classical complement pathway by the bacterial outer-membrane protein A (OmpA) (Wooster *et al*., 2006). NMEC has also been shown to interact with immune cells: invasion of macrophages and monocytes prevents apoptosis and chemokine release, providing a niche for replication before dissemination back into the blood. Maturation of dendritic cells is inhibited by NMEC (Mittal and Prasadarao, 2008). A lambdoid phage that encodes O acetyltransferase have been discovered, which acetylates the O antigen to provide phase variation and diversity to the capsule (Doszo *et al*., 2005), and may therefore hide the bacteria from host defences.

2.3 Fluoroquinolone Antibiotics

2.3.1. Chemistry of fluoroquinolone antibiotics

Fluoroquinolones comprise a group of synthetic antimicrobials, which have been derived from 1,4-dihydro-4-oxoquinoline-3-carboxylic acid. More than 10,000 compounds have been designed from the parent bicyclic 4-quinolone molecule. Initially, fluoroquinolones used for treatment of diseases had a carboxylic group in position 3, a keto group in position 4, fluorine in position 6, and a piperazinyl or methyl-substituted piperazinyl group in position 7. Further modifications of the molecular structure involved substitutions at the N-1 position, enhancing activity against Gram-negative and Gram- positive bacteria and improving drug kinetics in mammalian species. (Neu, 1990; Grohe, 1998; Petersen and Schenke 1998). Recently, the group of 8-methoxy quinolones has been recognised, having an increased activity against Gram-positive bacteria and being effective also against wild-type and first-step *gyr A* mutants (Fung-Tomc *et al.*, 2000).

2.3.2 Antibacterial spectrum of fluoroquinolones

The first generation of quinolones (nalidixic acid, flumequin, oxolinic acid) were effective particularly against Gram-negative bacteria (*Salmonella* spp. *E. coli*, *Bordetella* spp. and *Yersinia* spp.). With the introduction of enrofloxacin, the prototype of the second generation of quinolones (denoted fluoroquinolones), the spectrum was broadened towards Gram-positive bacteria *(*Staphylococci spp, Streptococci spp and *Listeria monocytogenes*) and includes also Campylobacter spp., *Pseudomonas aeruginosa* and *Mycoplasma* spp., as well as anaerobic Gram-positive and Gram-negative bacteria. Recently introduced fluoroquinolones have an even improved efficacy, being designed to meet specific requirements of human and veterinary therapy (Maxwell and Critclow, 1998).

2.3.3. Mechanism of Action of fluoroquinlone antibiotics

The fluoroquinolones selectively inhibit bacterial DNA synthesis in the presence of competent RNA and protein synthesis. More specifically, these agents target the action of topoisomerase II (also called DNA gyrase) and topoisomerase IV, which belong to a group of related enzymes known as DNA topoisomerases found in all organisms (Drlica and Malik, 2003). DNA gyrase, which is a tetramer that consists of two subunits (GyrA and GyrB) is involved in the supercoiling of the bacterial DNA and thus being essential for replication and transcription. It has been identified as primary target for most fluoroquinolones (Maxwell and Critchlow, 1998) while topoisomerase IV is a primary target in certain bacteria such as *Staphylococcus aureus* and Streptococci spp (Kaats and Seo, 1998).). DNA gyrase also repairs small single-strand breaks in DNA that occur during replication. Binding of fluoroquinolones to bacterial topoisomerase-DNA complexes will generally result in a bactericidal effect, which is concentration dependent. A 100% bactericidal effect is achieved at drug concentrations exceeding 8 times the minimum inhibitory concentration (MIC) (Maxwell and Critchlow, 1998). The A subunit (encoded by *gyr*A gene) is involved in breakage and reunion of DNA, while the B subunit (encoded by *gyr*B gene) is the site of ATP hydrolysis and conformational changes in the complete enzyme to allow DNA strand passage as new molecules are produced (Drlica and Malik, 2003). The role of topoisomerase IV in bacteria is to separate the

daughter chromosomes following the replication process. Similar to DNA gyrase, topoisomerase IV is comprised of two subunits, ParC and ParE, which are encoded by *par*C and *par*E genes, respectively. Ultimately, the quinolones exert their antibacterial effect by binding to complexes of DNA and topoisomerase II or IV, which leads to interference in the DNA replication process. The affinity of fluoroquinolones to bacterial gyrases is significantly higher than their affinity to eukaryotic DNA-topoisomerases, which explains their broad safety margin in human and animal therapy (Robinson *et al.*, 1991).

2.3.3.1 **Pharmacokinetics.**

The fluoroquinolone antibiotics are known for their rapid oral absorption, blood and urine concentrations that markedly exceed the MICs for many common bacterial pathogens, wide distribution into body tissues with serum and tissue concentrations above the MIC for most Gram-negative and many Gram-positive aerobic organisms, and half-lives sufficiently long to permit dosing every 12 to 24 h. The pharmacokinetic parameters of the newer fluoroquinolones have many similarities, although there are differences in half-life, degree of absorption, metabolism, and elimination. In general, quinolones exhibit linear pharmacokinetics, with increases in serum concentrations directly proportional to dose size, and pharmacokinetic properties (serum half-life, total body clearance, etc.) independent of dose. Renal clearance mechanisms are the most important for removal of ofloxacin, levofloxacin, and gatifloxacin. Renal excretion of these compounds occurs via both tubular secretion and glomerular filtration, with glomerular filtration as the major component. Hepatic mechanisms of elimination are more important for removal of trovafloxacin, and multiple mechanisms of elimination contribute to norfloxacin, ciprofloxacin, moxifloxacin and gemifloxacin elimination. Fluoroquinolones are excreted across the

bowel wall into the intestinal lumen, which also explains their efficacy in diarrheal diseases.

2.3.3.2 **Absorption.**

The excellent bioavailability of the quinolones allows oral dosing in place of the more traditional parenteral administration. With most of the new fluoroquinolones, oral absorption is sufficient to achieve adequate serum bactericidal activity for systemic infections (Nix and Schentag, 1988). Fluoroquinolones are absorbed primarily in the duodenum and the proximal jejunum. Absorption does not require acidity or an alkaline environment and fluoroquinolones are absorbed to a similar extent in a fasting state or with a meal (Nix and Schentag, 1988). The fluoroquinolones are rapidly absorbed after oral dosing, reaching peak serum concentrations in 1 to 2 h. Peak plasma levels differ for each drug. Elderly and critically ill individuals absorb the drugs normally, but peak concentrations in these individuals are generally delayed and are usually higher, since such patients frequently have a concomitant decrease in renal function.

2.3.3 .3. Distribution

Fluoroquinolones have a large volume of distribution, ranging from 1 to more than 4 L/kg. Clearly, the apparent volume of distribution of all fluoroquinolones exceeds the 0.6 L/kg that corresponds to total body water. However, the derived values in the literature vary considerably, even for the same quinolone, presumably because few studies used intravenous forms of these drugs to determine precise volumes of distribution. The accuracy of the derived value depends upon knowing bioavailability accurately enough to factor it out. For example, after intravenous administration of ciprofloxacin, the apparent volume of distribution was 2.2 to 2.7 L/kg. If an oral dose

were used to assess this parameter, it would appear to be higher (in the range of 3.2 L/kg). All of the newer fluoroquinolones are widely distributed throughout the body. Interstitial fluid concentrations range from 50 to 100% of peak plasma levels after 2 h, and between 4 and 24 h they generally exceed serum concentrations. Concentrations significantly above those in serum are attained in the kidney, liver, and lung; levels in saliva, bronchial secretions, and prostatic fluid are lower than those in serum (Gootz and Brighty,1996; Nix and Schentag, 1988).

Urine drug concentrations are high and remain above the MICs of common urinary pathogens. In most instances, they exceed inhibitory levels for urinary pathogens for a full 24 h. Urinary concentrations above 10 mg/L often can be detected up to 48 h after ingestion of a single dose. The lowest concentrations of fluoroquinolones in urine are seen with trovafloxacin (Teng *et al*.,1995; Teng *et al*., 1996), and moxifloacin (Stass and Kubitza, 1999). The highest urinary concentrations are noted with gatifloxacin (Gootz and Brighty,1996), and levofloxacin (Fish and Chow, 1997), because these compounds are well absorbed and are excreted by the kidney completely unchanged. Most fluoroquinolones continue to achieve adequate therapeutic concentrations in the urine, even when renal function is greatly reduced. Consistent with transintestinal elimination (Ritz *et al.*, 1994), the fecal levels of most quinolones are sufficient to inhibit most gastrointestinal bacterial pathogens. The cerebrospinal fluid levels of ciprofloxacin and ofloxacin in patients with inflamed meninges are 40 to 90% of serum concentrations. The levels of ciprofloxacin and ofloxacin in human aqueous humor range from 3.8 to 25% and 44 to 88% of serum levels, respectively. The total areas under the blister fluid–concentration-time curves exceed serum levels by 120% for ciprofloxacin, norfloxacin, and ofloxacin (Wise *et al.*, 1986). Ciprofloxacin, ofloxacin, and other quinolones appear to penetrate into prostate tissue and seminal

fluid reaching concentrations exceeding those achieved in serum. Ciprofloxacin penetrates well into pancreatic tissue. The penetration ratio in one study was 1.0 for pancreatic tissue and 0.83 for pancreatic juice (Isenmann *et al*., 1994). Biliary concentrations also exceed those in serum (Edmiston *et al*., 1996).

After a single 200-mg intravenous dose, concentrations of ciprofloxacin in cortical bone and cancellous bone were 6.9 and 9.7 ug/g. Other quinolones also appear to penetrate bone. However, these values should be interpreted cautiously because tissue:serum ratios change in relation to time after administration, and study designs differ among investigations. Bone marrow tissue concentrations are excellent and in almost every case exceed MICs for infecting bacteria (Nix and Schentag, 1988). Quinolones also reach high concentrations inside many cells. Fluoroquinolones enter polymorphonuclear cells, alveolar macrophages, peritoneal macrophages, and phagocytic cells within the liver, producing concentrations ranging from 3 to 10 ug/mL (Wise *et al*., 1996). An anionic transport mechanism removes the compounds from white blood cells (Nix and Schentag, 1988). Tissue concentrations are generally higher in infected tissues than in uninfected tissues, because of WBC accumulation. There are more white blood cells in infected tissue, and these compounds are probably present intracellularly in concentrations higher than those in extracellular fluids, though the degree of antimicrobial activity of these drugs at intracellular sites has not been well studied. Most of the fluoroquinolones have relatively low protein binding of 14 to 45% (Okezaki *et al*., 1989). Ciprofloxacin, levofloxacin, gatifloxacin, and ofloxacin are 10 to 25% protein bound (Nix and Schentag, 1988). Thus any compromise of antimicrobial activity by the presence of serum protein should be minimal.

2.3.3.4. Routes of Elimination

Metabolism

Hepatic metabolism is essential for clearance of several fluoroquinolones. In the case of fluoroquinolones such as norfloxacin and ciprofloxacin, most metabolism occurs at the piperazine substituent on ring position 7. In contrast to the pathway for the earlier quinolone compounds, metabolic alteration of the newer fluoroquinolones does not typically occur on the position 7 ring. And whereas the metabolic products of earlier quinolones had antimicrobial activity, glucuronides formed at position 3 are clearly inactive because this part of the molecule is essential for antimicrobial activity. As with most oxoquinolone metabolites, the oxo- metabolite of ciprofloxacin is active, although less so than ciprofloxacin. In urine, 36% of a 500-mg oral dose is recovered as unchanged ciprofloxacin, 9.6% as the oxo- metabolite, 2 to 4% as the dioxo-metabolite, and less than 2% as other metabolites (Westphal and Brogard, 1993).

Renal Excretion

Quinolones are eliminated by renal mechanisms, including glomerular filtration and tubular secretion, as well as by nonrenal routes, such as hepatic metabolism and transintestinal transport (Rohwedder *et al.*,1990). Fluoroquinolones excreted primarily by hepatic metabolism (e.g., trovafloxacin, moxifloxacin) have longer half-lives in many cases (Galante *et al.*, 1986, Westphal and Brogard 1993), than quinolones excreted primarily by renal mechanisms. Renal clearance usually exceeds the glomerular filtration rate, suggesting that tubular secretion plays a major role in the elimination of these drugs. The fact that most of these antibiotics interact with probenecid (Nix and Schentag, 1988; Shimada, *et al*., 1993), is further evidence that these compounds undergo renal tubular secretion. Renal clearance of the fluoroquinolone ranges from 140 to 425 mL/min in patients with normal renal

function. Administration of probenecid reduces the renal clearance of ciprofloxacin by 50% and total urine recovery by 24%.

Hepatic metabolism

Compared to the other fluoroquinolones, drug metabolism occurs to the greatest extent for trovafloxacin and moxifloxacin (Teng *et al.*,1995; Teng *et al.*, 1996). Metabolites constitute between 15 and 30% of norfloxacin and ciprofloxacin recoverable from urine. As with all drugs subject to excretion by combinations of renal and metabolic pathways, patients with multiple organ failure and resulting impairment of both pathways would show extreme prolongations of serum half-life. In this case, neither elimination pathway can compensate for failure of the other, and marked accumulation would occur. Severe hepatic disease also would be expected to prolong the serum half-lives of trovafloxacin, moxifloxacin and norfloxacin. In fact, ciprofloxacin and norfloxacin may accumulate in patients with hepatic failure, particularly with concomitant renal impairment (Galante *et al.*, 1986, Westphal and Brogard, 1993).

2.3.4. Drug interactions involving fluoroquinolones

Some significant and potentially significant interactions are summarized below.

Anticoagulants

Studies on the interactions between quinolones and warfarin demonstrate that norfloxacin prolongs the elimination half-life of (R)-warfarin, while not affecting (S)-warfarin. Because the (R)-enantiomer is five to eight times less active than the (S)-isomer, the overall norfloxacin-warfarin interaction should be of little clinical significance. In clinical trials of newer agents, no significant interaction with warfarin was described. However, several anecdotal cases have implied interactions between warfarin and commonly-prescribed quinolones (Jones and Fugate, 2002 ; O'Connor

and O'Mahony,2003). Any patient receiving a quinolone along with warfarin anticoagulation should have prothrombin time closely monitored.

Divalent Cations

Fluoroquinolones form chelates with divalent cations, particularly aluminum and magnesium and, to a lesser degree, iron, zinc, and calcium. Thus, co-administration of fluoroquinolones with antacids or agents such as sucralfate reduces their bioavailability by as much as 85%, which can result in therapeutic failures. Iron preparations behave similarly to antacids, and adequate time should be allowed between doses. Multivitamin preparations that contain minerals should be avoided as well. Allowing a 4- to 6-h interval between the administration of antacids or sucralfate and fluoroquinolones will likely avoid the interaction, but this is not always a suitable alternative for patients on long-term antacid treatment. Histamine-2 antagonists do not affect the oral absorption of fluoroquinolones and can be used for acid control when the quinolones must be used in the presence of acid-reducing medications.

Theophylline, Caffeine, and the Xanthines.

Clearance of theophylline and caffeine is inhibited by some of the quinolones. Given the different affinity for the cytochrome P-450 isozyme 1A-2, the fluoroquinolones vary in their relative degree of interaction with theophylline. The effect is strongest with enoxacin, which, in combination with theophylline, results in an approximate doubling of theophylline levels. Norfloxacin and ciprofloxacin interact with theophylline to a lesser extent than enoxacin and raise the serum concentration of theophylline by 2 to 5 µg/mL. No clinically significant interaction was demonstrated upon coadministration of theophylline with moxifloxacin, gatifloxacin, gemifloxacin, or trovafloxacin (Davy *et al*., 1999; Niki *et al*., 1999). Caffeine, a chemical analogue

of theophylline, interacts similarly when coadministered with quinolones. Patients receiving certain fluoroquinolones should be advised against excessive caffeine intake, and if CNS effects develop, they should be instructed to cease caffeine intake.

Tizanidine

A potentially dangerous interaction was recently found between ciprofloxacin and tizanidine, which is used for the treatment of muscle pain associated with spasticity or muscle tension (Granfor *et al*., 2004). In healthy volunteers, compared to placebo, concentrations of tizanidine in blood were increased 10-fold after receipt of 500mg of ciprofloxacin twice daily for 3 days. The interaction resulted in a severe decrease in blood pressure and enhanced central nervous system effects. The proposed mechanism is an inhibition of the liver metabolism of tizanidine. The combination of these is now contraindicated.

Probenecid

Probenecid administration increases peak plasma concentrations and prolongs the half-life of quinolones primarily excreted by the renal route, such as ciprofloxacin, ofloxacin, levofloxacin, gatifloxacin, and gemifloxacin. The mechanism of this effect is inhibition of renal tubular secretion, most likely secondary to inhibition of renal transport proteins by probenecid. Accordingly, trovafloxacin and moxifloxacin are less affected, since they are excreted primarily by hepatic clearance mechanisms.

2.3.5. Mechanisms of resistance to fluoroquinolones

2.3.5.1 Mutation in QRDR of the DNA GYRASE

In *E. coli* and many other Gram-negative bacteria, quinolone resistance is conferred by point mutations in the *gyrA* gene. All mutations described have been found to reside in the quinolone determining region (QRDR) of the A-subunit of DNA gyrase (topoisomerase II), corresponding to amino acids 67-122. Amino acid changes at Ser-

83 (to Phe, Tyr, or Ala) or at Asp 87 (to Gly, Asn, or Tyr) are the most frequently observed changes in nalidixic acid resistant strains. Double mutations in both residues Ser-83 and Asp-87 have been found in fluoroquinolone resistant clinical isolates of *E.coli* and *Salmonella* spp. (Heisig *et al.*, 1995; Everett, *et al.*, 1996; Griggs *et al.*, 1996; Deguchi *et al.*, 1997; Taylor and Chau, 1997). A QRDR has also been identified in the *gyrB* gene of *E. coli,* but the overall contribution of *gyrB*-mutations to fluoroquinolone resistance remains to be elucidated. In Gram-positive bacteria (for example *Staphylococcus aureus*), topoisomerase IV, of which ParC and ParE are homologous to GyrA and GyrB, respectively, is the primary target for fluoroquinolones. Mutations in the genes *parC* and *parE* at positions equivalent to those identified in *gyrA* and *gyrB* participate in the high level resistance to fluoroquinolones (Reyba *et al.*, 1995; Vila *et al.*, 1996). At present, topoisomerase IV has been recognised also as second target for quinolones in Gram-negative bacteria such as *E. coli*. Evidence suggests that *parC* mutations occur frequently when Gyr A is already resistant.

2.3.5.2. Efflux Pump mediated fluoroquinolone resistance

Active efflux has become recognized as a major component of microbial resistance to most classes of antibiotics. They contribute to both intrinsic and acquired resistance to many different antimicrobials. This mechanism of resistance is mediated by efflux pumps, which are membrane-associated transporters promoting antibiotic extrusion from the cell via an energy-dependent process. Some efflux pumps selectively expel specific antibiotics, while others, referred to as multidrug resistance (MDR) pumps, export a broad array of structurally unrelated compounds (Li and Nikaido, 2004; McKeegan *et al.*, 2004). MDR is conferred mainly by the efflux systems of the major facilitator superfamily (MFS) of gram-positive bacteria, with the most studied pump

being NorA of *Staphylococcus aureus*, while the resistance/nodulation/division (RND)efflux systems are the major contributors in gram-negative bacteria (Li and Nikaido,2004; McKeegan *et al.*, 2004). They reduce the intracellular accumulation of antimicrobials in bacterial cells, thus reducing antimicrobial activity. Genomic analysis has demonstrated the existence of multiple, putative efflux pumps in many bacteria as products of distinct genes (*emrAB, acrAB, norA, nfxB, nfxC* and others). The efflux pump, AcrAB, extrudes quinolones out of the *E. coli* bacteria. The pump is partly controlled by the multiple antibiotic resistance (mar) genes and appears to be the major mechanism of resistance for mar mutants (Okusu *et al.*, 1996). The mar genes regulate accumulation and thus intracellular concentrations of quinolones by altering the expression of porins and efflux pumps (Everett *et al*., 1996). The mar genes cause an efflux of a variety of chemically unrelated compounds including different drug classes of antibacterials (Goldman *et al.*,1996) and are affected by a variety of chemically unrelated substances. Salicylate and tetracycline induce MarA production, a positive regulator of acrAB transcription, so that salicylate stimulates fluoroquinolone resistance selection. Resistance may be seen with mar expression alone or in combination with type II topoisomerase mutations (Goldman *et al*.,1996). The combination of AcrAB overexpression with topoisomerase mutations causes high level fluoroquinolone resistance; over 60% of high-level ciprofloxacin-resistant isolates had an increased production of AcrA (Mazzariol *et al*., 2000; Oethinger *et al*., 2000). Cross-resistance between fluoroquinolones and antibacterials of chemically unrelated drug classes is associated with the increased expression of efflux pumps because of their limited substrate specificity. For example, MexAB confers resistance to nonfluorinated and fluoroquinolones, tetracycline, and chloramphenicol, Mex CD confers resistance to fluoroquinolones, erythromycin, trimethoprim, and triclosan,

Mex EF confers resistance to the latter plus chloramphenicol, imipenem, and triclosan, and Mex XY confers resistance to fluoroquinolones, erythromycin, and aminoglycosides. Several comprehensive reviews have summarized the impact of fluoroquinolone-extrusion and resistance (Hooper, 2005; Van Bambeke *et al*., 2010; Piddock, 2006). In addition, soxRS gene products, which are involved in bacterial adaptation to superoxide stress, affect fluoroquinolone activity, too (Oethinger *et al*., 1998). Various combinations of target enzyme alteration, diminished antibiotic accumulation, and efflux are often seen in fluoroquinolone-resistant *E. coli*, other Enterobacteriaceae and nonfermenters (Everett *et al.*, 1996). Consequently, a fluoroquinolone resistant or even multidrug-resistant phenotype can easily be selected by an exposure to a broad range of chemically unrelated drug classes, thus, representing the fourth type of cross-resistance. These examples illustrate the complexity of fluoroquinolone resistance mechanisms, selection by fluoroquinolones and coselection of resistance by chemically unrelated classes of antibacterials and antiseptics.

2.3.5.3. Membrane Permeability

A decrease in the permeability of the bacterial cell wall caused by alterations in the hydrophilic pores (outer membrane porins) has been described as third mechanism of acquired resistance. The nfxB gene codes for an altered outer cell membrane protein F, thereby decreasing fluoroquinolone entry into the cell (Truong *et al*., 1997). In addition, soxRS gene products, which are involved in bacterial adaptation to superoxide stress, affect fluoroquinolone activity, too. This is an additional nontopoisomerase resistance mechanisms that are not under mar control which can change quinolone resistance patterns.

1.3.3.6.4. Plasmid-mediated quinolone resistance (PMQR)

The most prominent of the several mechanisms of fluoroquinolone resistance that have been described is through chromosomal mutations and plasmid-mediated quinolone resistance (Hooper, 2000). More specifically, fluoroquinolone resistance among enteric and pathogenic bacteria is caused by chromosomal mutations that reduce membrane permeability and restrict antibiotic concentration within the bacterium. Recently, plasmid-mediated quinolone resistance (PMQR) genes were detected in Enterobacteriaceae . The PMQR genes confer low-level quinolone resistance and supplement the level of resistance caused by other resistance mechanisms. Four different PMQR determinants; Qnr, AAC(6′)-Ib-cr, QepA and OqxAB are commonly found among Enterobacteriaceae.

2.4 Antimicrobial Evaluation Techniques

The antimicrobial activity of an agent is measured *in-vitro* in order to determine its potency, its concentration in body fluids or tissues and the sensitivity of a given microorganism to a known concentration of the antimicrobial agent.

Investigation and determination of these quantities may be undertaken by one of these principal methods: agar diffusion, broth dilution, agar dilution and spore germination inhibition test.

2.4.1 Agar Diffusion.

This includes paper disc plate method, the cup-plate method and the cylinder-plate method. The principles of these agar diffusion methods are the same. As the test microorganism grows they are exposed to a continuous gradient of decreasing concentration of the antimicrobial agents at increasing distance from the reservoir. Zones of inhibition (zone of no growth of microorganism) are formed where the

concentration of the diffused antimicrobial agents is potent enough to inhibit the growth of the test microorganism. Large zones indicate more effective antimicrobial activity or greater diffusibility of the drug or both, no zone indicates complete resistance (Baron and Finegold, 1990).

2.4.2 Agar-plate-dilution method.

Graded dilutions of the antimicrobial agent are incorporated into agar plates, one plate for each dilution to be tested. Standard inoculum of the test organism is delivered to the agar surface of the plates containing different concentrations of the agent. The plates are incubated at the optimum temperature for the growth of the test organism and for the appropriate time. The test organisms will grow on those plates that do not contain enough antimicrobial agent to inhibit them. The minimum inhibitory concentration is the dilution that allows no more than one or two colony forming unit (cfu) or only a slightly haze to grow (Baron and Finegold, 1990).

2.4.3 Broth dilution method

This method is used mainly for quantitative test of antimicrobial agents. Dilutions of the antimicrobial agents are incorporated into appropriate liquid medium and then inoculated with a standardized suspension of the test microorganism. Usually, two-fold dilution series of the antimicrobial agent are prepared with the selected medium. Each tube receives about 0.05-0.1ml of the inoculum (5 x 10^6cfu/ml). Control tubes which do not receive any antimicrobial agent are set-up. The tubes are incubated at the optimium temperature for the growth of the test organism for the appropriate time. After sufficient incubation, the tubes are examined for turbidity indicating growth of the test microorganism. The organism will grow in the control tube and in any other tube that does not contain enough antimicrobial agent to inhibits growth of the organism, indicated by lack of visual turbidity. The

lowest concentration of the agent visibly inhibiting the growth of microorgainsm is taken as the minimum inhibitory concentration (MIC). (Jawetz *et al.*, 1989).

The limitation of broth dilution method for MIC is when the antimicrobial agent is not soluble in the broth and/or forms a cloudy precipitate with it. Estimation of the MIC of the antimicrobial agent by this method will be erroneous. In such a situation, the MIC is best determined in a solid medium where growth is manifested by formation of visible colonies of the microorganism. This method has been used by Olutimeyin and Onaolapo (1997) to determine the MIC of benzoic acid and sodium benzoate against bacterial strains isolated from orange drinks.

2.4.4. Evaluation of combined activity of antimicrobial agents

Antimicrobial agents are sometimes use in combination to achieve a certain effect. The following methods are used in evaluation of combined activity of antimicrobial agents. Checker board method (Scot *et al.*, 1995), Time-kill curve (Richard and Barbara, 1987) overlay inoculum susceptibility disc method (Chinwuba *et al.*, 1991)

2.4.4.1 CheckerBoard method

In this method, dilution plate and diluters are used. An isolate of a test microorganism is exposed to multiple ratio concentrations of two antimicrobial agents. The MICs for each agent alone and in the combinations are determined in the plate after incubation at 37 ^{0}C for 24 h. The effect of the combined activity can be expressed as the Summed fractional inhibitory concentration which is calculated as follows (Alan and Derek, 1989)

$$\frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}} + \frac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$$

2.4.4.2 Overlay inoculum susceptibility disc method

Paper discs are impregnated with one of the antimicrobial agents needed for the combination activity. The second antimicrobial agent is incorporated into a molten agar plate to form base antimicrobial agar layer. The standardized test microorganism suspended in about 5ml molten agar is added to the plate as thin overlay inoculum agar layer and then allowed to solidify. The antimicrobial disc are then placed asceptically on the solidified surface. A control is set up in which only the molten agar is poured on a second plate to produce an antimicrobial agent free base agar layer. The antimicrobial discs are then placed asceptically on the solidified surface of the control plate. The inhibition zone diameters are recorded after incubation at 37 ^{0}C for 18-24 h. The antimicrobial combinations which produce 19% increment or more correspond to synergism, increments less than 19% corresponds to additivity while no difference in the inhibition zone diameters exhibit indifference in activity (Chinwuba *et al*., 1991).

2.4.4.3. Interpretation of combined effect of antimicrobial

Combination of two or more antimicrobial agents in the treatment of an infection could result into any of the following interactions antagonism, indifference, additivity and synergism. Antagonism is an interaction where the combined effect is less than that of the more effective agent when used alone. Indifference is an interaction where the combined action is no greater than that of the more effective agent when use alone. Additivity is an interaction where the combined effect is equivalent to the sum of the actions of each antimicrobial agent when used alone. Synergism is an interaction where the combined effect is significantly greater than the sum of actions of each agent when used alone (Alan and Derek, 1989).

2.5. Essential Oils.

Essential oils also called ethereal oils, volatile oils, plant oils or aetheroleum are natural, volatile, complex plant compounds, oily or lipid – like in nature and frequently characterized by a strong fragrance. They are stored in specialized plant cells, usually oil cells or ducts, resin ducts, glands or trichomes (glandular hairs). The essential' part of the term 'essential oil' is thought to be derived from a phrase attributed to Philippus Aureolus Theophrastus Bombastus von Hohenheim (1491-1541), or Paracelsus as he became known, a Swiss physician who named the active component of a drug preparation 'quinta essentia' (Edris, 2007). Essential oils make up only a small proportion of the wet weight of plant materials, usually approximately 1% or less. Essential oils are often described as secondary plant metabolites, have been all those compounds synthesized by the plant which do not appear to be essential for plant growth and development and/or those compounds without an obvious function (Croteau, 2000). They are also not universally synthesized in all plants. In contrast, primary metabolites are produced by all plants and usually have an obvious function and are part of the essential metabolic processes in respiration and photosynthesis (Theis and Lerdau, 2003). Essential oils are synthesized by plant organs such as the buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark and are stored in secretory cells, cavities, canals cells cavities, canals epidermic cells or glandular trichomes.

2.5.1. Chemistry of essential oils and their chemical components

The classification and nomenclature of essential oil compounds is complicated by the fact that many were isolated and studied before the instigation of systematic chemical nomenclature. Consequently, many are known by non-systematic or trivial or common names (Obst, 1998). These are sometimes not always based on their sources, such as eucalyptol, limonene, pinene and thymol, names which hint at historical botanical origins of these compounds. In terms of shedding light on their chemistry, nature and characteristics of essential oils and their components (Christine and Katherine, 2011).

In general, the essential oils consist of chemical mixtures involving several tens to hundreds of different types of molecules depending on the oil in question. These main groups include terpenes and terpenoids and aromatic and aliphatic constituents, all characterized by low molecular weight only a few have a high percentage of a single component. These chemical constituents can be subdivided into two distinct groups; the **Hydrocarbons** which are made up almost exclusively of terpenes (monoterpenes, sesquiterpenes, and diterpenes) and the **oxygenated compounds** which are mainly esters, aldehydes, ketones, alcohols, phenols and oxides. They may also contain Nitrogen and Sulfur.

2.5.2 Factors affecting oil production and antimicrobial activities of plants

The presence, yield and composition of essential oils may be influenced by many factors, including climate, plant nutrition and stress (Croteau *et al*, 2000). In commercial production settings selection and breeding programmes are often instigated to improve yields and foster desired compositions (Figue-iredo *et al*., 2008). Great variation exists amongst antimicrobial essential oils in terms of both the

diversity of plant from which they may be derived and the chemical composition of each oils.

unfairly skews data in favour of the greater susceptibility of Gram-positive bacteria, studies testing a larger number of essential oils against a wide variety of bacteria tend to identify no such pattern.

2.5.3. Antimicrobial activities of Essential oil

While the spectrum and scale of the antimicrobial activity of essential oils are becoming better characterized, a deeper understanding of the precise effects of essential oils and their components on microorganism has just been gained over the last decade or two. Many of the described effects involve interactions with biological membranes. For example, in bacteria, carvacrol has been shown to cause collapse of the proton-motive force and depletion of the ATP pool, leading to death (Ultee *et al*., 2002; Ultee *et al*., 1999; Gill and Holley, 2006), while tea-tree oil (*M. alternifolia*) and its major component terpinen -4-ol increase membrane permeability to potassium ions (Cox *et al.,* 2000). Carvacrol has also been shown to inhibit the synthesis of flagellin, the protein that make up flagella used for bacterial motility, in the important foodborne pathogen *Escherichia coli* 0157:H7. Specific effects on bacterial virulence factors (the products by which bacteria establish infection and produce disease) have also been identified. Examples include that cinnamaldhyde interferes with quorum sensing communication processes mediated by two different types of signaling compounds, acyl homoserine lactones and a group known collectively as autoinducer -2 (Al-2) (Brackman *et al*., 2008).

2. 5.4 **Review of *Cymbopogon citratus* plant and its essential oil**

Fig 1: *Cymbopogon citratus* showing the leaf

Cymbopogon citratus (DC.) Stapf, commonly known as lemongrass and other Cymbopogon species is a tall, coarse grass with a strong lemon taste. Lemongrass is a perennial herb widely cultivated in the tropics and sub-tropics, designates two different species, East Indian *Cymbopogn flexuosus* (DC.) Stapf and West Indian, *Cymbopogon citratus* (DC.) Stapf. (Naik *et al*., 2010). *Cymbopogon citratus* (DC) Stapf. has been cultivated over many years for medicinal purposes in different countries through out the world. The use of lemongrass was found in folk remedy for coughs, consumption, elephantiasis, malaria, ophthalmia, pneumonia and vascular disorders (Naik *et al.*, 2010). Researchers have found that lemongrass holds antidepressant, antioxidant, antiseptic, astringent, bactericidal, fungicidal, nervine and sedative properties (McGuffin *et al.*, 1997). Further, many workers had reported about the antibacterial activity of lemongrass oil against a diverse range of organisms

comprising Gram-positive and Gram-negative organism, yeast and fungi (Shigeharu *et al*.,2001; Cimanga *et al*., 2002; Nguefack *et al*., 2004; Pereira *et al*., 2004). Some workers had observed that Gram-positive organisms were more sensitive to the oil than Gram-negative organisms.

2.5.5. Other names of lemongrass

Lemongrass is known by the scientific names *Cymbopogon Citratus* or *Andropogon Citratus*. Locally, different names are given to *Cymbopogon citratus* and they include *serai* in Malaysia and Brunei *serai* or *sereh* in Indonesia,, and *salai* or *tanglad* in the Philippines, Malabar grass in India, other common names are Capim-cidrao or Capim-santo in **Brazil**, Tej-sar **in Ethiopia**:, Sera in **Hindi**: Sereh **in Indonesian**:, **Italian**: in Cimbopogone, Sakumau **in Malaysia**:, Zacate limon in **Mexico**:,**Swedish**:, Citrongräss **in Thailand**: **Turkish**: Citronella in **USA**: **lemongrass**, **barbed wire grass**, **silky heads**, ***cha de Dartigalongue***, **fever grass**, ***hierba Luisa***, and ***gavati (grass)chaha*** (tea), amongst many others.

2.5.6 Botanical Profile of *Cymbopogon citratus*

Kingdom	Plantae
Order	Poales
Family	Poaceae
Subfamily	Panicoideae
Tribe	Andropogoninae
Subtribe	Andropogoneae
Genus	Cymbopogon
Specie	*Cymbopogon citratus*

2.5.7. Ecology and geographical Distribution of lemon grass

Lemongrass is a genus of Asian, African, Australian, and tropical island plants in the grass family (Naik *et al*., 2010). They are mainly cultivated as culinary and medicinal herbs because of their scent, resembling that of lemons (*Citrus limon*).

2.5.8. Folkloric/ Traditional uses of Lemongrass

As the name implies, lemongrass smells just like lemons, but it is milder, sweeter, and far less sour. This grass is used in countless beverages (including tea), desserts, soups, and curries and other forms of culinary creations as a flavoring agent, where fresh lemon is not available. It is widely used in Chinese and Thai recipes. It is also suitable for use with poultry, fish, beef, and seafood. Lemongrass oil is used as a pesticide and a preservative. Despite its ability to repel some insects, such as mosquitoes, its oil is commonly used as a "lure" to attract honey bees.

The health benefits of Lemongrass Essential Oil can be attributed to its many beneficial properties as an analgesic, antidepressant, antimicrobial, antipyretic, antiseptic, astringent, bactericidal, carminative, deodorant, diuretic, febrifuge, fungicidal, galactogogue, insecticidal, nervine, sedative and tonic substance

2.5.9 Review of coconut plant and its oil

Fig2a Coconut plant

Fig 2b Coconut oil

2.5.9.1, **COCONUT PLANT AND COCONUT OIL**

The coconut tree (*Cocos nucifera*) is a member of the family Arecaceae (palm family) and the only accepted species in the genus *Cocos*. The term **coconut** can refer to the entire coconut palm, the seed, or the fruit, which, botanically, is a drupe. The *Cocos nucifera* is a large palm, growing up to 30 m (98 ft) tall, with pinnate leaves 4–6 m (13–20 ft) long, and pinnae 60–90 cm long; old leaves break away cleanly, leaving the trunk smooth. Coconuts are generally classified into two general types: tall and dwarf. On fertile soil, a tall coconut palm tree can yield up to 75 fruits per year, but more often yields less than 30, mainly due to poor cultural practices. Given proper care and growing conditions, coconut palms produce their first fruit in six to ten years, taking 15 – 20 years to reach peak production.

Coconuts are known for their great versatility, as evidenced by many traditional uses, ranging from food to cosmetics. They form a regular part of the diets of many

people in the tropics and subtropics. Coconuts are distinct from other fruits for their large quantity of "water", and when immature, they are known as tender-nuts or jelly-nuts and may be harvested for their potable coconut water. When mature, they still contain some water and can be used as seednuts or processed to give oil from the kernel, charcoal from the hard shell, and coir from the fibrous husk. When dried, the coconut flesh is called copra. The oil and milk derived from it are commonly used in cooking and frying, as well as in soaps and cosmetics. The husks and leaves can be used as material to make a variety of products for furnishing and decorating. The coconut also has cultural and religious significance in certain societies, particularly in India, where it is used in Hindu rituals (Patil, 2016).

Coconut oil, also known as copra oil, is an edible oil extracted from the kernel or meat of mature coconuts harvested from the coconut palm. It has various applications. Because of its high saturated fat content, it is slow to oxidize and, thus, resistant to rancidification, lasting up to six months at 24 °C (75 °F) without spoiling.

2.5.9.2. **Local names of coconut plant**

The coconut and coconut plant are locally named based on the uses, physical appearance and /or origin. In Igboland, it is generally called Aki Beeke or Aki Oyibo (meaning English Kernel). In Nsukka and some part of Enugu state , it is called Aki Oba. In Hausaland it is called KwaKwa. In India, it is *kalpa vriksha* ("the tree which provides all the necessities of life"). In the Malay language, it is *pokok seribu guna* ("the tree of a thousand uses"). In the Philippines, the coconut is commonly called the "tree of life"(Margolis, 2006).

2.5.9.3 Botanical profile of coconut Plant

Kingdom : Plantae

Order: Arecales

Family: Arecaceae

Subfamily: Arecoideae

Tribe: Cocoeae

Genus: Cocos

Species: Cocos nucifera

2.5.9.4. Ecological and Geographical distribution of coconut plant

The Coconut trees grow in rainforests, tropical and subtropical areas. They are widely spread in Malaysia, southern Asia, India, South America, the Pacific Islands. The coconut palm grows well on sandy soils and can tolerate saline condition.

2.5.9.5. Uses of Coconut Oil

The coconut palm is grown throughout the tropics for decoration, as well as for its many culinary and nonculinary uses; virtually every part of the coconut palm can be used by humans in some manner and has significant economic value. The coconut seed provides oil for frying, cooking, and making margarine. Some studies have shown some antimicrobial effects of the free lauric acid. (Mary, 1996). Lauric acid is also prominent in the saturated fat of human breast milk, giving vital immune building properties to a child's first stage of life. Outside of human breast milk, nature's most abundant source of lauric acid is coconut oil. It can also be used as a mild form of sunscreen (Korać and Khambholja, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Test micro organisms

Total of 2449 *E.coli* and 958 *S.aureus* isolates recovered from both human and animal subjects in Enugu State were used for the study. Control strains were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. They were obtained from Nigerian Institute of Medical Research ,(NIMR) Lagos.

3.1.2 Culture Media

The following culture media used were purchased from Oxoid chemical, (Cambridge, UK) and they include MacConkey agar, Eosin methylene blue {EMB} Mannitol salt agar, Nutrient agar and broth, Muelleur Hinton agar and broth, Blood agar (B.A) and Kliglier Iron agar .

3.1.3 Solvent for extraction

Distilled water (Water Resources Management Laboratory Limited, University of Nigeria, Nsukka, Enugu State).

3.1.4 Antibiotics and antibiotic sensitivity discs.

Pure samples of ciprofloxacin (PCCATm Houston- USA) and gentamicin (Lek Pharmaceutical d.d. Slovenia), Erythromycin (Medisca Inc. USA).

Antibiotic discs used were purchased from Oxoid, (Cambridge,UK). and they include 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)

3.1.5 Plant oils used

Freshly harvested leaves of lemongrass plant were obtained from Nsukka and Enugu (Enugu State). The identity of the plant leaves was authenticated by Mr. A. O. Ozioko (Botanist) of the Bioresource Development and Conservation Programme (BDCP) Nsukka, Enugu State. *Cocos nucifera* oil (Virgin Coconut Oil) was purchsed from Ogige market in Nsukka, Nigeria.

3.1.6 Laboratory materials

These include 100mm diameter glass Petri dishes (Borosil^{R}, china), glass test tubes (Pyrex, U.S.A), test tube racks (Chikpas instrumentation, Nigeria), automatic micropipette (Perfect, china), conical flask (Techmel, U.S.A), flat bottom flask (Simax, Czechoslovakia), bijou bottles, glass measuring cylinders (Model UL, China), Triple beam balance (MB 2610, China), binocular light microscope (Micron BI-KG-7A, U.S.A), Dixon non-electric autoclave (Model ST 18, U.K), Chikpas water bath, hot-air oven and incubator (Chikpas instrumentation 2010 ,Nigeria), GCMS-QP2010 PLUS (SHIMNADZU, JAPAN). thermocycler (A & E Laboratories, UK Model Cyl-005-1.) and Primer pairs.

3.1.7 Study Area:

Enugu State is one of the 36 States in Nigeria and is located in the south east geopolitical zone of the country. The state shares borders with Abia State and Imo State to the south, Ebonyi State to the east, Benue State to the northeast, Kogi State to the northwest and Anambra State to the west. The State operates district health system which is made up of seven (7) District Hospitals at Enugu Urban, Udi, Agbani, Awgu, Ikem, Enugu-Ezike,

and Nsukka. The District Health System (DHS) is a form of decentralised provision of health care where health facilities, health care workers, management and administrative structures are organised to serve a specific geographic region or population. In Enugu State, Nigeria, the DHS was introduced following the election of a new democratic government in 1999. The Enugu DHS delivers a range of health care services to population groups ranging from 160,000 and 600,000 people through a structured management system (the district health management team) which integrates primary and secondary health services. In addition to the aforementioned district hospitals, there are many health institutions in the State comprising of the University of Nigeria Teaching Hospital (UNTH), Ituku-Ozalla, National Orthopaedic Hospital, Abakaliki road, Enugu, Enugu State University Teaching Hospital, Parklane Enugu , numerous private hospitals and clinics and at least one health center or cottage hospital in every one of the seventeen Local Government Areas and thirty nine Development Centres in the State.

3.2 Method

3.2.1 . EXPERIMENTAL DESIGN

This work consists of a case control study involving humans (symptomatic and asymptomatic) and animals; a surveillance for the distribution of fluoroquinolone resistance genes and antimicrobial evaluations involving some commonly used antibiotics, lemon grass essential oil and coconut oil.

3.2.2. SAMPLING SITE:

Using the geographical cluster sampling method, seven health district in the State were covered by randomly selecting the study subjects (both the humans and animals) from at least two local government area (LGA) of each district. The districts and the 17 local government are shown in Figure 3a

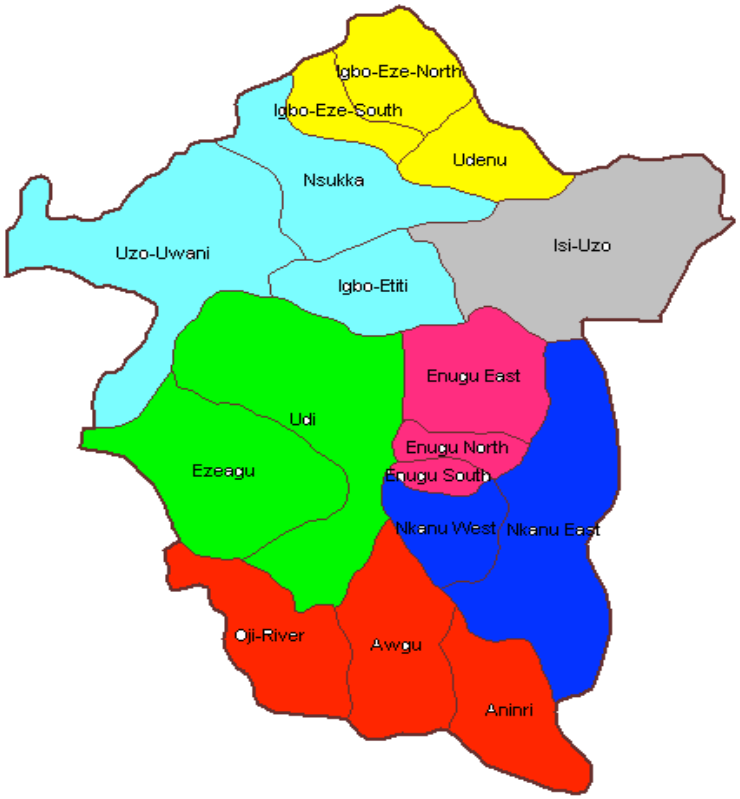

Fig 3a. Map of Enugu State showing the sampling site (7 health districts)

Key

Enugu-Ezike district

Enugu urban district

Nsukka district

Agbani district

Ikem district

Udi district

Awgu ditrict

3.2.3. ETHICAL APPROVAL:

Ethical approval was obtained from the Ethical committee of the State Ministry of Health and the clearance reference number is MH/MSD/EC/O218 (the clearance is attached as appendix 7). Besides, signed informed consent of all the grown up subjects and the parents of the minors were obtained following the approval of the head of institution/facility/schools used for the study. The consent of the proprietors/ keepers of the animal farms used were also obtained as they were helpful in the selection of the animals used.

3.2.4. SAMPLING TECHNIQUES AND CRITERIA:

3.2.4.1 INCLUSION CRITERIA: These include the following subjects:

- Age 0- 60 years.
- Apparently healthy volunteers not on any form of antimicrobial therapy at least 3 months preceeding the sample collection (negative control subjects)
- In- or out-patient who has been on antibiotics at least 4 days preceeding the sample collection.
- Orthopaedic patients who have been on antibiotics due to chronic wound for at least 1 week preceeding the wound swab collection.
- Apparently healthy pigs (age > 4 months), Cattle (age > 8 months), chicken (age > 8 weeks).

-

3.2.4.2. EXCLUSION CRITERIA:

These include the following subjects:

- Age > 60 years.
- Apparently healthy volunteers on any form of antimicrobial therapy at least 3 months preceeding 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.
- Sick animals of all ages or apparently healthy pigs (age < 4 months), Cattle (age < 8 months), chicken (age < 8 weeks).

3.2.4.3. SAMPLE SIZE:

The sample size was calculated using the following equation (Araoye, 2004):

$$N = \frac{(z)^2 pq}{(l)^2}$$

Where N=sample size

z= score for a given confidence interval usually set at 1.96 for 95% C.

p= prevalence value of 50% (0.5)

q= (l-p)=1- 0.5 = 0.5

l= permissible error of estimation which is taken as 0.05 (5%)

$$N = \frac{(1.96)^2 \times 0.5 \times 0.5}{(0.05)^2}$$

= 384

This value was rounded up to 420 samples from each specimen source collected for the study.

3.2.5 Sample collection and storage:

A total of 7980 specimens of urine, faecal matter, and nasal, wound and skin swabs were collected using sterile containers and swab-sticks by random sampling techniques from humans and farm animals in Enugu State. Eight hundred and forty samples each of urine, faecal matter and nasal swabs were collected from both healthy carriers and patients, and 420 wound swabs were collected from patients alone. Futher, 1680 samples of nasal, meat/vendors tables, skin and anal swabs were collected from cattles, pigs and chickens.

At the respective health care facilities, arrangement was made with a physician, matron or laboratory scientist/ technician whereby the parent/caregiver of any child who satisfies the study inclusion criteria was requested to provide the child's stool, urine and nasal swab specimens after consultation. Prior to collection of specimen, the parent/caregiver 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. For animal specimens, arrangement was made with the proprietor and animal keepers who helped in the sample collection. All the specimens collected were transported in icebag to the Microbiology Laboratory of the Department of Pharmaceutical Microbiology, University of Nigeria, Nsukka for immediate culture and sensitivity tests. The FQ-resistant isolates were transported in nutrient agar slants to the Anaerobe Laboratory, Molecular Biology and Biotechnology Division, Nigerian Institute of Medical Research Yaba Lagos (NIMR) for molecular studies.. Methodology was based on PCR and metagenomics analysis. DNA sequencing analysis was done at Inqaba Biotechnology Pty South Africa.

3.2.6. ISOLATION AND CHARACTERIZATION OF FQREC AND FQRSA.

The specimens were inoculated on MacConkey, Eosin methylene blue {EMB} and Mannitol salt agar for the isolation of *Escherichia coli* and *S. aureus* respectively. These isolates were identified using conventional methods as described previously (Cheesbrough 2000; Arora and Cheng 1977). Isolates that fermented lactose and developed greenish metallic sheen were suspected to be *Escherichia coli and* were subjected to IMVIC (indole production, methyl red, voges proskae and citrate utilization test) as described elsewhere (Arora 1999) and the representative colonies were confirmed to be *Escherichia coli* by DNA sequencing. Similarly 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 to be *Staphylococcus. aureus* by conventional PCR.

3.2.7 MOLECULAR CHARACTERIZATION OF THE ISOLATES.

3.2.7.1. CONFIRMATION OF *STAPHYLOCOCCUS AUREUS* ISOLATES BY POLYMERASE CHAIN REACTION (PCR).

Chromosomal DNA was extracted from the isolates by boiling according to the method of Zheng *et al.,* (2004). The identity of the isolates was confirmed based on PCR using the primer set Foward 5 - AAC TCT GTT ATT AGG GAA GAA CA - 3, and reverse: 5 - CCA CCT TCC TCC GGT TTG TCA CC -3 (Inqaba Biotechnical, South Africa) with an expected band of 756 bp according to McClure *et al*., (2006). The PCR reaction mixture was prepared by adding into a sterile eppendorf tubes, 16µl of sterile distilled deionised water, 0.5ul each of the forward and backward primers and 3µl of extracted DNA from the isolates. PCR was carried out in a thermal cycler (A&E, Laboratories UK, Model 005 Gradient thermocycler) with the reaction

cycles consisting of an initial denaturation at 94ºC for 5 min; 35 cycles of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min. A final extension step at 72ºC was continued for another 10 min. The PCR products were resolved on 2% agarose gels containing 0.5 µg/mL ethidium bromide and documented using a gel documentation system (Clinix Science, Japan).

3.2.7.2 CONFIRMATION OF *ESCHERICHIA COLI* ISOLATES BY DNA SEQUENCING

DNA extraction was from a 24 h growth of microbial isolates in BHI broth harvested by centrifugation at 14, 000 x g for 10 minutes. The cells were washed three times in 1 ml of Ultra pure water by centrifuging at 12,000 rpm for 5 min. DNA extraction and purification was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps. Model D6005 (Zymo Research, California, USA). Hundred milligram of bacterial cells was resuspended in 200 µl of sterile water. This was transferred into a ZR BashingBead™ Lysis Tube. Exactly 750 µl Lysis solution was added to the tube. The bead containing the solution was secured in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 5 minutes. The ZR BashingBead™ Lysis Tube was centrifuged in a micro-centrifuge at 10,000 x g for 1 minute. 400 µl of the supernatant was pipeted into a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000 x g for 1 minute. This was followed by the addition of 1,200 µl of Fungal/Bacterial DNA binding buffer into the filtrate in the collection tube. After this 800 µl of the mixture was transferred into a Zymo-Spin™ IIC column in a collection tube and centrifuge at 10,000 x g for 1 minute. The flow through was discarded from the collection tube and the process was repeated to obtain the remaining products. The 200 µl DNA pre-wash buffer was added into the Zymo-Spin™ IIC Column in a new collection tube and centrifuge at 10,000 x g for 1 minute. This was followed by

the addition of 500 µl Fungal/Bacterial DNA Wash Buffer into the Zymo-Spin™ IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC column was transferred into a clean 1.5 ml micro-centrifuge tube and 100 µl of DNA Elution Buffer was then added directly to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The Ultra-pure resulting filtrate (DNA) obtained was used as a template during the assay. This was transported in ice to the laboratory for sequencing. DNA sequencing was performed by Sanger (dideoxy) Sequencing Technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems (Russell, 2002). Primer used was bacteria: 16S, 27-F. This result was obtained as nucleotides. Sequence analysis from resultant nucleotides base pairs was performed by BLAST analysis by using CLO Bio software and by direct blasting on http://blast.ncbi.nlm.nih.gov. . For every set of isolate, a read was BLASTED and the resultant top hits with minimum E-score for every BLAST result showing species name was used to name the specific organism.

3.2.7.3. MAINTENANCE OF THE STOCK CULTURES.

The stock culture of each confirmed isolate was stored in nutrient agar slants at 4^0C. Prior to use, the cultures were activated by successive daily sub-culturing first onto mannitol salt agar and MacConkey agar plates and then into nutrient agar slant, to ensure there was no contaminant, for a period of 3 days.

3.2.8. ANTIMICROBIAL EVALUATION

3.2.8.1.STANDARDIZATION OF TEST ISOLATES FOR SENSITIVITY TEST

The standardization of innoculum was carried out according to the method described by Arora (1999). The tops of 5-10 similar appearing, well isolated colonies on an agar plate were touched with a sterilized straight wire and then inoculated in a nutrient broth medium. These broth bottles were incubated at 37 ^{0}C for 4 – 6 h to obtain the growth at logarithmic phase. The density of the organisms was adjusted to approximately 10^8 colony – forming units (CFU)/mL by comparing its turbidity with that of 0.5 McFarland opacity standards.

3.2.8.2. ANTIBIOTIC SENSITIVITY TESTS

Antibiograms were prepared for the *S. aureus and E. coli* isolates against 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) by disk diffusion method described by Bauer *et al* (1996). Within 10 – 20 min after adjusting the turbidity of the innoculum suspension to that of standard, a sterile nontoxic cotton swab was dipped into the innoculum and rotated several times with firm pressure on the inside wall of the tube to remove excess fluid. The dried surface of Mueller-Hinton agar plate 100mm in diameter containing 20 ml Mueller-Hinton agar was inoculated by streaking the swab three times over the entire agar surface. The lid of the dish was then replaced and the dish was allowed to stand at room temp for 3 min to allow the surface of the agar to dry before the antibiotics discs were applied using sterile forceps. After placement, the disc on the surface of medium was pressed to provide uniform contact. The plates were allowed to stand at

room temperature for 30 minutes to allow for diffusion of drug and then incubated aerobically at 37 ^{0}C for 24 h and the zones of inhibition developed were measured and recorded. The *S. aureus and E. coli* isolates were considered as sensitive or resistant to the test antibiotics based on the inhibition zone diameter (IZD) they produced on the guidelines of the CLSI (2013). *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as the reference strain for the susceptibility studies.

3.2.8.3. EVALUATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF SOME ANTIBIOTICS AGAINST THE TEST FQREC AND FQRSA

The MIC of the antimicrobial agents were determined using agar dilution method (NCCLS, 1999). Twelve (12) different concentrations of pure sample of the ciprofloxacin (PCCATm Houston- USA) and 14 different concentrations of pure sample of gentamicin (Lek Pharmaceutical d.d. Slovenia) and erythromycin (Medisca Inc. USA) were prepared by two-fold dilutions. The ranges of the concentrations of the drugs against FQREC were 1.0 – 2096 μg/ml and 0.065 - 512 μg/ml for ciprofloxacin and gentamicin respectively. Similarly, the ranges of the concentrations of the drugs against FQRSA were 1.0 – 2096 μg/ml and 0.0325 - 128 μg/ml for ciprofloxacin and erythromycin respectively.

With an automatic micropipette, 1.0 ml each of these different dilutions (one dilution per plate) of a single agent was introduced into individual agar plates. The molten agar at 48^{0}C and the antimicrobial agents were mixed carefully and thoroughly and allowed to set. With the aid of a sterile wire loop, the standardized test isolates were streaked on the agar surface of the plates containing different concentrations of the antibiotics. This was done by streaking (about 8-10 different strains of the isolates per

plate) on the surface of the set agar. These inoculated agar plates were incubated at 37^0C for 24 h for bacteria. At the end of the incubation, the MICs were determined as the lowest concentration of the antibiotics that allowed not more than two colony forming units (cfu) to grow in it (Baron and Finegold, 1980).

3.2.8.4. MULTIPLE ANTIBIOTIC RESISTANCE INDEX (MARI):

Multiple antibiotic resistance index (MARI) was evaluated to investigate whether the isolates have been exposed to antibiotics. To determine resistant profile of the FQREC, and FQRSA isolates, MARI was evaluated using the following formular:

MARI = a/b, where "a" is the number of antibiotics to which the FQREC or FQRSA is resistant to and "b" is the total number of antibiotics to which the FQREC or FQRSA isolates was evaluated against.

3.2.9. EVALUATING FLUOROQUINOLONE RESISTANCE MECHANISMS IN FQRSA AND FQREC

3.2.9.1. PCR detection of gyr A, qnr A and Nor A genes in the test isolates

3.2.9.1.1. DNA extraction by boiling.

Staphylococcus aureus and *Escherichia coli* strains stored in slants were sub-cultured on nutrient agar plates and then further on nutrient broth (Oxoid, UK) prior to DNA extraction. The pure single colony of genotypically confirmed isolates was further subcultured in 5 ml of Nutrient broth and incubated at 37 ºC overnight for DNA extraction. The whole chromosomal DNA was extracted by boiling according to the method of Queipo-Ortuño *et al*., (2008). One millilitre of the broth culture of the test isolates was added into a pre-labelled micro tubes. This was centrifuged at 7000g for 3 min (equivalent to 10,000rpm for 2 min) and then the supernatant was discarded. This was followed by the addition of 200µl of sterile distilled water and vortexed to homogenize the pellets. The mixture was boiled at 100^oC for 10 min using a heating

block (Thermo Fisher, UK). This was allowed to cool and then vortexed and centrifuged at 10,000g for 5 min. The supernatant was carefully transfered into another pre-labelled micro tube using micropipette. About 1µl of the DNA obtained was quantified to determine concentration and integrity using a Nano spectrophotometer (Model ND 1000, Thermo Scientific). This was used as template DNA for molecular anaysis.

3.2.9.1.2. PCR Amplifications and cycling conditions

PCR amplifications were performed on a thermocycler (A & E Laboratories, UK Model Cyl-005-1.). The primer pairs used for screening the three fluoroquinolone-resistant genes- *gyrA. qnrA* and *NorA genes*- are shown in Table 1.

The reaction volume was 25 µl and consisted of 10X PCR buffer, 25 mM MgCl2, 10 mM dNTP's mixture, 5 U/µl of Taq DNA polymerase (Fermentas, USA), 10 pmol of each primer set, and 5 ng of extracted bacterial DNA. Amplifications were performed following an initial denaturation temperature at 95 ^{o}C for 10 minutes, followed by 25 amplification cycles of denaturation for 30 seconds at 94 ^{o}C, annealing for 30 seconds at 50 ^{o}C, polymerization for 30 seconds at 72 ^{o}C, and a final extension cycle for 5 minutes at 72 ^{o}C.

For *qnrA,* the reaction volume was 25 µl and consisted of 10X PCR buffer, 25 mM $MgCl_2$, 10 mM dNTP's mixture, 5 U/µl of Taq DNA polymerase (Fermentas, USA), 10 pmol of each primer set, and 5 ng of extracted bacterial DNA. Amplifications were performed following an initial denaturation temperature at 95 ^{o}C for 10 minutes, followed by 30 amplification cycles of denaturation for 30 seconds at 94 ^{o}C, annealing for 30 seconds at 52 ^{o}C, polymerization for 30 seconds at 72 ^{o}C, and a final extension cycle for 5 minutes at 72 ^{o}C.

For *NorA* gene screening, all conventional PCR reactions were performed in a total volume of 25 µl comprising 3 µl of bacterial DNA template and 22 µl reaction mixture containing PCR buffer (10 mM Tris/HCl pH 9.0, 50 mM KCl,1.5 mM $MgCl_2$, 0.1% Triton X-100,), 1.0 U Taq DNA polymerase (Promega, USA), 0.2 mM dNTPs and each primer at a concentration of 0.2 UM. The reaction was carried out in a thermal cycler Model 005 (A & E Laboratory, UK). The PCR program was set at an initial denaturation temperature of 94^{o}C for 4 min, followed by 35 cycles of denaturation at 94 ^{o}C for 30 s , annealing at 45 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min, followed by a final extension at 72 ^{o}C for 5 min for *Nor* A gene. A negative control without a DNA template was included in each PCR run.

3.2.9.1.3 Gel Electrophoresis

Amplified products (10 µl) were separated using 1.5 % agarose gel electrophoresis in TAE buffer (40mM Tris-acetate, 2 mM EDTA [pH 8.3]) performed at 70 V for 1 hour. Gels were stained with 0.5 µg/ml of ethidium bromide for 45 min and destained with water for 20 min. Stained gels were examined under ultra-violet (UV) transilluminator in a photodocumentation system (Clinix Japan). A major bands corresponding to the expected band size was considered in the analysis. A DNA ladder digest of 1 kb (Fermenters USA) was used as a molecular weight marker

TABLE 1: PRIMER USED FOR THE STUDY

Primers	Oligonucleotide sequence (5' to 3')	Reference	Expected Size (bp)
gyrA	5'- AATGAACAAGGTATGCACC-3' 5'-TACGCGCTTCAGTATAACGC-3'	(Schmitz *et al*., 1998)	222
qnrA	F: ATTTCTCACGCCAGGATTTG R: GATCGGCAAAGGTTAGGTCA	(Stephenson *et al*., 2010)	516
NorA	5' TTCACCAAGCCATCAAAAAG'3 5' CTTGCCTTTCTCCAGCAATA'3	(Kaatz and Seo, 1995	705

3.2.9.2. Plasmid profiling

The test fluoroquinolone-resistant isolates were evaluated for the presence of plasmid DNA as described by Gohar *et al*., 2015. One ml of 24 h cultures of test organisms in Trypcate Soy Broth (TSB) medium (Merck, Germany) was transferred into 1.5ml sterile Eppendorf micro-centrifuge 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^{o}C for 1 h. 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 1 h in refrigeration condition (4^{o}C). Plasmid DNA would then be precipitated in each tube by centrifugation at 12.00g for 10mins. The precipitated DNA was dissolved in 50 µl of 10mM Tris EDTA – buffer (TE) containing 10 µl of RNASE. The isolated plasmids were separated on 1.5% agarose gel electrophoresis

and visualized under UV light transilluminator and photographed as described by Farshad *et al.*, (2012).

3.2.9.3.. Plasmid curing experiment:

Plasmid curing was done using acridine orange as given previously (Esimone *et al.*, 2010). Plasmid curing was conducted by treating FQREC and 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 (Esimone *et al.*, 2010). The tubes were incubated at 37^{o}C for 24 h. After incubation, the broth was shaken properly for homogenization of the culture, and loopful of the broth culture was taken and inoculated onto drug-free Mueller-Hinton agar plates and incubated for 24 h at 37^{o}C. Susceptibility tests were carried out on the FQREC and FQRSA treated isolates using the commonly used antibiotic disks in the study area, and then, the diameter of zones of inhibition were taken after incubation. FQREC and 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).

3.2.9.4. Evaluating the effect of efflux pump inhibitor- Omeprazole- on the susceptibility of FQRSA AND FQREC to fluoroquinolones

The preliminary antibiotic susceptibilities of FQRSA AND FQREC in the presence of EPI (omeprazole) were determined by the agar diffusion method using MH agar alone and supplemented with EPIs at 32 μg/ml, 64 μg/ml and 128 μg/ml. Four fluoroquinolone antibiotics were tested including the following disks: ciprofloxacin (5 μg), ofloxacin (5 μg), levofloxacin (5 μg), and pefloxacin (5 μg). After 24 h incubation at 37°C, the inhibition zone diameters were measured. Results are expressed as the mean values of triplicate independent experiments. In the second step of the experiments, the effects of the combination of omeprazole and fluoroquinolone against FQRSA AND FQREC were confirmed by MIC evaluation. MICs of ciprofloxacin and omeprazole were determined for FQRSA and FQREC by agar dilution method (NCCLS, 1999). Bacterial suspensions were prepared from an MH broth culture obtained after incubation at 37°C in a water bath for 6 h and further diluted to 0.5 opacity standard. Ciprofloxacin concentrations ranged between 1024 and 1 μg/ml, and the EPI concentrations ranged between 512 and 1 μg/ml to ensure that the test omeprazole does not exhibit antibacterial activity even at high concentration. In this second series of experiments, antibiotic solutions were combined with EPI at a final concentration of 128 μg/ml. After an 18 h incubation period at 37°C, the MIC was defined as the lowest concentration that inhibited any visible growth. All tests were done in at least triplicate, and the mode values were retained.

3.2.10 Conjugation experiments.

3.2.10.1 Conjugation studies on FQREC

The transfer of fluoroquinolone resistance determinants (eg *qnr A*) gene in FQREC was studied by carrying out conjugation experiments as described previously (Wang *et al*., 2003). Conjugation experiments were performed using *E.coli* BL21 as the recipient . The recipients were selected on M-H with streptomycin (2000 mg/L) and the donors were selected on M-H with ciprofloxacin (0.1 µg/ml) (PCCA^{Tm} Houston-USA). Both recipients (0.09ml) and donors(0.01ml) were added to 2ml of fresh MH broth and incubated for 24 h. Half millilitre of recipient cells in logarithmic phase were added to 4ml of fresh MH broth and incubated for 24 h without shaking. Transconjugants were selected on MacConkey agar plates supplemented with streptomycin (2000mg/L) and ciprofloxacin (0.1 µg/ml). To find out if FQ-resistance determinants co-transferred, colonies were picked from the selection agar and evaluated by PCR and antibiotics susceptibility tests.

3.2.10.2. Antimicrobial susceptibility test on FQREC donors and transconjugants.

MIC of various antibiotics (ciprofloxacin, ofloxacin, levofloxacin, pefloxacin, gentamican, ceftriaxone and doxycyline) were determined for the *qnrA* gene-positive donors and the recipient transconjugants using the agar dilution method according to CLSI guidelines (CLSI, 2013) and using *E. coli* 25922 as a control.

3.2.10.3. Conjugation experiments with FQRSA .

The transfer of plasmid mediated resistance (PMQR) was studied in FQRSA by performing conjugation experiments as described previously (Wang *et al*., 2003) The experiments were performed with *Staphylococcus cohnii subsp urealyticum* (Gen Bank Accession number NR.037046.1) as the recipient. The recipients were selected on M-H agar supplemented with streptomycin (2000mg/L) and the donors (FQRSA) were selected on M-H agar supplemented with ciprofloxacin (0.06 µg/ml:). Both recipients (0.09 ml) and donors (0.01 ml) were added to 2ml of fresh M-H broth and incubated for 16 to 24 h. Culture of donor and recipient cells (0.5ml each) were added to 4 ml of fresh MH broth and incubated for 24 h without shaking. Transconjugants were selected on mannitol salt agar plates supplemented with streptomycin (2000 mg/L)) and ciprofloxacin 0.06 µg/ml). Transconjugants growing on the selection agar were subjected to susceptibility studies to confirm the transfer to resistance markers.

3.2.10.4. Antibiotics Susceptibility test on FQRSA donors and transconjugants

MICs of various antibiotics (ciprofloxacin (CPX) oflxacin (OFX) levoflxacin (LEV), gentamicin (GN), ceftriaxone(CT), and doxycyline(DOXY)) were determined for some FQRSA (habouring plasmids) and recipients transconjugants as well as the pre-conjugated recipient *S. cohnii subsp urealyticum* using the agar dilution method according to CLSI guidelines (CLSI, 2013) and using *S. aureus* 25923 as the control.

3.2.11. Antibacterial evaluation of the essential oil of lemongrass and coconut oil on both FQRSA AND FQREC isolates

3.2.11.1. Extraction Of Essential Oil Of Lemon Grass

Freshly harvested leaves of lemongrass plant were hydro-distilled in a Clevenger type apparatus for 3 h to get essential oil. according to the method used by Reis *et al.*, (2006). Clevenger apparatus is an all glass apparatus which consists of a round bottom flask, condenser and extraction burette. A 1.0 kg of the plant material were chopped and loaded into a 4 litre round bottom flask with sufficient quantity of water. On heating the flask, essential oil glands present in the plant material got ruptured. The steam essential oil vapour generated in the flask passed through a condenser to remove the energy which finally converts the vapour into liquid. The temperature of the condenser was kept low by connecting it to a water circulators loaded with ice blocks. The condensate (mixture of essential oil and water) was collected in an extraction burette. Since the water and essential oil have different densities, essential oil floated on the surface of the water in the extraction burette. The essential oil was measured directly in the extraction burette. The oil yield was calculated in percentage of volume per weight (v/w) of plant samples. The oil samples were stored in an air-tight container at 0^0C before antibacterial evaluation and GC-MS analysis without any further treatment.

3.2.11.2. Procurement of Coconut oil

The coconut oil used for the study was purchased from Ogige Market in Nsukka, Enugu State of Nigeria. Nature's Grove Virgin Coconut Oil is made using a healthy method of extraction "wet milling",this is a non-thermal process of extracting oil which ensures that nutrients/ components are not lost during production.

3.2.11.3 Gas Chromatography/Mass Spectrometry Analysis

The essential oil of lemon grass and the coconut oil were analysed by using GCMS-QP2010 PLUS SHIMNADZU, JAPAN. The capillary column type was DB-IMS [30,0m (length) X0.25µm (diameter) X0.25µm (film thickness)]. The carrier gas used was helium at constant flow rate of 0.99,l/min and average velocity of 36.2cm/s; the pressure was 56.2KPa. The initial column temperature was set at 60^0C for 1 min and increased by 3^0C/min up to 180^0C and to the final temperature of 280^0C at the rate of 6^0C/min; volume injected was 1.0µl at 250^0C and split ratio was 41.6:1. The relative amount of individual components of the total oil was expressed as percentages peak relative to total peak area. Qualitative identification of the different constituents was performed by comparison of the GC-MS data with published mass spectral database (NIST02.L) and the data from literature.

3.2.11.4. ANTIBACTERIAL ACTIVITY OF THE TWO OILS

The lemon grass essential oil and coconut oil were evaluated for their antibacterial activity against the confirmed fluoroquinolone resistant *E. coli* and *S. aureus* by the method of the agar well diffusion as described by Esimone and Adikwu, (2002) with little modification. Two fold dilutions of the two oils were prepared in DMSO ranging from 25 to 3.13% and 100(neat) to 3.13 % for the EO of lemongrass and coconut oil respectively. Molten Mueller-Hinton agar (15 ml) was seeded with 0.1 ml of standardized broth cultures of the test bacteria. A total of 6 wells, 8 mm in diameter were made in the agar using a sterile cork borer. A 0.05 ml each of the two-fold dilutions of the two oils was added into each labeled hole using a sterile pipette. As a control, a 0.05 ml DMSO was put in the centre well. Similarly, two fold dilutions of 0.120 mg/ml of gentamicin were added into respective agar-wells for comparison. The plates were left for 1 h at room temperature for diffusion after which they were

incubated at 37^0C for 24 h. Diameters of the zones of inhibition were measured at the end of the incubation period. The mean of triplicate determinations was taken.

3.2.11.5. EVALUATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF THE TWO OILS AGAINST FQRSA AND FQREC

The MIC of the two oils were determined using agar dilution method (NCCLS, 1999). Eight (8) different dilutions of each of the oils in DMSO were prepared by two-fold dilution. The ranges of the concentrations of the oils against the test isolates were 0.0013 – 0.16 % (lemon grass essential oil) and 0.78 - 10 % (for coconut oil).

With an automatic micropipette, 0.064 ml and 1.0 ml each of these different dilutions (one dilution per plate) of the Lemon grass essential oil and coconut oil was introduced into individual agar plates respectively. The molten agar and the oils were mixed carefully and thoroughly and allowed to set. With the aid of a sterile wire loop, the standardized test isolates were delivered on the agar surface of the plates containing different concentrations of the agent. This was done by streaking (about six-eight different strains of the isolates per plate) on the surface of the set agar. These inoculated agar plates were incubated at 37 ^{0}C for 24 h. At the end of the incubations, the MICs were determined as the lowest concentration of the oil that allowed not more than two colony forming units (cfu) to grow in it (Baron and Finegold, 1990).

3.2.11.5.1 Evaluation of minimum bactericidal concentration (MBC) of the lemongrass essential oil against test isolates.

After taking the MIC readings, the plates with no visible growth were used to determine MBC. Culture on the plate without visible growth were picked using a sterile wireloop and then sub-cultured on freshly prepared Mueller–Hinton agar by streaking method. The culture media were incubated appropriately for 24 h and then observed for growth. After 24 h, the lowest concentration from which the microorganisms did not recover and grow when transferred to the fresh media was recorded as the minimum bactericidal concentration (MBC) (Cheesbrough, 2002).

3.2.11.7 *In-Vitro* interactions of ciprofloxacin and the oils.

3.2.11.7.1. Combined effects of ciprofloxacin and the lemongrass essential oil against the test isolates.

The combined effects of the ciprofloxacin and the lemongrass essential oil against the test isolates were evaluated using the agar dilution checker board method as described by Mandal *et al.,* (2004). The two agents used for the study were combined and incorporated into molten agar at concentration 4 x MIC. For each isolate of the test organism, different concentrations of the two combined agents were prepared and evaluated by combining them at different ratios starting from 0:10 (that is, zero part of the ciprofloxacin to 10 parts of the oil), then moving through 1:9, 2:8, 3:7,… and 10:0. The same procedure was repeated for all the test isolates. For each isolate, the fractional inhibitory concentrations (FIC) of all the ratios of the combined agents were determined and then combined. Their sum gives the combined effect. The FIC value for each agent was calculated using the formula.

$$FIC\ (A) = \frac{\text{MIC of the extract A in Combination}}{\text{MIC of A alone}}$$

The addition of FIC_A and FIC_B gives the FIC index from where an inference can be drawn.

FIC (index) = ΣFIC = FIC (A) + FIC (B..

The effects of the combinations were classified as synergistic, additive, indifference and antagonistic if the FIC index is ≤ 0.5, >0.5 to 1, > 1 to 2 and >2 respectively (Hayami *et al*, 1999).

3.2.11.7.2 Combined effects of fluoroquinolones and coconut oil against the test isolates

3.2.11.7.2.1. Preparation of drug stock solutions and discs

Stock solution containing 100 mg/ml of ciprofloxacin was prepared by weighing out accurately 0.5 g each of the drugs and dissolving in 5 ml sterile water. Two fold serial dilutions were carried out to obtain 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 mg/ml of the drug solutions. These solutions were used to prepare the antibiotic discs using Whatmann No 1 filter paper in accordance with the NCCL standards (NCCLS, 1999)

3.2.11.7.2.2. The combined effects of the ciprofloxacin and coconut oil

The combined effects of the ciprofloxacin and the coconut oil against the test isolates were evaluated using the thin overlay innoculum susceptibility disc method as described elsewhere (Chinwuba *et al.*, 1991) with little modification. The coconut oil was incorporated into molten agar plates at 0.5 and 2.0 % concentration respectively to form base antimicrobial-agar layer. Ciprofloxacin and ofloxacin paper discs of various concentrations prepared as described above were used for the study. The

standardized test isolates (0.1 ml) suspended in 5.0 ml molten agar was added to the plate to form a thin overlay inoculums agar layer and then allowed to solidify. The antibiotics discs were then placed asceptically on the solidified surface. A control is set up in which only the molten agar was poured on a second plate to produce an antimicrobial agent free base agar layer. The antimicrobial discs were then placed asceptically on the solidified surface of the control plate. The inhibition zone diameters were recorded after incubation at 37 ^{0}C for 18-24 h. The antimicrobial combinations which produce 19% increment or more correspond to synergism, increments less than 19% corresponds to additivity while no difference in the inhibition zone diameters exhibit indifference in activity (Chinwuba *et al.,l* 1991).

3.2.11.8. Data analysis:

Statistical analysis: Data resulting from the study were analyzed and evaluated on the basis of averages and percentage values. Tables and bar charts were used (where appropriate) for the presentation of results. Statistically, a descriptive analysis was performed, and variables were analyzed with the statistical package for social sciences (SPSS) version 16.0 for windows. A p-value less than 0.05 was considered to be statistically significant for all the analysis.

CHAPTER FOUR:

4.0 Result

4 .1 Prevalent rate and molecular confirmation of *Escherichia coli and Staphylococcus aureus* isolates

The isolation or prevalence rates (%) of *Staphylococcus aureus* and *Escherichia coli* isolates from human subjects in this study are shown in Table 2 and Table 3 respectively. A total of 2487 human *E. coli and S. aureus* isolates were recovered from 420 samples each of urine, faecal matter, nasal and wound swabs of both human patients and healthy volunteers. This total sum (2487) comprises 669 and 531 healthy volunteer *E. coli and S. aureus* isolates, and 792 and 495 patients' *E. coli and S. aureus* respectively. In healthy volunteers, 154, 415 and 281 *E. coli* strains were isolated from urine, stool and nasal swab specimens at isolation rates of 36.7, 98.8 and 24.8% respectively. In the test human patients, 287, 384, 95 and 26 *E. coli* isolates were recovered from urine, stool, nasal and wound swab specimens at isolation rates of 68.3, 91.4, 21.7 and 6.2% respectively. The test *S. aureus* isolates were recovered from urine and nasal swabs of the healthy volunteers at the isolation rates of 50.5 and 76.0% respectively. In human patients, the prevalence rates of *S. aureus* isolates obtained from specimen of urine, nasal and wound swabs were 62.1, 45.5 and 10.2% respectively.

Similarly, a total of 920 animal test isolates comprising 559 *E. coli* and 361 *S. aureus* were recovered from 420 samples each of nasal, fecal, vendors tables and skin swabs collected from pig, cattle and chicken. Based on the specific specimen used, the isolation rate of *E. coli* strains recovered from nasal, skin, meat sellers table and anal swabs of the three farm animals were: pig (9.0, 9.3, 9.5, 30.3%), cattle(6.0, 6.4, 13.7 , 16.2%) and chicken (0, 6.0, 9.3, 19.8%) respectively. The isolation rates of *S. aureus*

strains recovered from nasal, skin, and vendors tables' swabs were 11.0, 10.2 and 18.3% respectively. The isolation rates of *S. aureus* from specimens collected from cattle and chicken are shown in Table 2

Table 2: Isolation rates of both *Escherichia coli* and *Staphylococcus aureus* in human subjects

Isolate Source	Sample size (n)	*Escherichia coli*		*Staphylococcus aureus*	
		Healthy volunteer (%)	Patients (%)	Healthy volunteer (%)	Patients (%)
Urine	420	154 (36.7)	287 (68.3)	212 (50.5)	261 (62.1)
Stool	420	415 (98.8)	384 (91.4)	N.S	N.S
Nasal swab	420	100 (24.8)	95 (21.7)	319 (76.0)	191 (45.5)
Wound swab	420	N.S	26 (6.2)	N.S	43 (10.2)
Total	$1680\left(\sum PT\right)+1260\left(\sum HV\right)$ = 2940	669	792	531	495
		1461		1026	

NB: Values in parenthesis represent the isolation rate.

Key: N = total number of specimen from both healthy volunteer and patients

NS = not sampled

∑HV = Total number of samples from healthy volunteers

∑PT = Total number of samples from patients.

Table 3: Isolation rates of both *Escherichia coli* and *Staphylococcus aureus* in animal subjects

Isolate source	Sample size(n)	Number(%) of *Escherichia coli*			Number (%) of *Staphylococcus aureus*		
		Pig	Cattle	Chicken	Pig	Cattle	Chicken
Nasal swab	420	38 (9.0).	25 (6.0)	N.S	46 (11.0)	27 (6.4)	N.S
Skin swab	420	39 (9.3)	27 (6.4)	25 (6.0)	43 (10.2)	40 (9.5)	23 (5.5)
Faeces	420	127 (30.2)	68 (16.2)	83 (19.8)	N.S	N.S	N.S
Meat /vendors table	420	40 (9.5)	48 (13.7)	39 (9.3)	77 (18.3)	53 (12.6)	52 (12.4)
Total	168x3* = 5040	244	168	147	166	120	75
			559			361	

NB: Figures in parenthesis represent isolation rate.

*Three farm animals were used. So that the total sample size in animals was

1680 x 3 =5040

Key: NS: not sampled

4.1.1. Confirmation Of *Staphylococcus aureus* isolates By PCR

The identity of *S. aureus* as confirmed by PCR is represented in Fig 3b below. The identity of the isolates was confirmed based on PCR analysis.

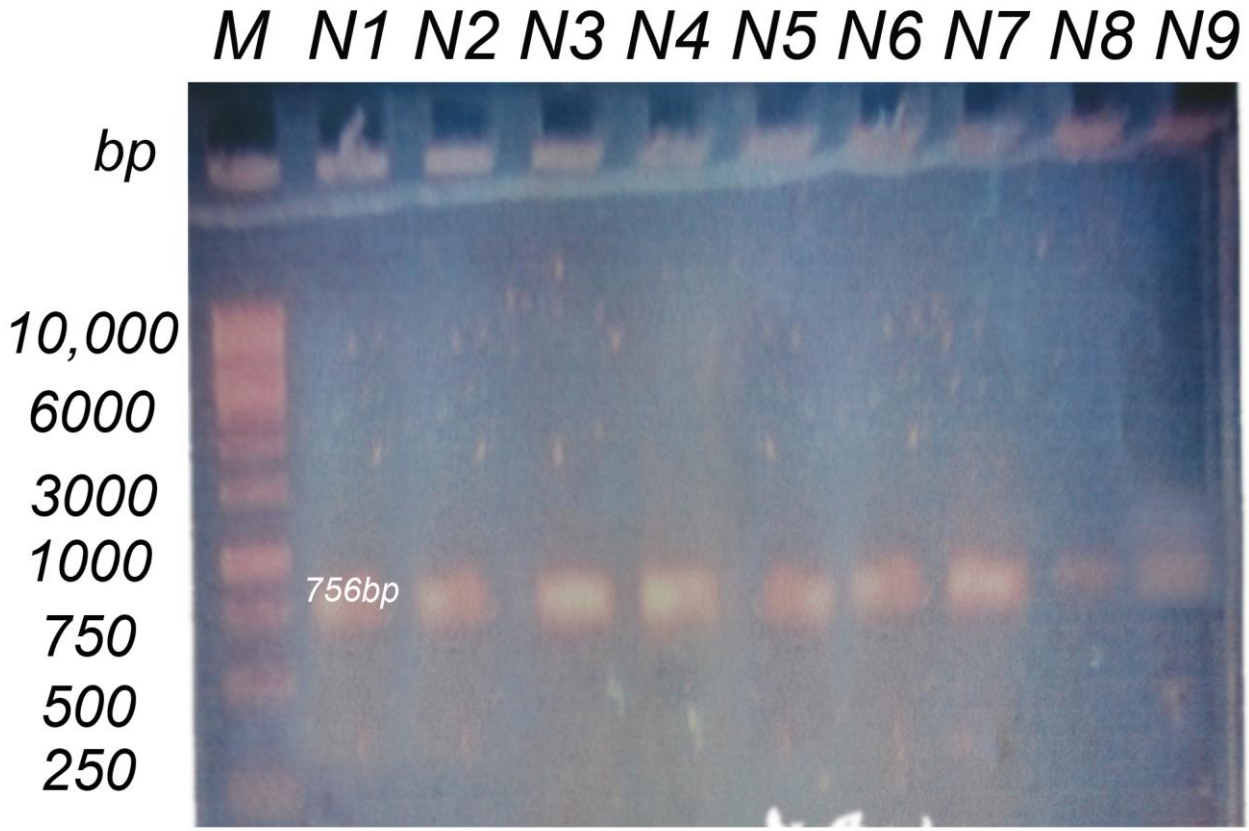

Fig 3b; Identification of *Staphylococcus aureus* isolates by polymerase chain reaction

Key: M = 1kb DNA maker (Appendix 8)

N1 = *S. aureus* positive control

N2-N5 = human isolates confimed to be *S. aureus*

N6–N9= animal isolates confirmed to be *S. aureus*

4.1.2. CONFIRMATION OF *ESCHERICHIA COLI* ISOLATES BY DNA SEQUENCING .

Table 4 . DNA sequencing result of the representative fluoroquinolone resistant *E coli* isolates with their corresponding accession numbers.

Sequencing Lab No	Lab No	Sample Type	DNA Type	Gene bank Accession Number	Name of organism
1	Ec 1	DNA	Genomic	Gu594312.1	*Escherichia coli*
2	Ec 10	DNA	Genomic	CP008805.1	*Escherichia coli 0157*
3	Ec 65	DNA	Genomic	CP008805.1	*Escherichia coli0157*
4	Ec 16	DNA	Genomic	KJ803893.1	*Escherichia coli*
				CP010816.1	*Escherichia coli*
5	CS17	DNA	Genomic		
6	CS18	DNA	Genomic	KJ210328.1	*Escherichia coli*
7	CE71	DNA	Genomic	KJ847237.1	*Escherichia coli*
8	CE73	DNA	Genomic	CP007592.1	*Escherichia coli 0157*
9	CE75	DNA	Genomic	CP007592.1	*Escherichia coli 0157*
10	CE76	DNA	Genomic	FR715025.1	*Escherichia coli*
11	CE77	DNA	Genomic	KJ477001.1	*Escherichia coli*
12	CE78	DNA	Genomic	CP002729.1	*Escherichia coli*
13	CE79	DNA	Genomic	JN162446.1	*Escherichia coli*
14	CE82	DNA	Genomic	CP0108816.1	*Escherichia coli*
15	CE83	DNA	Genomic	KJ831499.1	*Escherichia coli* ESBL88B15_13_1E
16	CE84	DNA	Genomic	HQ169124.1	*Escherichia coli*
18	CA	DNA	Genomic	CP007393.1	*Escherichia coli*
19	CB	DNA	Genomic	CP009166.1	*Escherichia coli*
17	CE102	DNA	Genomic	KJ803896.1	*Escherichia coli*
20	CD	DNA	Genomic	CP002729.1	*Escherichia coli*

NB: The sequencing codons of some *E.coli* strains identified are shown as appendix 1

4.2 Antibiotics susceptibility patterns and prevalence of test FQREC and FQRSA Isolates.

The antibiotics susceptibility patterns and the prevalence of fluoroquinolone resistance in *E. coli and S. aureus* strains isolated from humans and animals are shown in Tables 5-12. The comparison of the antibiotic resistance patterns of these tests isolates according to source, location and age are presented in Figures 4 - 30

For non fluoroquinolone antibiotics, the 1461 human, 244 pig, 168 cattle and 147 chicken *E .coli* isolates tested showed the following percentage resistance to gentamicin, ceftriaxone, amoxycillin, erythromycin and doxycycline in human (24.0, 17.8, 84.7, 94.8 and 72.1%) , in pig (4.9, 2.9, 64, 73.0 and 63.5%), in cattle (3.6, 1.8, 57%, 90.5 and 64.9%), and in chicken (16.3, 10.9, 88.0, 96 and 87.1%) respectively.(Tables 5 and 6). In this study, high antibiotics resistance to erythromycin, amoxycilin and tetracycline were recorded among human and animal *E.coli* isolates.

The prevalence of fluoroquinolone resistance among the *E.coli* isolates from the human test subjects is (20.8, 20.5, 20.7, 21.5%), pig (5.7, 6.1, 5.7 and 7.8%), cattle (0, 0, 0 and 0%), and chicken (13.6, 14.3, 11.6 and 17.7%) for ciprofloxacin, ofloxacin, levofloxacin and pefloxacin respectively. In human, the prevalence of of1oxacin resistant *E. coli* was the least while that of pefloxacin resistance was highest. The prevalences of FQREC isolates according to specific specimen source of the isolates are shown in Table 12. No FQREC was isolated from the nasal swabs of the pigs and all the specimens from cattle. The mean prevalence of FQREC from the *E coli* isolates from the nasal swab, fecal specimen, skin swab and meat/vendor's table swab of pig were 0, 9.2, 10.3 and 11% respectively. For chicken *E. coli* isolates, the mean prevalence of FQREC from the fecal specimen, skin swab and meat/vendor's table swab were 13.2, 14.0 and 16.7% respectively.

For non-fluoroquinolone antibiotics against the *S. aureus* tested the percentage resistance to gentamicin, ceftriaxone, amoxycillin, erythromycin and doxycycline were: for human 23.9, 18.3, 88.5, 27.7 and 84.4%; pig 36, 16, 86, 24.7 and 86.3% ; cattle 13.3, 10, 90, 19.2 and 83.3%, and chicken 13.3, 5.3, 88, 16 and 82.3% respectively. The resistance rates of the *S. aureus* isolates (from human and animals tested) to gentamicin, ceftriaxone and erythromycin were low when compared with other antibiotics tested. Like *E. coli* isolates, the high resistance of *S. aureus* to amoxycillin (78.3% in humans, 64.8% in pig, 57.0% in cattle and 88% in chicken)), observed in this study is of public health concern.

The prevalence of ciprofloxacin, ofloxacin, levofloxacin and pefloxacin resistance among *S. aureus* isolated were for human, 21.1, 21.6, 19.4 , 22.5 %, pig 4.1, 3.4, 2.7 , 4.8 %, cattle 7.5, 7.5, 5.8 ,13.3% and chicken 13.3, 13.3, 13.3, 13.3% respectively (Tables 7 and 8). The present data show that the prevalence of FQ-resistance *S. aureus* isolates from human range from 19.4- 22.5%. while in animal, the range is 3.4- 13.3% depending on the source of the isolate and the structure of the fluoroquinolone under study. Based on the sex of the test subject (healthy and human patients), 91 of 236 (39%) FQREC isolates were from male subject while 145 (61%) were from female subject under study (Table 9**)**.

The prevalence of fluoroquinolone resistance among *S. aureus* isolates from different specimens of pig origin are: nasal swab ,0.0, 0.0, 0.0, 0.0% , skin 6.1, 6.1, 3.0, 9.1 % and Meat/ vendor;s table 5.9, 4.5, 4.5 , 5.9% for ciprofloxacin, ofloxacin, levofloxacin and pefloxacin respectively(Table 10). Other specific prevalence are shown in the same table.

Table 5. The antibiotics Resistance Profile of *E.coli* isolates from Human subject.

Specimen source	Specimen	Number of isolates	Cpx	Ofx	Lev	Pef	Gn	Cef	Amx	Ery	Doxy
Healthy volunteer	Stool	415	38 (9.1)	35 (8.41)	39 (9.4)	40 (9.6)	60 (14.5)	46 (11.1)	292 (70.4)	415 (100)	398 (95.9)
	Urine	154	19 (12.3)	19 (12.3)	22 (14.3)	24 (15.6)	23 (14.9)	14 (9.1)	119 (77.3)	109 (7.8)	109 (70.8)
	Nasal swab	100	33 (33)	33 (33)	28 (28)	33 (33)	32 (32)	15 (15)	81 (81)	78 (78)	78 (78)
Patient	Stool	384	93 (24.2)	94 (24.5)	94 (24.5)	94 (24.5)	130 (33.9)	100 (26.0)	384 (100)	384 (100)	384 (100)
	Urine	287	71 (24.7)	68 (23.7)	69 (24.4)	73 (25.4)	71 (24.4)	43 (14.9)	248 (86.4)	285 (99.3)	225 (78.4)
	Nasal swab	95	35 (36.8)	35 (36.8)	35 (36.8)	35 (36.8)	30 (31.6)	35 (36.8)	95 (100)	95 (100)	95 (100)
	Wound swab	26	15 (57.7)	15 (57.7)	15 (57.7)	15 (57.7)	4 (15.4)	7 (26.5)	18 (69.2)	19 (73.1)	17 (65.4)
Total mean % prevalence		1461	304 (20.8)	299 (20.5)	302 (20.7)	314 (21.5)	350 (24.0)	260 (17.8)	1237 (84.7)	1385 (94.8)	1306 (89.4)

NB: Figures in parenthesis represent the prevalence(%) of antibiotics resistance isolates

KEY: Cpx = Ciprofloxacin, Ofx = ofloxacin, Lev = levofloxacin, Pef = pefloxacin, Gn = Gentamicin, Cef = Ceftriaxone, Amx = Amoxicillin, Ery = erythromycin, Doxy = Doycyline.

Table 6. The antibiotics resistance profile of *E.coli* isolates from the animals tested.

Number of *E.coli* isolates (%) prevalence

Animals	**Number of isolate(y)**	**CPX**	**Ofx**	**Lev**	**Pef**	**Gn**	**Cef**	**Amx**	**Ery**	**Doxy**
Pig	244	14	15	14	19	12	7	158	178	155
	(14.5)	(5.7)	(6.1)	(5.7)	(7.8)	(4.9)	(2.9)	(64.8)	(73)	(63.5)
Cattle	168	0	0	0	0	6	3	96	152	109
	(10.0)	(0)	(0)	(0)	(0)	(3.6)	(1.8)	(57)	(90.5)	(64.9)
Chicken	147	20	21	17	26	24	16	130	141	128
	(8.8)	(13.6)	(14.3)	(11.6)	(17.7)	(16.3)	(10.9)	(88)	(96)	(87.1)

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

KEY: Cpx = Ciprofloxacin, Ofx = ofloxacin, Lev = levofloxacin, Pef = pefloxacin, Gn = Gentamicin, Cef = Ceftriaxone, Amx = Amoxicillin, Ery = erythromycin, Doxy = Doycyline.
Y: values in parenthesis under the number of isolates represent isolation rate of *E. coli*

Table 7. The antibiotics Resistance Profile of *S.aureus* isolates from Human subject.

Specimen source	Specimen used	Isolate number	Number of *S.aureus* isolates (%) Prevalence)								
			Cpx	Ofx	Lev	Pef	Can	Cef	Amx	Ery	Doxy
Healthy volunteer	Nasal swab	319	36 (11.30)	33 (10.3)	36 (11.3)	35 (10.5)	25 (7.8)	30 (9.4)	279 (87.2)	64 (20.0)	257 (80.3)
	Urine specimen	212	42 (19.8)	42 (19.8)	47 (22.2)	50 (23.3)	52 (24.5)	57 (26.9)	192 (90.6)	55 (25.9)	189 (35.8)
	Nasal swab	191	43 (22.5)	45 (23.6)	43 (22.5)	56 (29.3)	69 (36.1)	48 (25.1)	191 (100)	66 (34.6)	191 (100)
Patient	Urine specimen	261	83 (31.8)	83 (31.8)	70 (26.8)	83 (31.8)	80 (30.7)	38 (14.6)	203 (77.9)	78 (29.9)	195 (74.7)
	Wound swab	43	28 (65.1)	28 (65.1)	26 (60)	30 (70)	19 (44.2)	15 (34.9)	43 (100)	21 (48.8)	34 (79)
Total mean % prevalence		1026	232 (22.6)	231 (22.5)	222 (21.6)	254 (24.8)	245 (23.9)	188 (18.3)	908 (88.5)	284 (27.7)	866 (84.4)

Table 8. The antibiotics Resistance Profile of *S. aureus* isolates from the animals tested

Animals	**isolate No. (y)**	**CPX**	**Ofx**	**Lev**	**Pef**	**Gn**	**Cef**	**Amx**	**Ery**	**Doxy**
Pig	166 (13.2)	6 (4.1)	5 (3.4)	4 (2.7)	7 (4.8)	38 (36)	23 (16)	126 (86)	36 (24.7)	126 (86.3)
Cattle	120 (9.5)	9 (7.5)	9 (7.5)	7 (5.8)	16 (13.3)	16 (13.3)	12 (10)	108 (90)	23 (19.2)	100 (83.3)
Chicken	75 (8.9)	10 (13.3)	10 (13.3)	10 (13.3)	10 (13.3)	10 (13.3)	4 (5.3)	66 (88)	12 (16)	65 (82.7)

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

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

y: values in parenthesis under the number of isolates represent isolation rate of *Staphylococcus aureus.*

Table 9. Prevalence Of FQRSA According To Specimen Source, Subject Age And Sex.

Specimen Source	**Age Range (yr)**	**Total no *S. aureus***	**No of FQRSA(%)**	**Sex M (%)**	**F (%)**
HVU	0-5	20	4(20)	2(50)	2(50)
	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	0-5	71	5(7.0)	2(40)	3(60)
	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)
W/S	19-60	43	28(65.1)	19(68)	9(32)
Pig	0.5-2	146	6(4.1)		
Cattle		120	9(7.5)		
Chicken	1/12-4/12	75	10(13.3)		

Key : HVU= healthy volunteer urine specimen
PSU= Patient specimen of urine
HVns = healthy volunteer faecal specimen
Pns= Patient nasal swab PWS= Patient wound swab
M= male subject F= female subject

Table 10 : Prevalence (%) of fluoroquinolone –resistant *S. aureus* (FQRSA) isolates from the test animals according to the specimen source.

Animal.	**Source .**	**No of *S. aureus* isolate (y%)**	**Prevalence of FQRSA (%) Cpx**	**ofx**	**lev**	**pef**
Pig	Nasal swab	46(11.1)	0 (0)	0(0)	0(0)	0 (0)
	Skin swab	43(10.2)	2(6.1)	2(6.1)	1(3.0)	3(9.1)
	Meat/vendors table	77 (18.3)	4(5.9)	3(4.5)	3(4.5)	4(5.9)
Cattle	Nasal swab	27(6.4)	0 (0)	0 (0)	0 (0)	0 (0)
	Skin swab	40(9.5)	2 (5)	20 (5)	2 (5)	0 2(5)
	Meat/vendors table	53(12.6)	7 (13.2)	7 (13.2)	5 (9.4)	14 (26.4)
Chicken	Skin swab	23(5.6)	2(8.7)	2(8.7)	2(8.7)	2(8.7)
	Meat/vendors table	52(12.4)	8(15.4)	8(15.4)	8(15.4)	8(15.4)

NB; values in parenthesis under the number of isolates represent prevalence of *S.. aureus* (y) and FQRSA

Key: CPX= Ciprofloxacin, Lev= Levofloxacin , OFX = Ofloxacin, Pef = Pefloxacin

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Table 11. Prevalence of FQREC according to specimen source, subject age and gender.

Specimen Source	**Age Range(yr)**	**Total no, of *E.coli***	**No of FQREC%**	**Gender – FQREC**	
				M(%)	**F(%)**
HVU	0-5	37	3(8.1)	1(33)	2(27)
	6-18	52	5(9.6)	1(20)	4(80)
	19-60	65	11(16.9)	2(18)	9(82)
PSU	6-18	199	28(23.5)	10(36)	18(64)
	19-60	168	43(25.6)	23(53)	20(47)
HVFS	0-5	162	5(3.1)	3(60)	2(40)
	6-18	281	13(4.6)	3(23)	10(47)
	19-60	198	20(11.9)	7(37)	13(65)
PFS	6-18	373	38(10.2)	14(37)	24(62)
	19-60	320	55(17.2)	21(38)	34(62)
PWS	19-60	26	15(57.7)	6(40)	9(60)
Pig	0.5-2	244	14(5.7)		
Chicken	1-4months	147	20(13.6)		

Key:
HVU = Healthy Volunteer urine specimen
PSU = Patient specimen of urine
HVFS = Healthy volunteers faecal specimen
PFF = Patient faecal specimen.
M = male subject f= female subject

Table 12 : Prevalence (%) of fluoroquinolone –resistant *E. coli* (FQREC) isolates from the test animals according to the specimen source.

Animal.	**Source .**	**No of *E .coli* isolate (%)**	**Prevalence of FQREC (%)**			
			Cpx	**ofx**	**lev**	**pef**
Pig	Nasal swab	38(9.0)	0 (0)	0(0)	0(0)	0 (0)
	Fecal specimen	127(30.0)	7 (5.5)	8 (6.3)	7 (5.5)	10(7.9)
	Skin swab	39(9.2)	4(10.3)	4(10.3)	4(10.3)	4(10.3)
	Meat/vendors table	40 (7.6)	3(7.5)	3(7.5)	3(7.5)	3(7.5)
Cattle	Nasal swab	25(6.0)	0 (0)	0 (0)	0 (0)	0 (0)
	Fecal specimen	68(16.1)	0 (0)	0 (0)	0 (0)	0 (0)
	Skin swab	27(6.4)	0 (0)	0 (0)	0 (0)	0 (0)
	Meat/vendors table	48(11.4)	0 (0)	0 (0)	0 (0)	0 (0)
Chicken	Fecal specimen	83(19.8)	10(12.0)	11(13.2)	10(12.0)	13(15.7)
	Skin swab	25(6.0)	3(12.0)	3(12.0)	3(12.0)	5(20.0)
	Meat/vendors table	38(9.0)	7(17.9)	7(17.9)	4(10.3)	8(20.5)

NB; values in parenthesis under the number of isolates represent prevalence of *Escherichia coli* and FQREC

Key: CPX= Ciprofloxacin, Lev= Levofloxacin , OFX = Ofloxacin, Pef = Pefloxacin

Based on the district or the geographical region of isolation of the test bacteria, the resistance pattern of *E. coli* and *S. aureus* isolates from both the healthy volunteers and patients are shown in Figures 4- 10 and Figures 11 -17 respectively. It is clear from the figures that high level resistance were exhibited by these two bacteria against the antibiotics used. The level of antibiotics resistance was more with *E. coli* isolates from patients specimens than from the corresponding specimens obtained from healthy carriers. However, there are few exceptions in which the percentage resistance of both patients and the carriers are equal. In both Enugu-Ezike and Nsukka district, the resistance rate of *E. coli* isolates to erythromycin was 100% for both patients and the carriers. In Ikem, Agbani and Awgu districts, the resistance rates of *E. coli* isolates to both Gentamicin and Ceftriaxone were low when compared with other antibiotics tested including fluoroquinolones. Based on the age of the subjects, Figures 17 and 18 compare the antibiotic resistance pattern of *E. coli* isolates from stool and urine specimens respectively. The antibiotics resistance of *E.coli* isolates from stool and urine specimen of healthy volunteers assumed a non-uniform pattern for the most of the antibiotics tested. However, for the fluoroquinolones (FQ), there is increase in the percentage of antibiotics resistance as the age of the subjects increased.

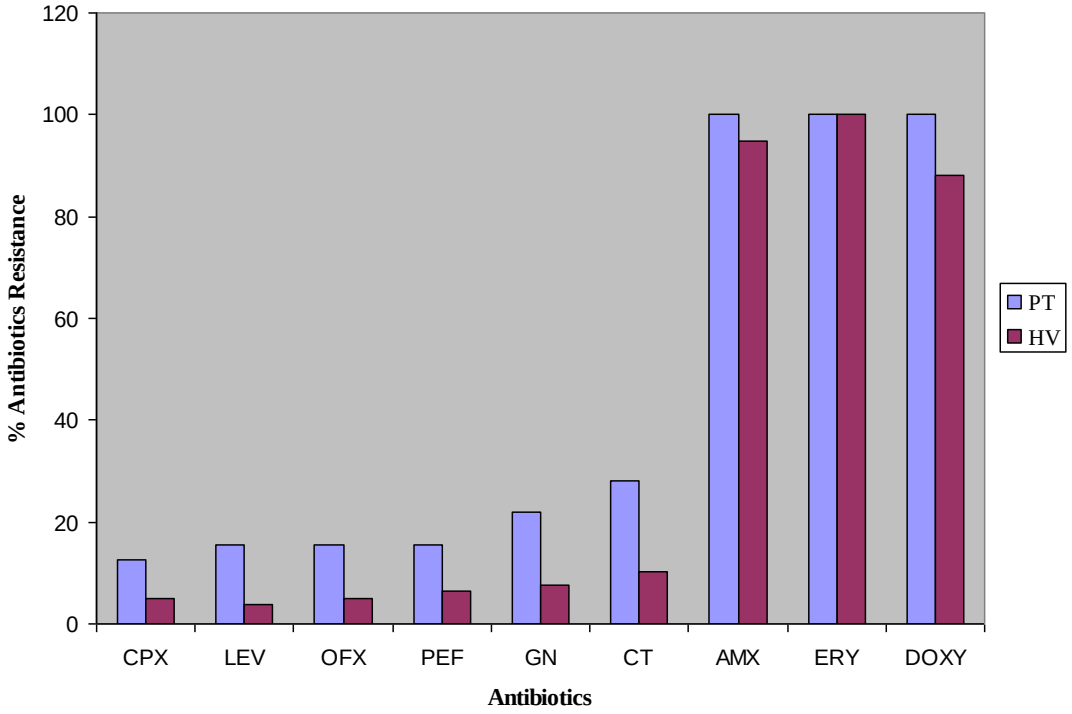

Fig. 4.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both healthy volunteer (HV) and patient (PT) in Enugu Ezike health district.
KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

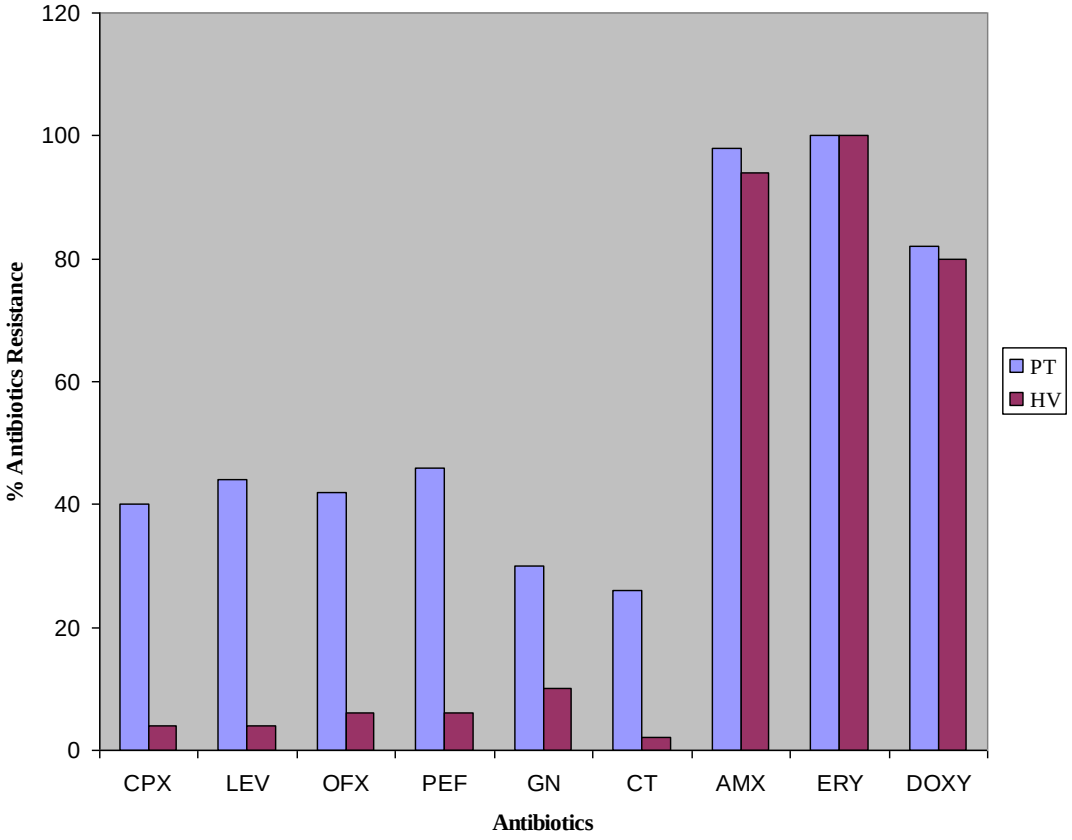

Fig. 5.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both healthy volunteer (HV) and patient (PT) in Nsukka health district.
KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

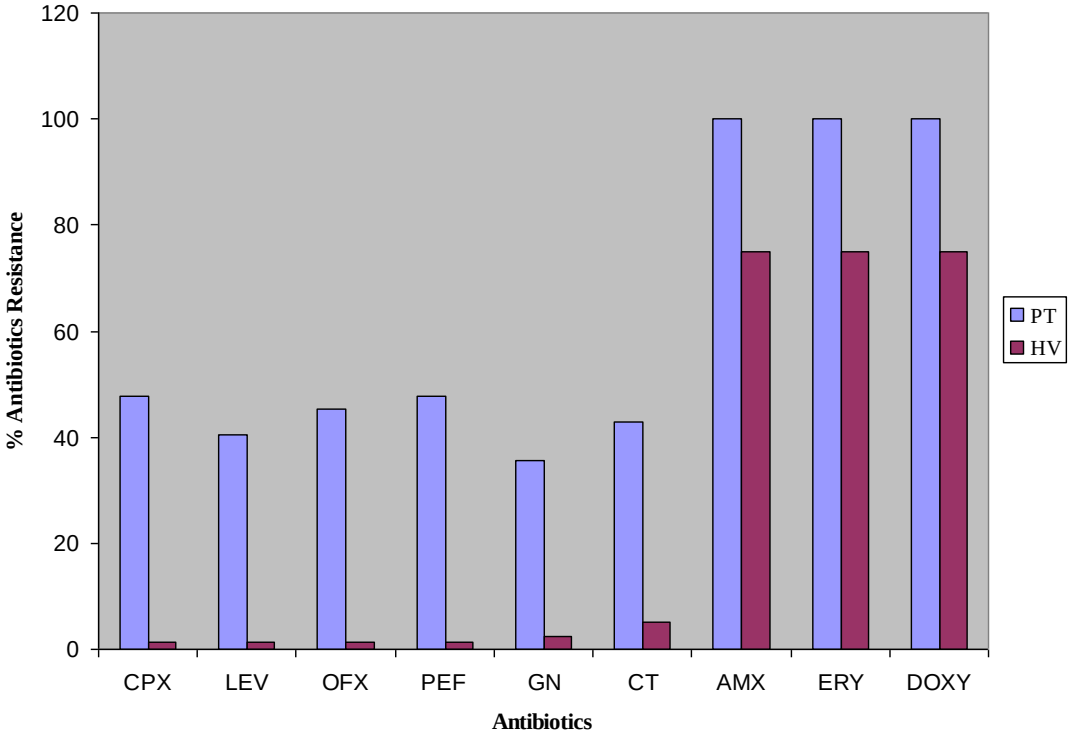

Fig. 6.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both Healthy volunteer (HV) and Patient (PT) in Enugu urban health district.
KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

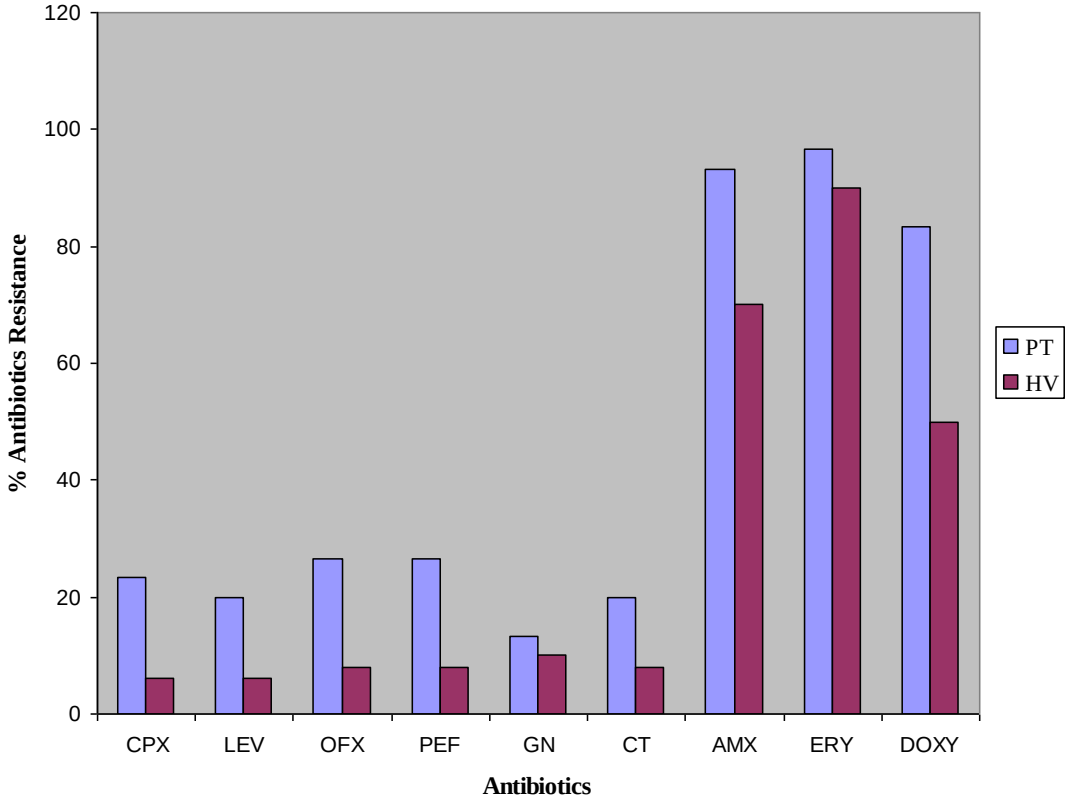

Fig. 7.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both healthy volunteer (HV) and patient (PT) in Udi health district.
KEY: HV healthy volunteer, PT Patient , Cpx ciprofloxacin, Ofx ofloxacin, Lev levofloxacin, Pef pefloxacin. Gn Gentamicin, Cef Ceftriaxone, Amx amoxicillin, Ery Erythromycin, Doxy Doxycycline.

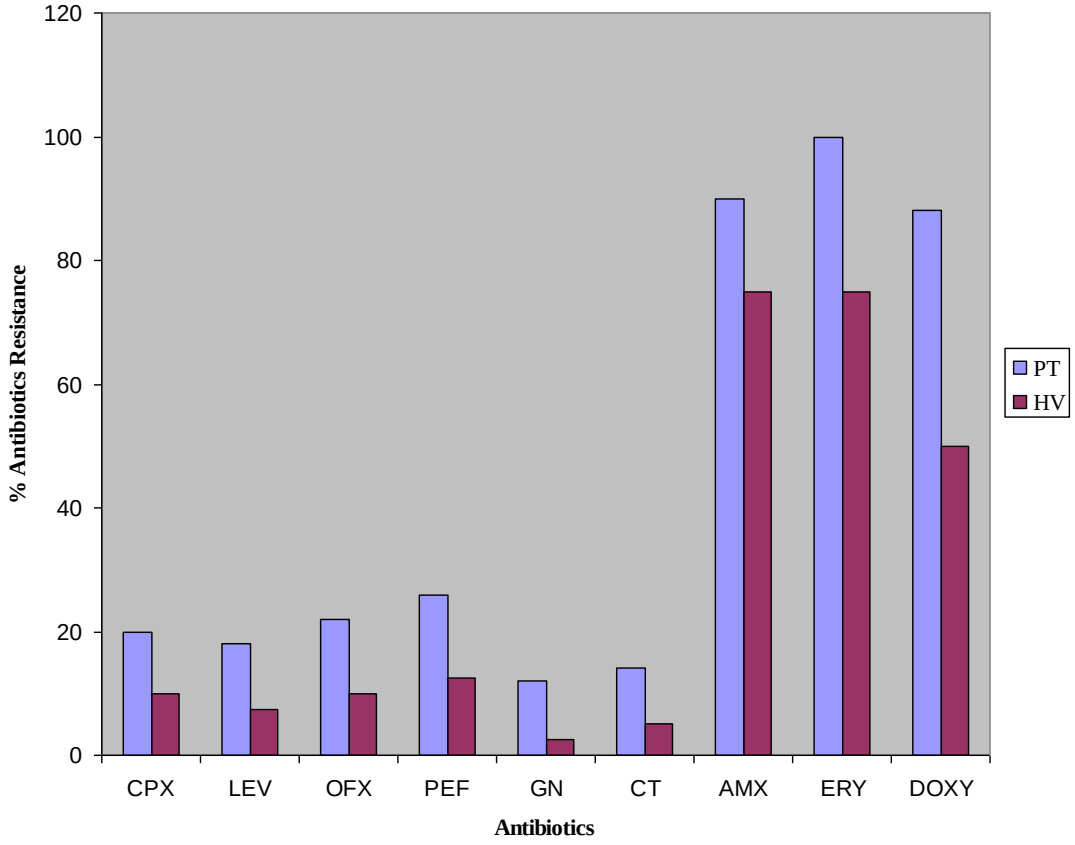

Fig.8.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both healthy volunteer (HV) and patient (PT) in Ikem health district.
KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

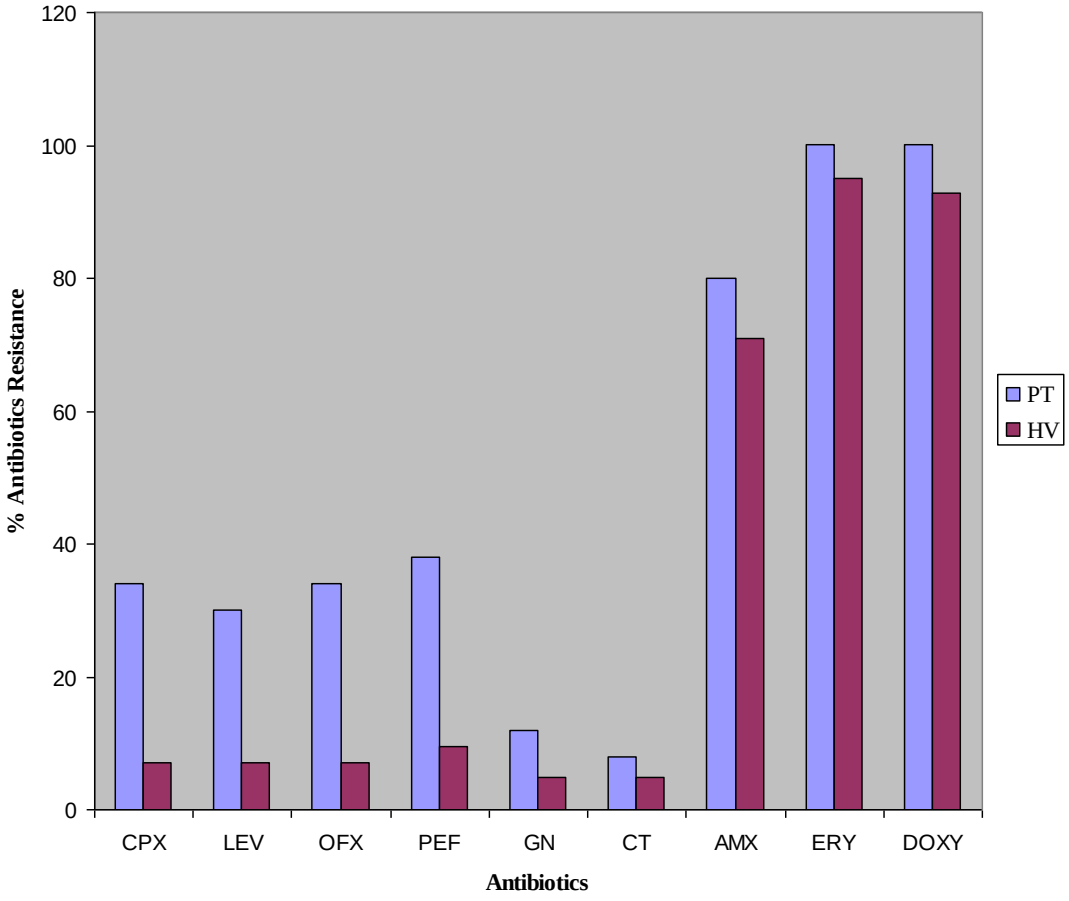

Fig. 9.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both healthy volunteer (HV) and patient (PT) in Agbani health district.
KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

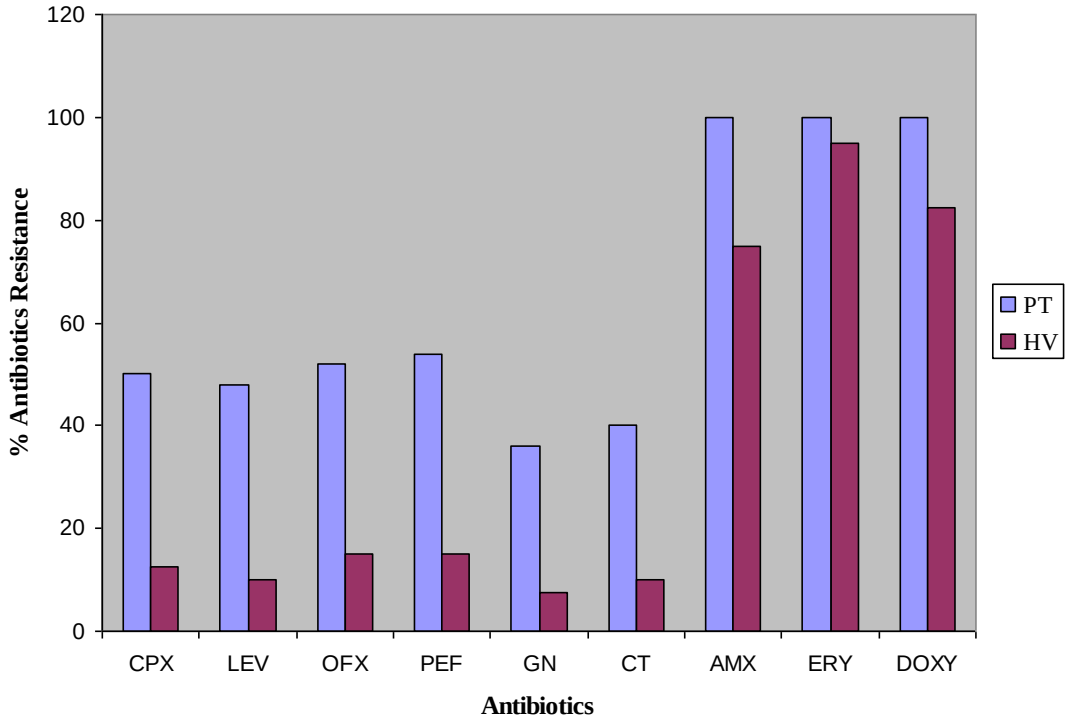

Fig. 10.0 The antibiotic resistance pattern of *Escherichia coli* isolates from both healthy volunteer (HV) and patient (PT) in Awgu health district.
KEY: KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

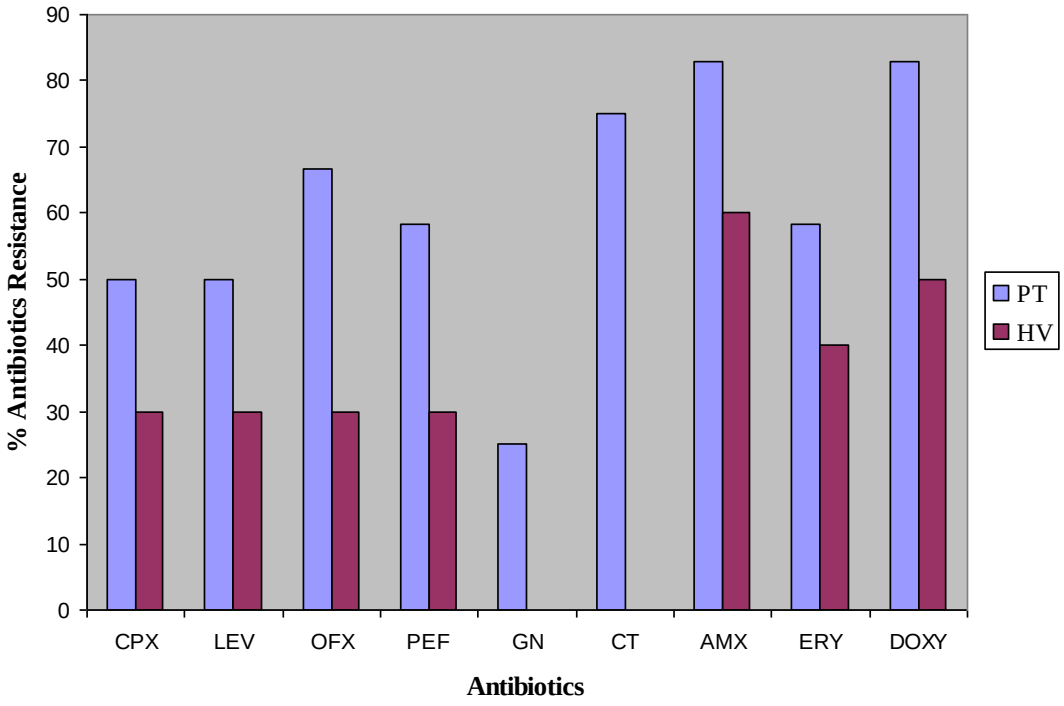

Fig. 11.0 The antibiotics resistance pattern of *S. aureus* isolates from both healthy volunteer (HV) and patient (PT) in Agbani health district.
KEY: KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

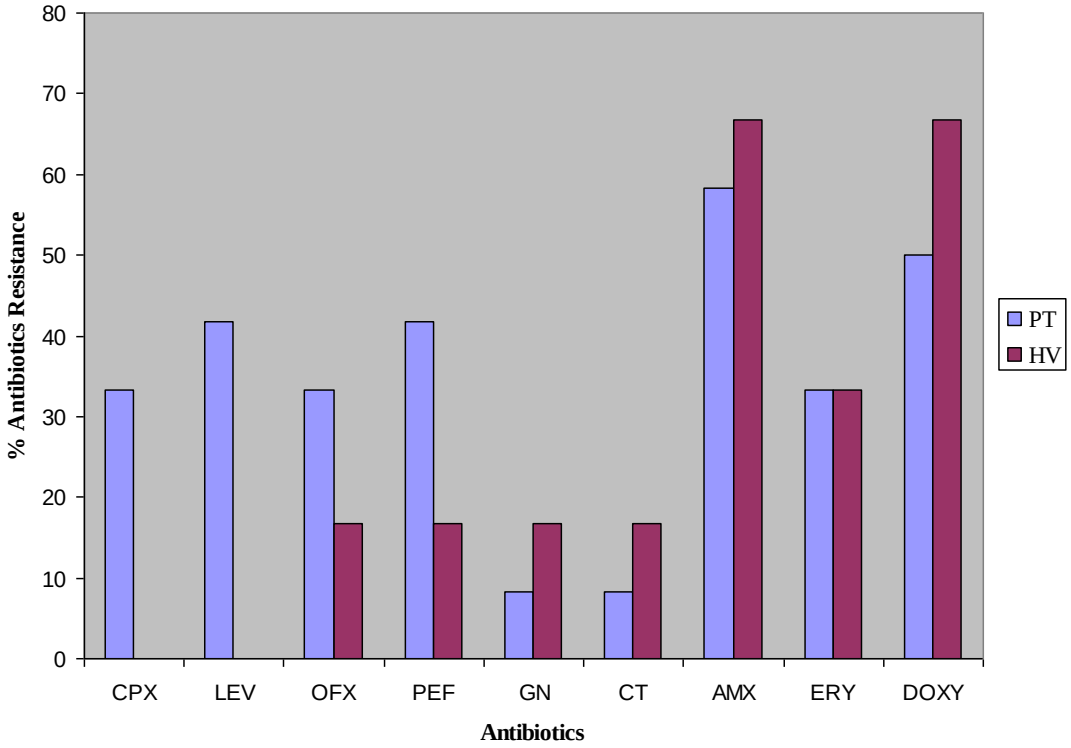

Fig. 12.0 The antibiotics resistance pattern of *S. aureus* isolates from both healthy volunteer (HV) and patient (PT) in Enugu Urban health district.
KEY: KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

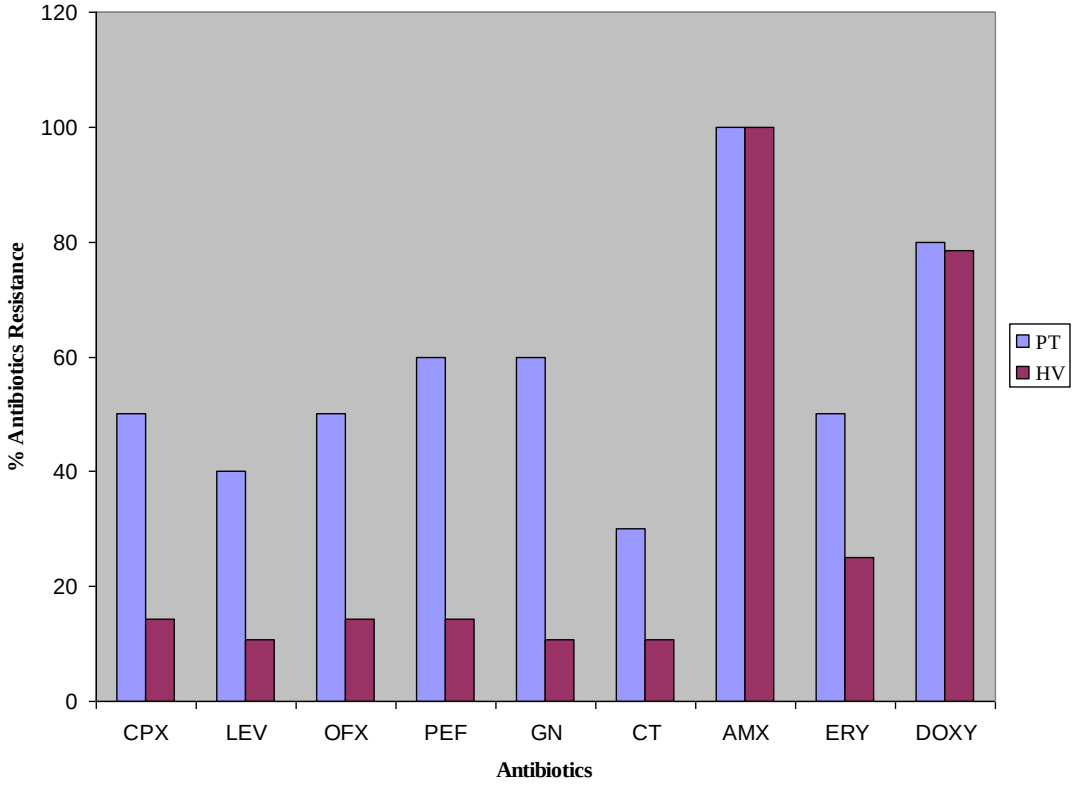

Fig. 13.0 The antibiotics resistance pattern of *S. aureus* isolates from both healthy volunteer (HV) and patient (PT) in Enugu Ezike health district.

KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

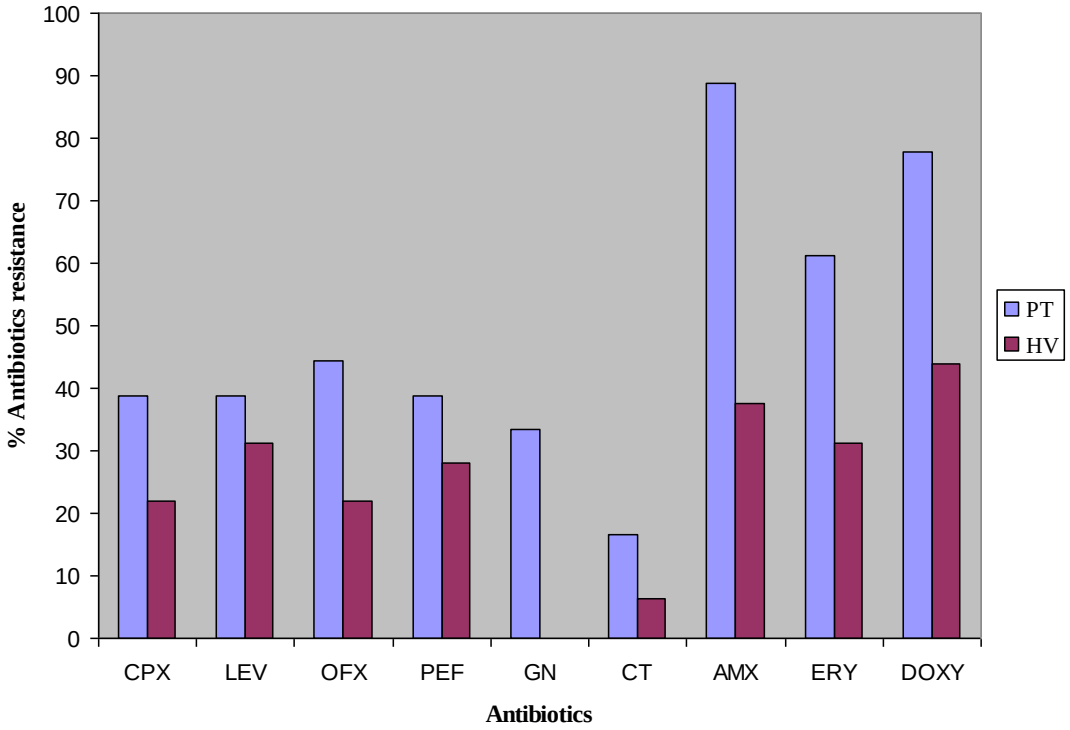

Fig. 14.0 The antibiotics resistance pattern of *S. aureus* isolates from both healthy volunteer (HV) and patient (PT) in Nsukka health district.

KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

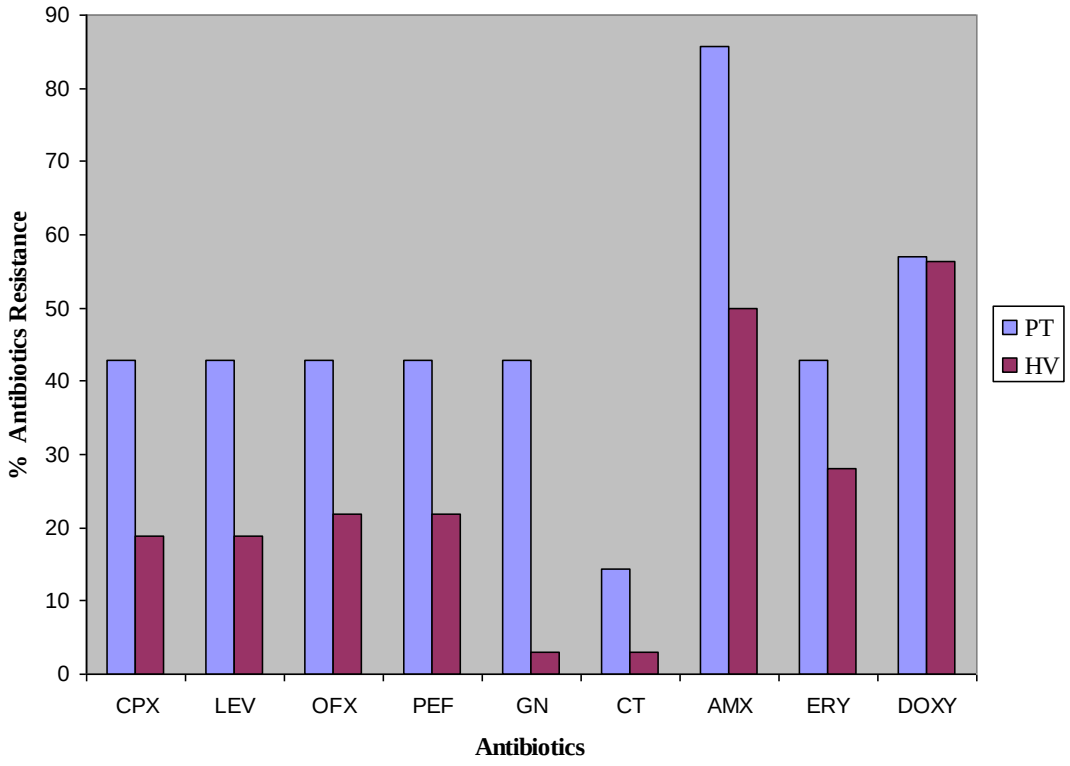

Fig. 15.0 The antibiotics resistance pattern of *S. aureus* isolates from both healthy volunteer (HV) and patient (PT) in Awgu health district.

KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

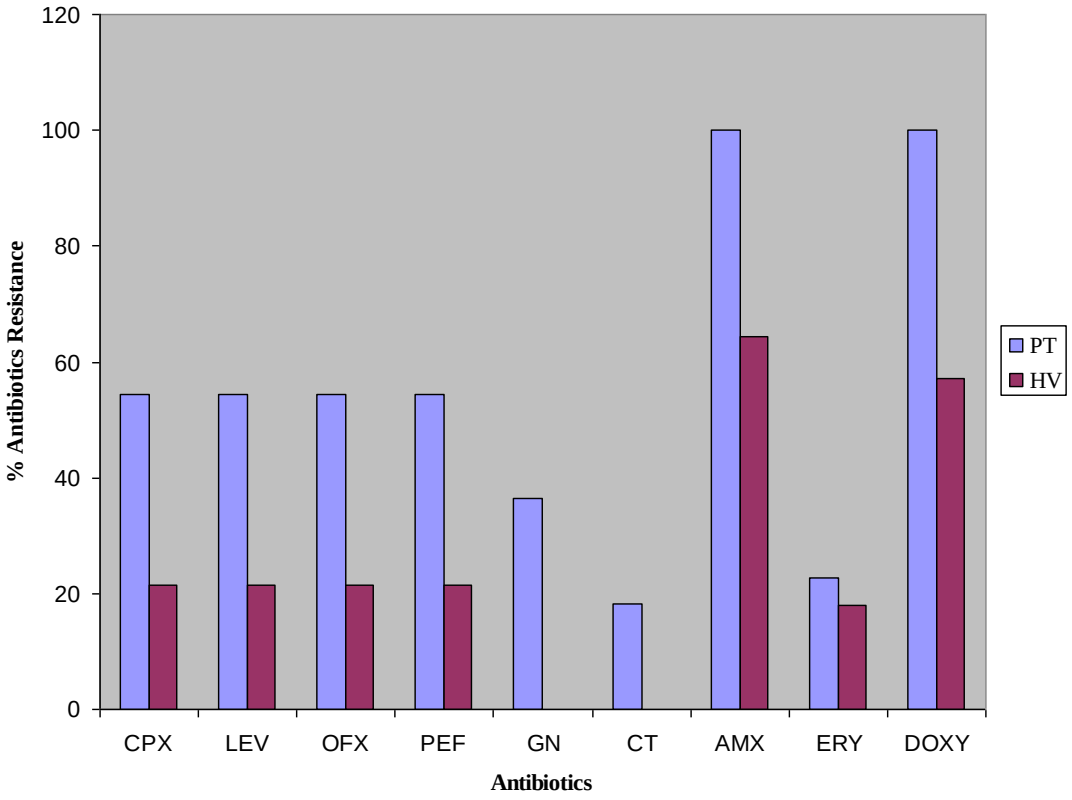

Fig. 16 The antibiotics resistance pattern of *Staph. aureus* isolates from both healthy volunteer (HV) and patient (PT) in Udi health district.

KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

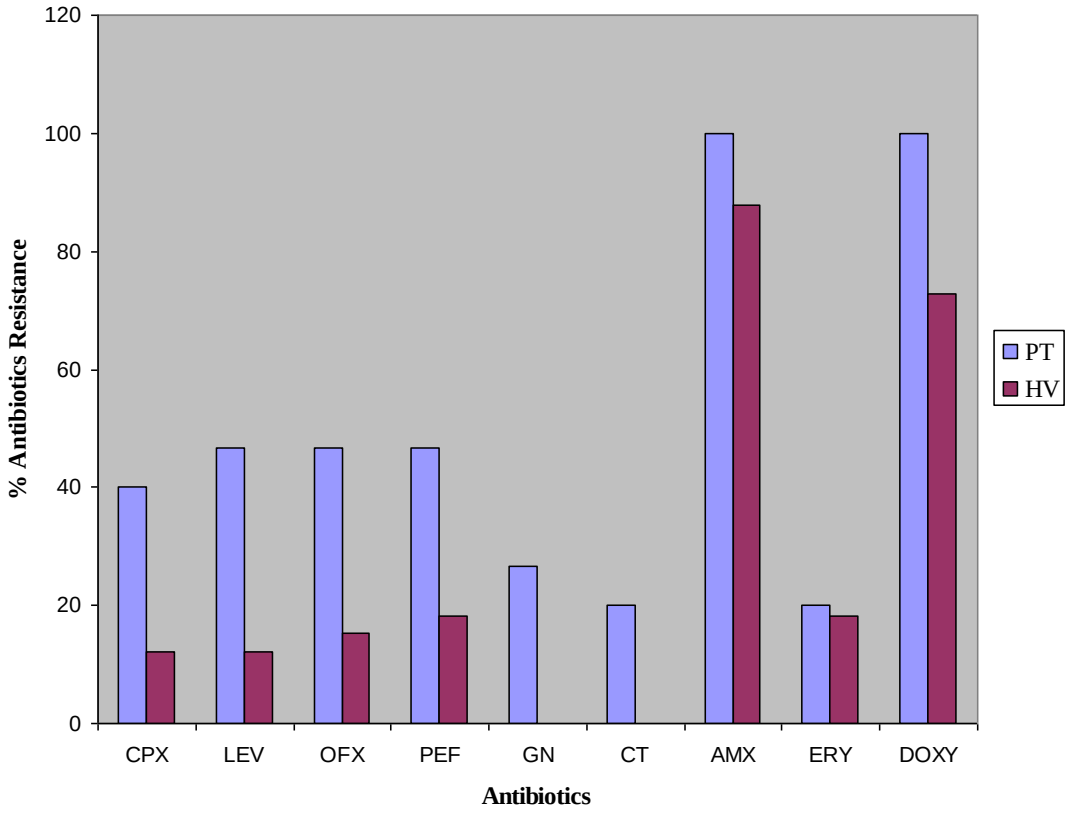

Fig. 17.0 The antibiotics resistance pattern of *S. aureus* isolates from both healthy volunteer (HV) and patient (PT) in IKEM health district.
KEY: HV= Healthy volunteer, PT= Patient , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

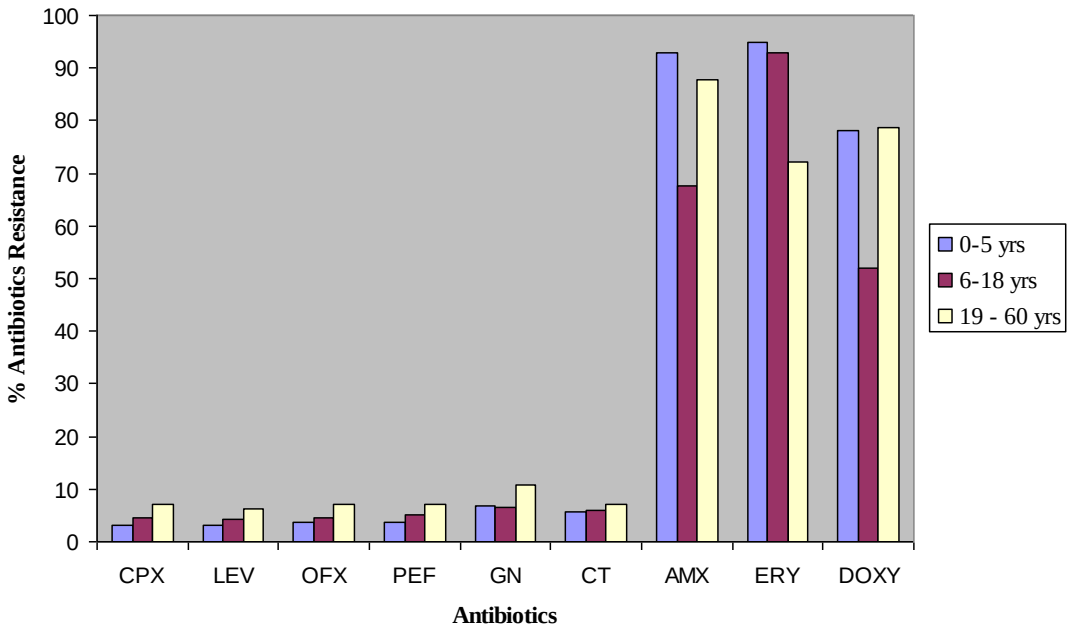

Fig. 18.0 Comparison of percentage antibiotics resistance pattern of *Escherichia coli* isolates from stool specimen of healthy volunteers based on the subjects age KEY: , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

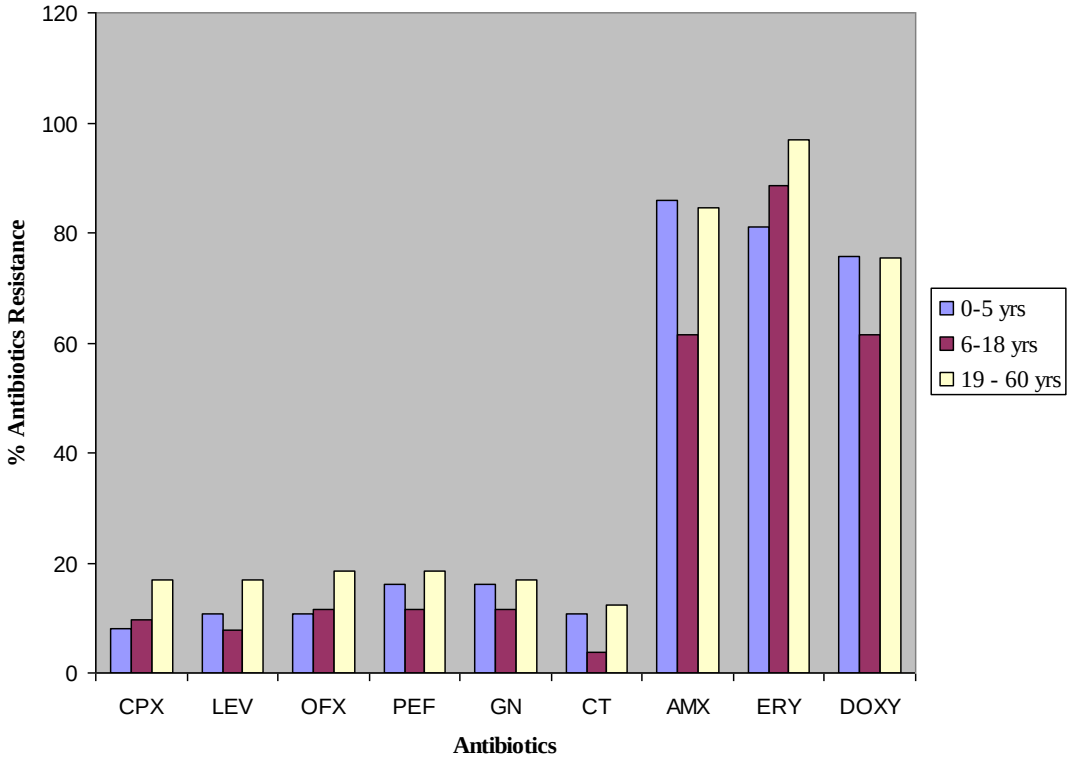

Fig. 19.0 Comparison of percentage antibiotics resistance pattern of *E. coli* isolates from Urine specimen of healthy volunteers based on the subjects age.
KEY: , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

Fig 20 and 21 compare the antibiotic resistance pattern of *S. aureus* isolates from nasal swab and urine specimens respectively according to the age of the subject. The antibiotics resistance of *S. aureus* isolates from nasal swab and urine specimen of healthy volunteers assumed a non-uniform pattern for the most of the antibiotics tested. However, for fluoroquinolones (FQ), there is increase in percentage antibiotics resistance for the test isolates with increase in the age of the subjects. For antibiotic resistance pattern of *S. aureus* isolates from urine specimen of healthy volunteers according to age (Fig 23), the level of FQ resistance is higher with the isolates from individuals within the age range 19-60 years old than with the isolates from individuals within the range 0-18 years old. A different pattern of antibiotics resistance was obtained with non-FQ antibiotics and urinary *S. aureus* isolates. The highest level of resistance was recorded with urinary *S. aureus* isolates from individuals within the age range 6-18 years.

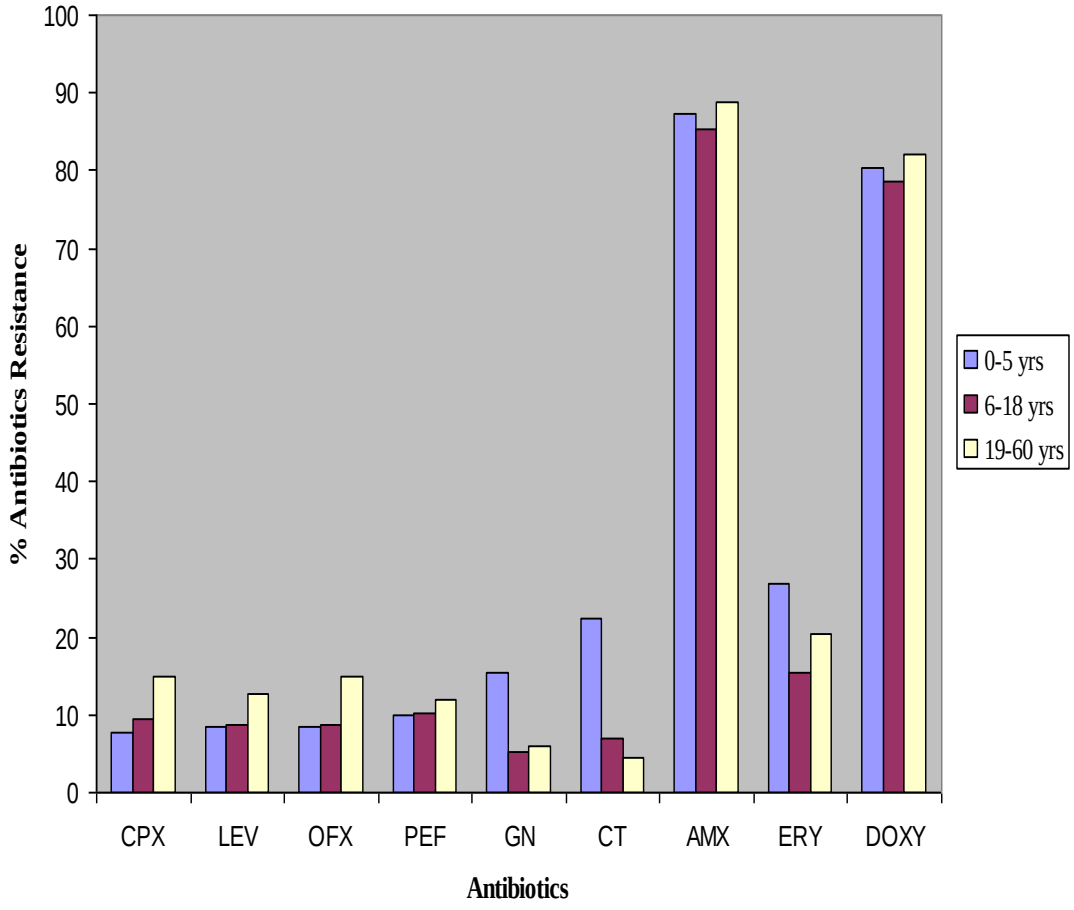

Fig. 20.0 Comparison of percentage antibiotics resistance pattern of *S. aureus* isolates from nasal swab specimen of healthy volunteers based on the subjects age. KEY: , Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

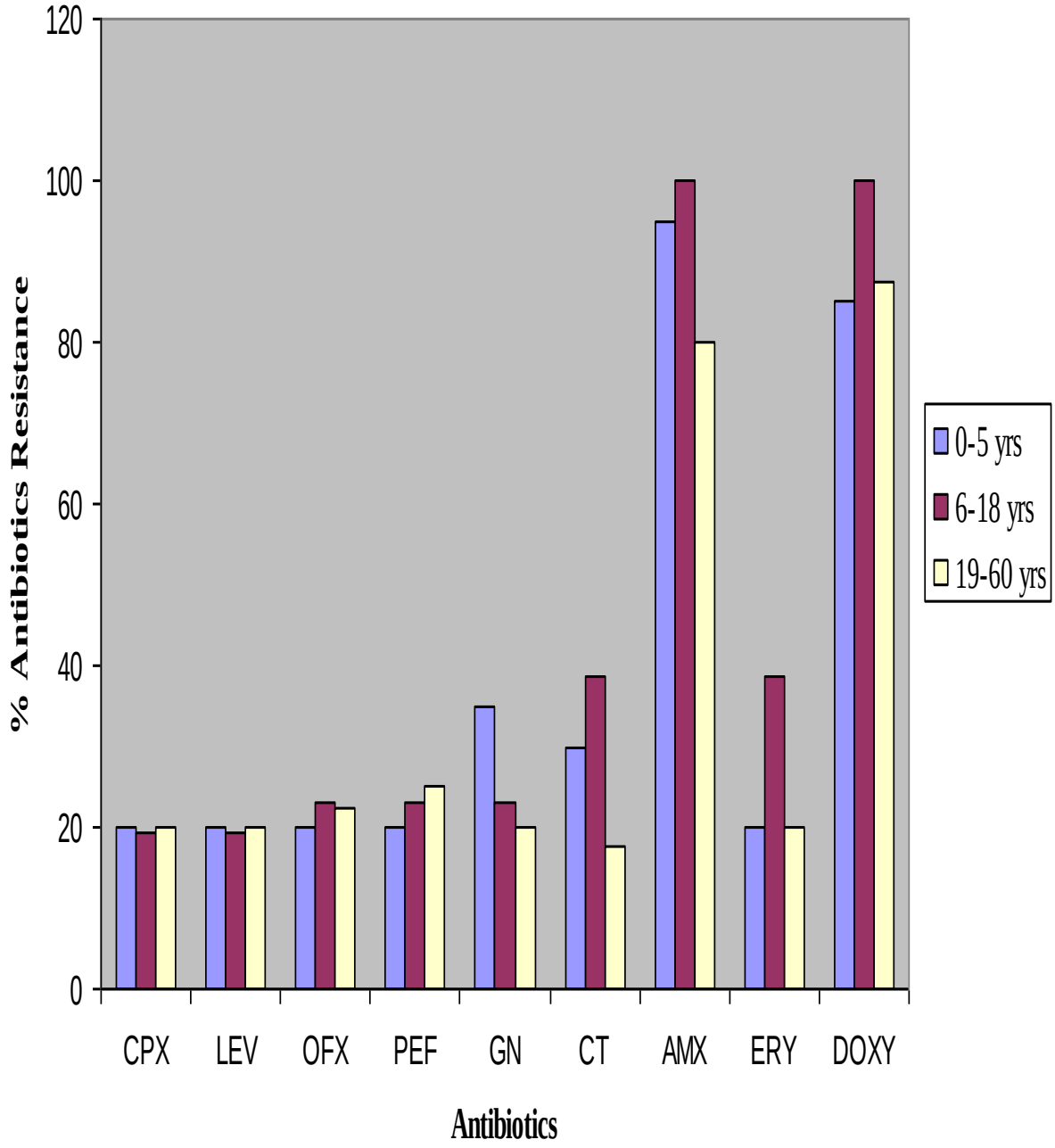

Fig. 21.0 Comparison of percentage antibiotics resistance pattern of *S. aureus* isolates from urine specimen of healthy volunteers based on the subjects age.
KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

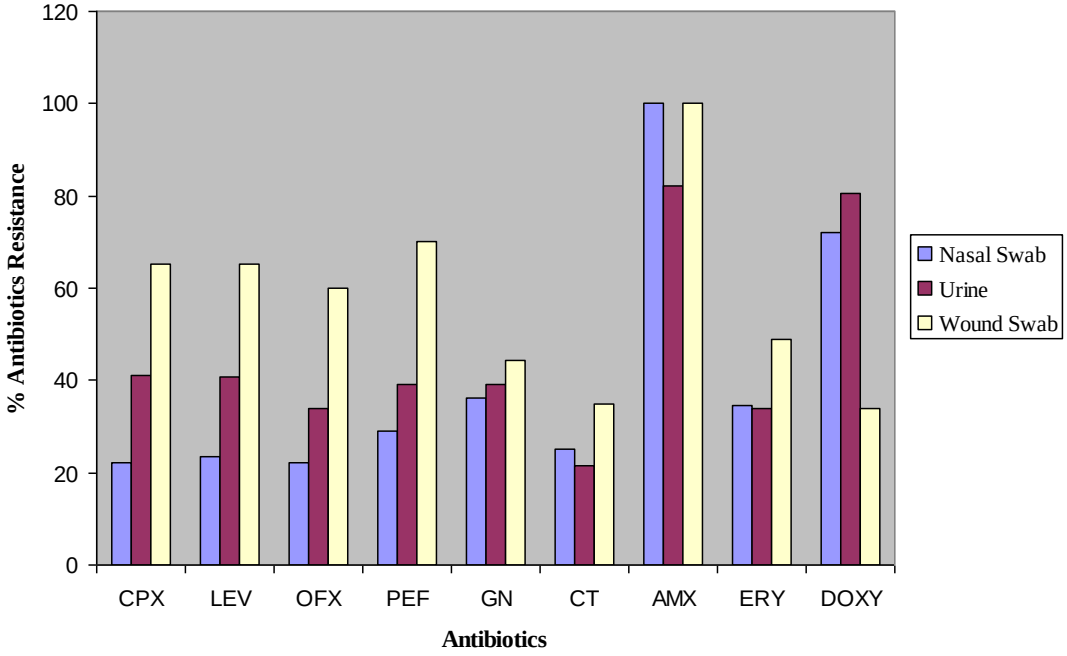

Fig. 22.0 Comparison of percentage antibiotics resistance pattern of *S. aureus* isolates from patients based on the isolates specimen source.
KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

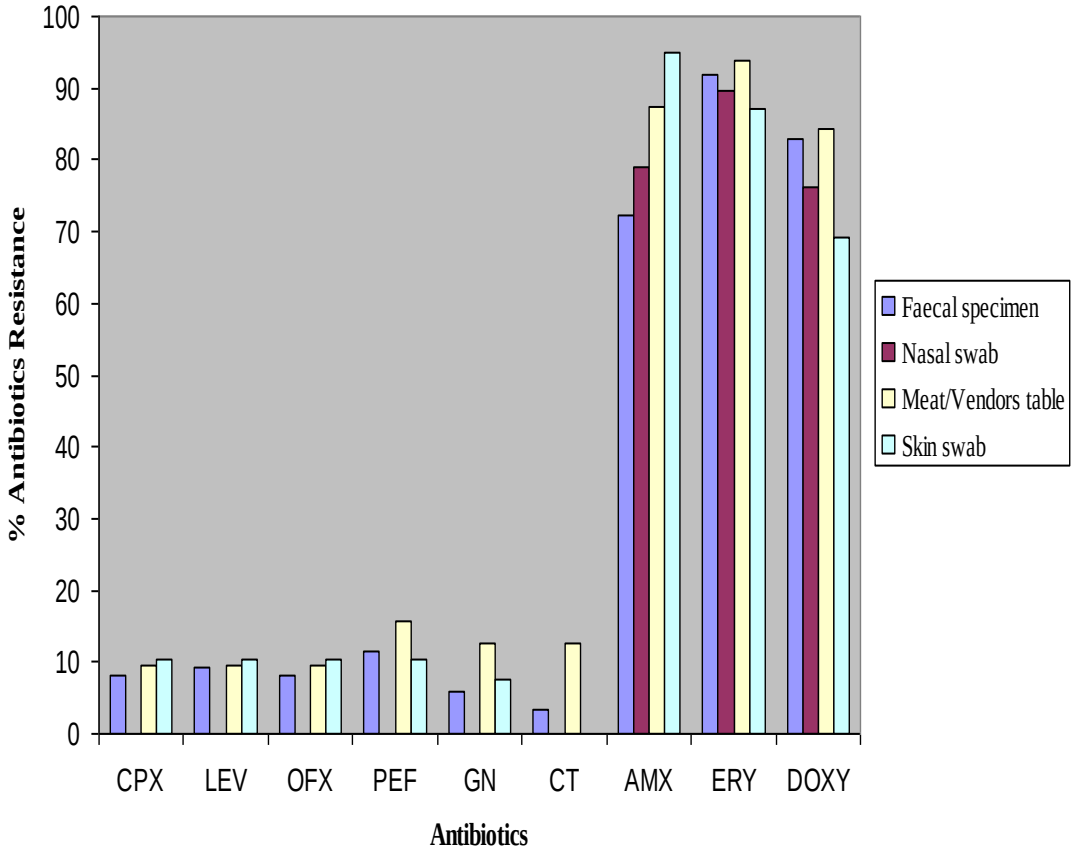

Fig. 23.0 The antibiotic resistance pattern of *Escherichia coli* isolates from pig in the study area.

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

Looking at the resistance pattern of antibiotics in the animals tested (Fig 23.0), in pig, fluoroquinolone, gentamicin and ceftriaxone resistant *E. coli* showed zero prevalence for isolates from nasal specimen. The *E.coli* isolates from meat/vendors table showed the highest resistance to gentamicin and ceftriaxone when compared to the isolates from other sources of the same animals. For the fluoroquinolones the isolates from skin swab showed the greatest resistance followed by that of meat/vendors table and the least was recorded for faecal specimen. For amoxycillin, erythromycin and doxycycline, the prevalence and pattern of antibiotics resistance is diverse and dependent on the antibiotics in question. The resistance rates of *E. coli* isolates from meat/vendors table and skin are higher than that of faecal or nasal specimen.

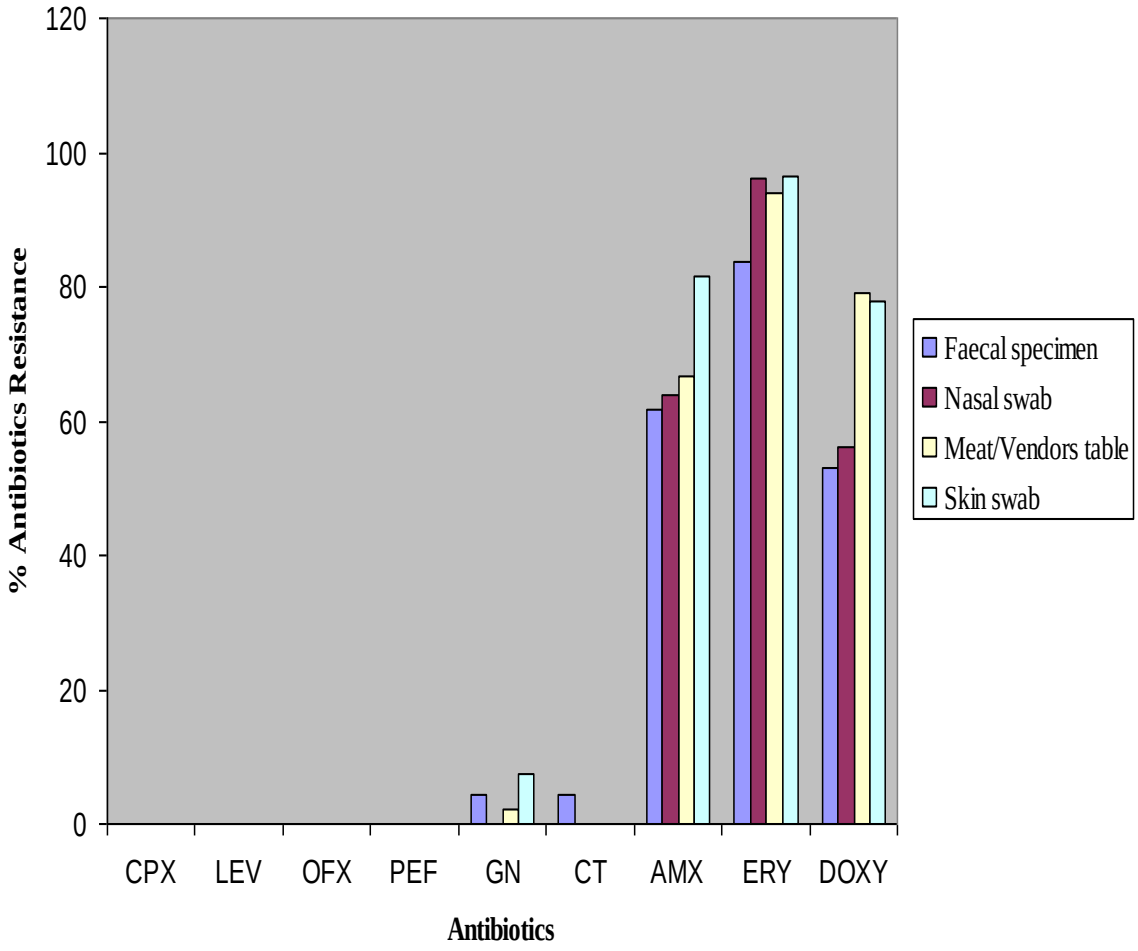

Fig. 24.0 . The antibiotic resistance pattern of *Escherichia coli* isolates from cattle in the study area.

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

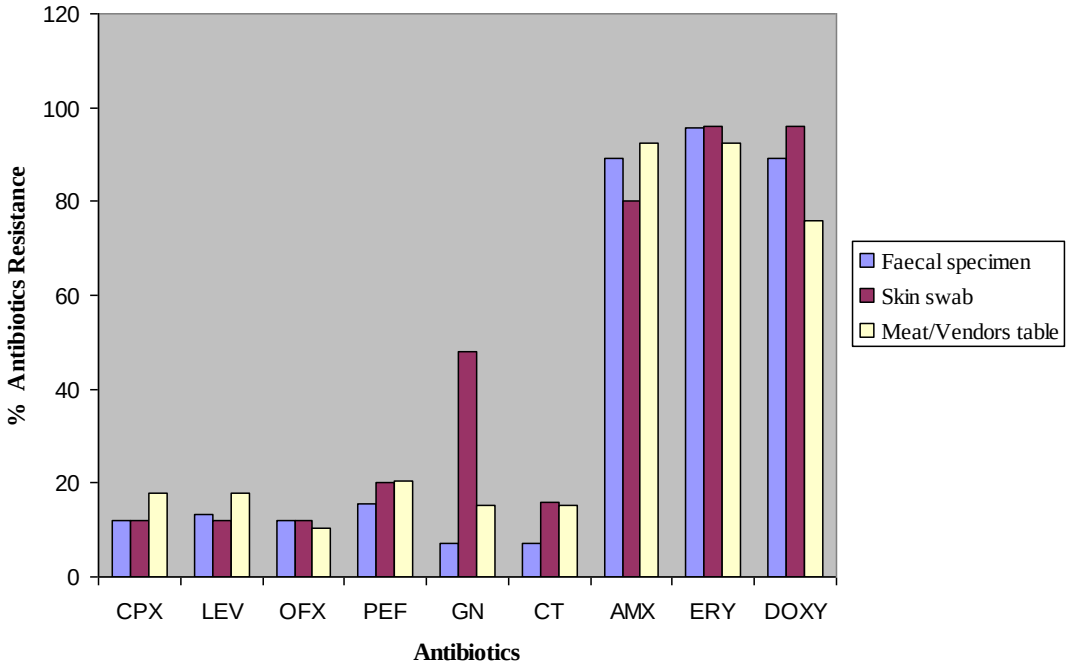

Fig. 25 .0 . The antibiotics resistance pattern of Escherichia coli isolates from chicken in the study area.

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

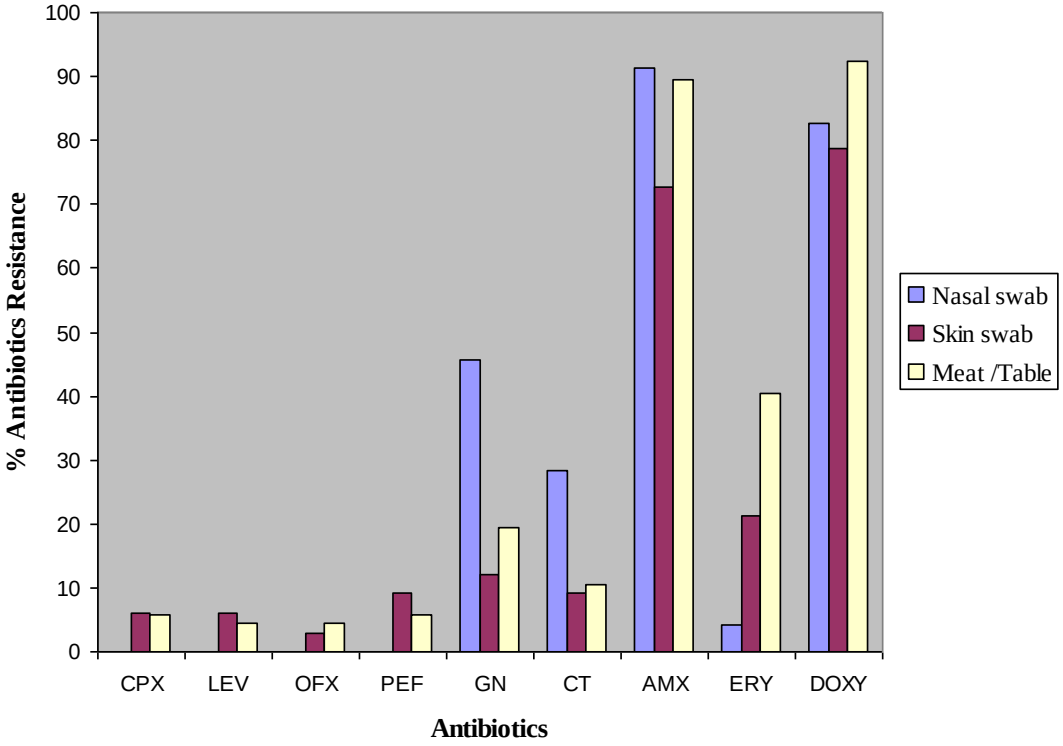

Fig. 26.0 The Antibiotic Resistance pattern of *Staphylococcus aureus* isolates from pig in the study area.
KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

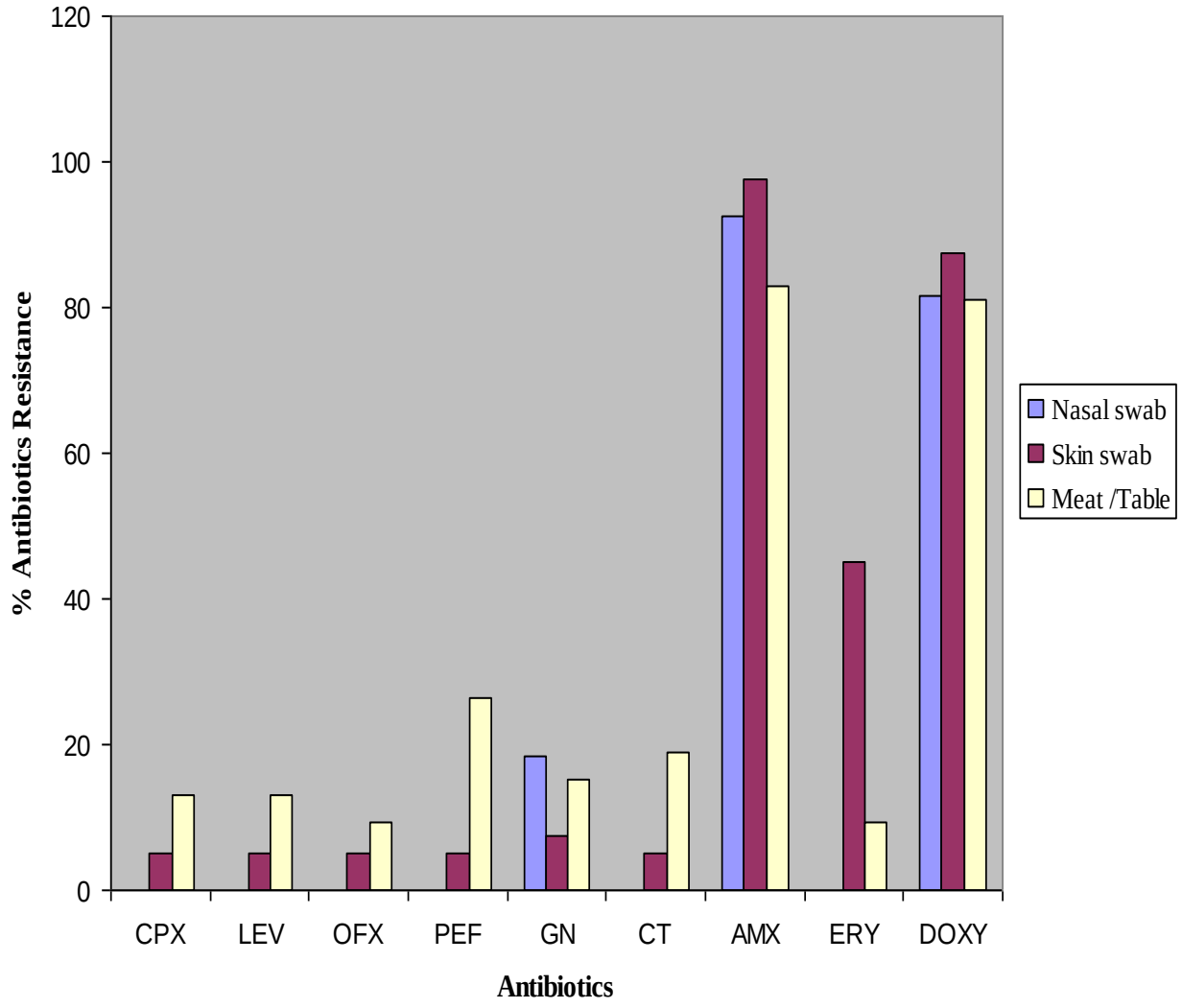

Fig. 27.0 The Antibiotic Resistance pattern *of Staphylococcus aureus* isolates from Cattle in the study area.

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

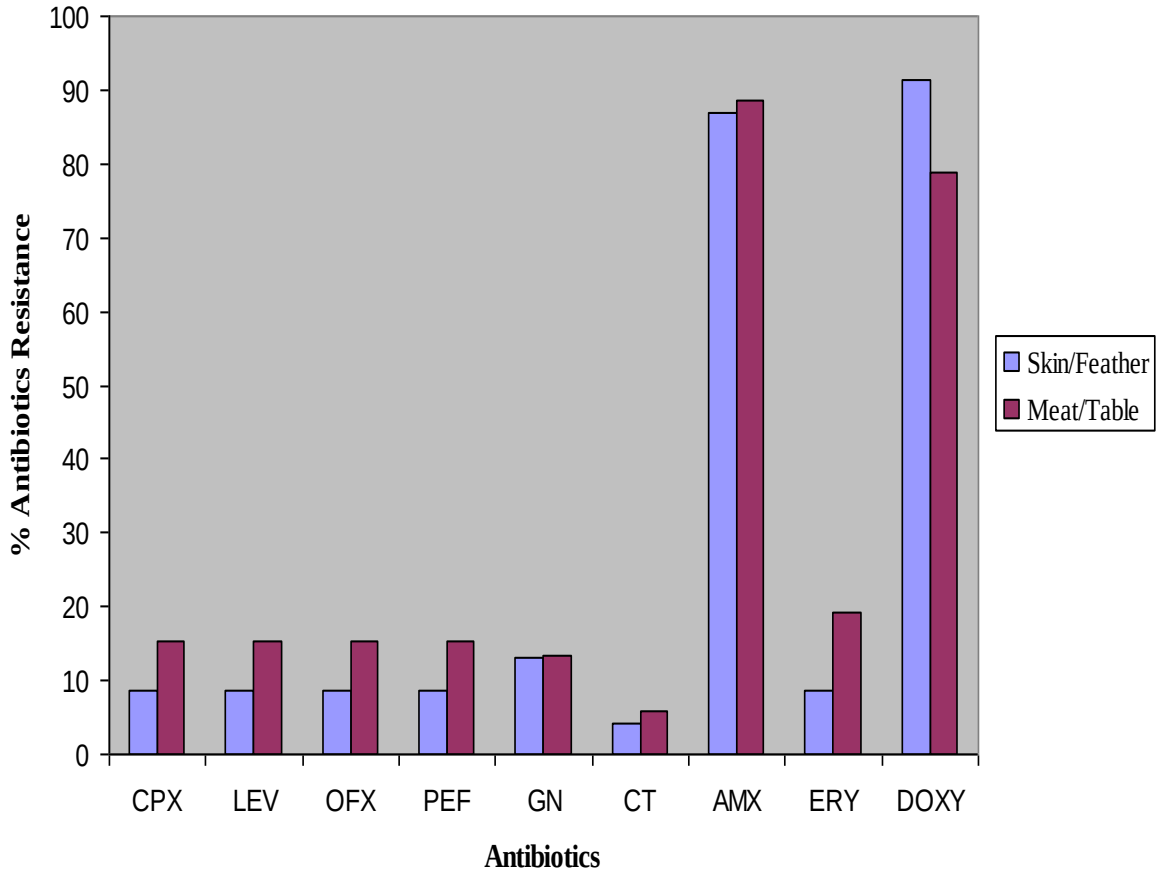

Fig. 28.0 The Antibiotic Resistance pattern of *Staphylococcus aureus* isolates from chicken in the study area.
KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

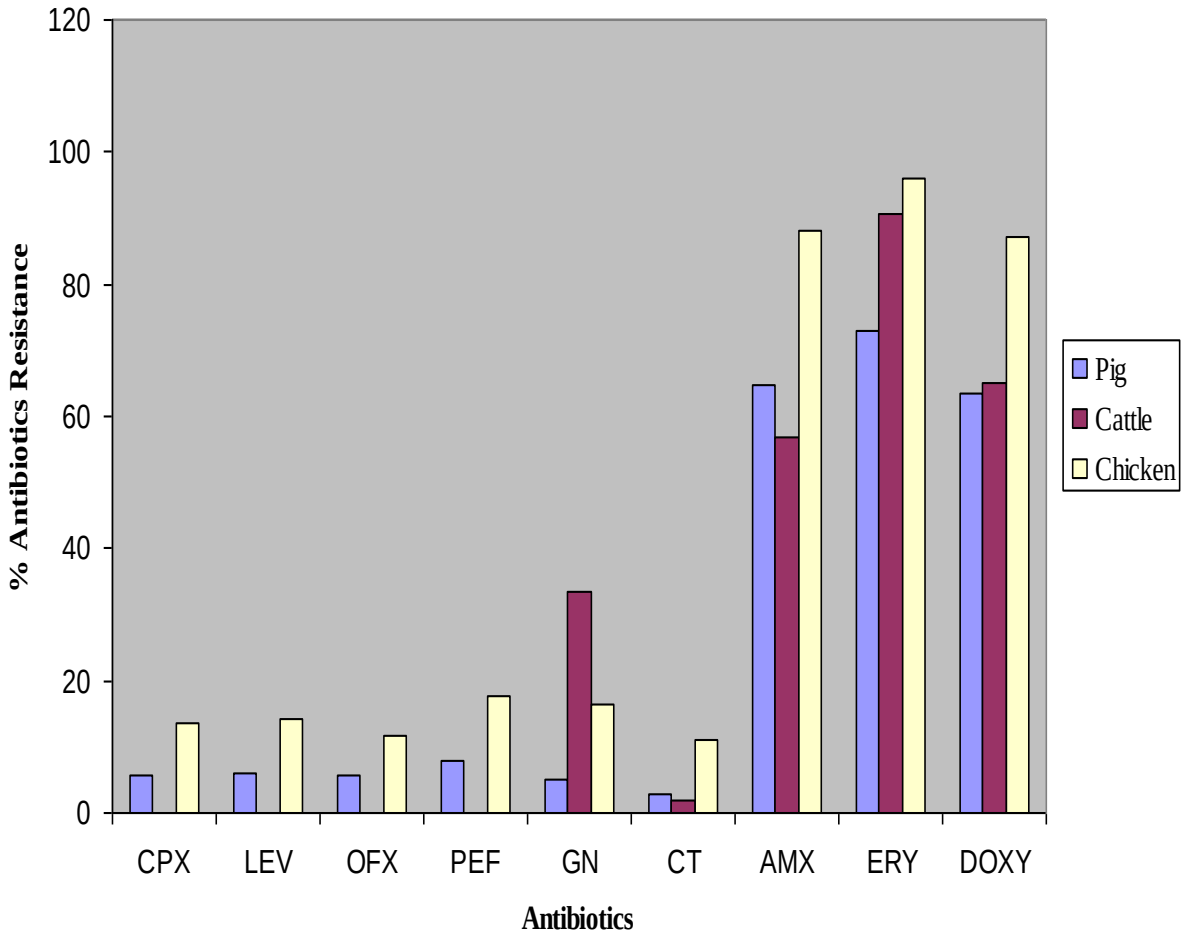

Fig 29.0 Comparison of percentage antibiotic resistance of *E. coli* isolates from animals

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

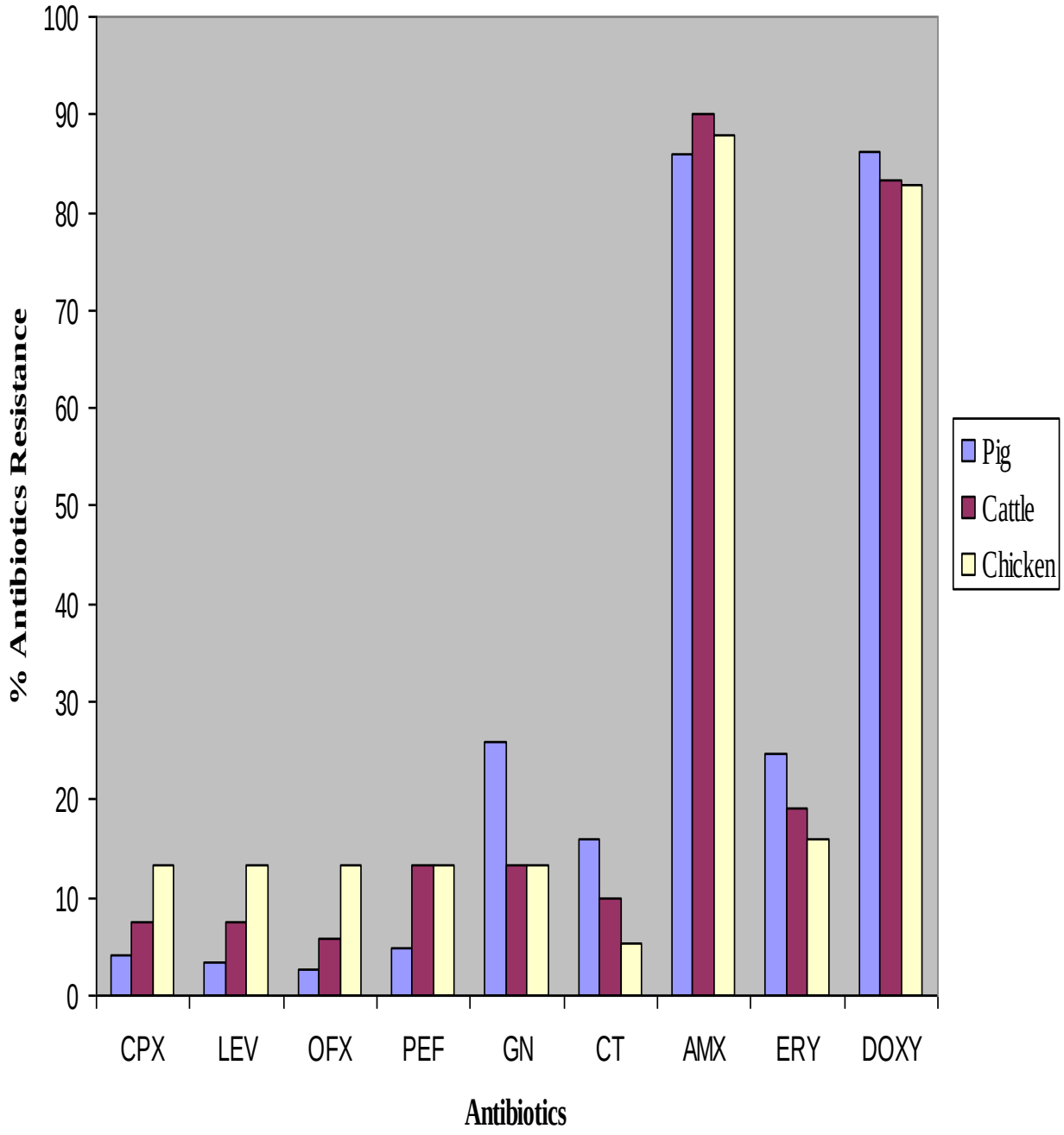

Fig 30.0 Comparison of percentage antibiotic resistance of *Staphylococcus aureus* isolates from animals

KEY: Cpx = Ciprofloxacin, Ofx = Ofloxacin, Lev = Levofloxacin, Pef= Pefloxacin. Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

4.3. Multiple antibiotic Resistance index (MARI).

The percentage frequency of the MARI of the test FQRSA and FQREC are shown in Table 13 and 14 respectively. Approximately 45 and 55.6% of FQRSA isolates from urine specimens of the healthy carriers and patient respectively, have MARI of 1.0. Similarly, apart from the FQRSA isolates from the nasal swab of healthy carriers with the modal value of 0.67, (corresponding to 36% of the isolates), the greatest proportions of FQRSA isolates from other sources (healthy volunteer urine, patient urine, patient nasal swab, wound swab, pig specimen, specimens of cattle and chicken) have their modal value of 1.0 (Table 13). Greater percentage of FQRSA isolates exhibited MARI of 0.89, 0.78 and 0.67 while few proportions of the isolates (8, 6.3 and 42.9%) from healthy carriers nasal swab, -patient nasal swab and cattle respectively have MARI of 0.56. No FQRSA isolates exhibited MARI of $\leq$0.45. For FQREC isolates shown in Table 13 the greatest proportions of the isolates from patients' urine, stool, wound swab and pig specimen have MARI value of one (1.0). Also, greater percentage of the isolates are also found to have MARI of 0.89 0.78 and 0.67. Few of the *E.coli* isolates (8.5% of the patient urine and 15.4% of the isolates from urine specimen of the healthy carrier).

The percentage of FQREC isolates that are resistant to others commonly used antibiotics in the area of study is shown in Table 15. In human isolates, all the FQREC (100%) tested are also resistant to erythromycin while 95, 53.3, 65.1 and 96.5% were resistant to amoxycillin, ceftriaxone, gentamicin and doxycycline respectively. For animal FQREC isolates, all (100%) of the FQREC were resistant to both amoxycillin and erythromycin, while 57.1 and 15%, 64.3 and 45%, and 92.9 and 90% from pig and chicken respectively, were resistant to ceftriaxone, gentamicin and doxycyline .

To confirm the multi-drug resistance properties of these isolates, the percentage of FQRSA isolates that are resistant to other antibiotics were calculated and their values are shown in **Table 14.** In healthy volunteer urine specimens, all the FQRSA (100%) isolates are resistant to doxycycline and amoxycillin, about 64% were resistant to gentamicin, ceftriaxone and erythromycin.

Table 13: Percentage frequency of Multiple antibiotic Resistance index (MARI) of test FQRSA based on the specimen source

MARI	HVUS	PSU	HVN	PN	W/S	Pg	C	CAT
1.0	5(45.4)	10(55.6)	6(24.0)	8(50.0)	14(70.0)	1(16.7)	4(80)	1(14.3)
0.89	2(18.2)	4(22.2)	5(20.0)	6(37.5)	1(5.0)	3(50)	0(0)	3(42.9)
0.78	2(18.2)	2(11.1)	3(12.0)	1(6.3)	1(5.0)	0(0)	0(0)	0(0)
0.67	2(18.2)	2(11.1)	9(36.0)	0(0)	4(20.0)	2(33)3	1(20)	0(0)
0.56	0(0)	0(0)	2(8.0)	1(6.3)	0(0)	0(0)	0(0)	3(42.9)
0.45	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Key: HVUS = Healthy volunteer urine specimen
PSU = Patient specimen of urine
HVN = Healthy volunteer nasal swab
PN = Patient swab
W/S = Wound swab
Pg = Specimen from pig
CH = Specimen from chicken
Cat = Specimen from cattle
() = Percentage

Table 14 :Percentage frequency of Multi Antibiotic Resistance Index (MARI) of Test FQREC Based on the Specimen source.

MARI	HVUS	PSU	HVSS	PS	W/S	PG	CH
1.0	1(7.7)	28(47.5)	7(41.2)	17(43.6)	4(28.6)	8(57.1)	2(10)
0.89	3(23.1)	12(20.3)	6(35.3)	14(35.9)	6(42.9)	2(14.3)	4(20)
0.78	6(46.2)	7(11.9)	3(17.6)	7(17.9)	2(14.3)	3(21.4)	8(40)
0.67	1(7.7)	7(11.9)	1(5.9)	1(5.9)	2(14.3)	(17.1)	6(30)
0.56	2(15.4)	5(8.5)	0(0)	0(0)	0(0)	0(0)	0(0)
0.45	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

Key:

HVUS = healthy volunteer urine specimen
PSU = patient specimen of urine
HVSS = healthy volunteer stool specimen
PS = patient stool specimen
W/S = wound swab
PG = Specimen from pig
CH = Specimen from chicken
() = Percentage

Table 15: The Percentage of FQREC isolate that are Resistant to other Commonly used antibiotics in the study area.

SPECIMEN source		**Test Antibiotics (%)**					
		FQ	GN	Cef	Am	Ery	Doxy
Animal	Pig:	100	64.3	57.1	100	100	92.9
	Chicken:	100	45.0	15	100	100	90
Human	HVUS	100	66.7	44.4	100	100.0	92.3
	PSU	100	61.0	54.2	96.6	100	93.0
Human	HVSS	100	82.4	47.1	94.1	100	100
	PS	100	79.5	56.4	92.3	100	97.4
	W/S	100	35.7	64.3	92.9	100	100

KEY:

HVUS = healthy volunteer urine specimen
PSU = Patient specimen of urine
HVSS = healthy volunteer stool specimen, PS = patients stool.
W/S = Wound Swab. Pg. = Specimen from pig
CH = specimen from Chicken
Fq = Fluoroquinolones, Gn= gentamicin, Cef = Ceftriaxone,
Amx – Amoxicillin, Ery = Erythromycin, Doxy = Doxycline.

Table 16: THE PERCENTAGE OF FQRSA ISOLATES THAT ARE RESISTANT TO THER ANTIBIOTICS IN USE IN THE STUDY AREA

SPECIMEN source		**Test antibiotics**					
		FQ	GN	Cef	Am	Ery	Doxy
Animal	Cattle:	100	44.5	22.2	100	55.6	100
	Chicken:	100	80	80	100	80	100
	Pig	100	83,3	33.3	100	78	100
Human	HVUS	100	64	64	100	64	100
	PSU	100	83	61	100	70	100
	HVnS	100	47.1	38.2	97.1	38.2	91.2
	PnS	100	68.8	75	100	93.8	100
	W/S	100	70	80	100	67.6	85

KEY:

Key: HVUS = healthy volunteer urine specimen , PSU = patient specimen of urine. HVnS= healthy volunteer stool specimen. PS = patient stool specimen, W/S = wound swab Pg = specimen from pig. CH = specimen from chicken. Fq = Fluoroquinolones, Gn= Gentamicin, Cef = Ceftriaxone, Amx =Amoxicillin, Ery= Erythromycin, Doxy = Doxycycline.

4.4 MIC OF SOME ANTIBIOTICS AGAINST THE TEST FQREC AND FQRSA`.

4.4.1. MIC of erythromycin against FQRSA isolates

The value of MIC of Erythromycin on FQRSA isolates fell within a very wide range for the most of the FQRSA isolates from the same specimen source in different subjects (**Table 17**). In isolates from patient urine and nasal swab, the MIC values for erythromycin ranged from 0.063-256 μg /ml . For wound swab isolates the MIC values ranged from 0.125-128 μg /ml. The range of MIC values for Erythromycin in animals was narrower than in humans. For Pig and Cattle, the range was 0.063-64 ug/ml while for chicken the range was 0.5-16 μg /ml.

The distribution of MIC of erythromycin on FQRSA isolates from both human and animals is shown in **Table 18.** In humans about 26.6% of FQRSA were highly susceptible to erythromycin and were within the MIC range of 0.125-0.5 μg /ml. These erythromycin- susceptible FQRSA isolates have their modal MIC value as 0.125 μg /ml. Those FQRSA isolates that were within the intermediate susceptibility to erythromycin (MIC value 1- 4 μg /ml) were few and were about 2% of all human FQRSA tested. The rest (71.4%) erythromycin resistant isolates were within the MIC range of 16-256 μg /ml. In animals, 17% of pig, 55% of cattle and 20% chicken FQRSA isolates were susceptible to erythromycin with the modal values of 0.063 μg /ml, 0.063 μg /ml and 0.5 μg /ml respectively. For erythromycin-resistant FQRSA, 83% of pig, 44% of cattle and 80% of chicken isolates were resistant to erythromycin with the modal MIC values of 16 μg /ml, 64 μg /ml and 16 μg /ml respectively. Since approximately 29% of human and 31% animal FQRSA isolates were susceptible to erythromycin, these erythromycin- susceptible FQRSA isolates have their modal MIC

value as 0.125 μg /ml. Those FQRSA isolates that were within the intermediate susceptibility to erythromycin (MIC value 1- 4 μg /ml) were few and were about 2% of all human FQRSA tested.

The frequency (%) of MIC values of ciprofloxacin against FQRSA isolates from different specimen are distributed in **Table 19.** The MIC value of FQRSA isolates from nasal swabs of both healthy carriers and patients are concentrated more within the MIC range of 4-64 μg /ml, with the MIC modal values of 4 μg /ml and 16 μg /ml for healthy carriers and patients respectively. For urinary FQRSA isolates, the MIC values of both healthy carriers and patients are widely distributed within 4 ug/ml through 128 and 512 μg /ml for healthy carriers and patients respectively. The modal MIC values for healthy carriers and patients are 128 μg /ml and 32 μg /ml . respectively. For wound swab isolates, the MIC values were concentrated within the range of 4-32 μg /ml.

In animals, most of the FQRSA isolates have their MIC values of ciprofloxacin within the range of 4-16 μg /ml with the modal ciprofloxacin value of 4 μg /ml for all the animal tested. Though high level ciprofloxacin resistance (MIC of 32-128 μg /ml) were recorded for nasal swab FQRSA isolates, the percentage of isolates having this high level was low (6-12.5%). Greater percentage of the nasal swab FQRSA isolates (12.5-37.5%) has low level ciprofloxacin resistance (MIC of 4-16 μg/ml). In animal, low level ciprofloxacin resistance isolates are more and there is no MIC of ≥128 μg /ml found in all the animal FQRSA isolates.

Table 17: MIC (ug/ml) of Erythromycin against FQRSA isolates from both Humans and Animal

Specimen	Subject	No of FQRSA	MeanMIC± SEM ug/ml	MIC Range	Number of FQRSA	
					E-Res	E-Susc
URINE	HTV	19	41.76 ± 17.75	0.125-128	10(53)	9(47)
	PTS	33	68.06 ± 17.66	0.125-256	19(58)	14(42)
NASAL	HTV	40	28.08 ± 1.84	0.063-256	26(65)	14(35)
swab	PTS	16	78.66 ± 21.23	0.125-256	12(75)	4(25)
WOUND SWAB	PTS	28	19.11 ± 5.76	0.125-128	13(46)	15(54)
Nares, Meat, Skin	PIG	6	24.02 ± 8.99	0.063-64	5(83)	1(17)
Nares, Meat, Skin	CATTLE	9	22.25 ± 10.44	0.063-64	3(33)	6(67)
Meat, Skin	CHICKEN	5	9.70 ± 2.91	0.5-16	4(80)	1(20)

KEY: HTV = Healthy Volunteer, PTS = patient specimen E-RES = Erythromycin-Resistant-FQRSA, E-SUBC = Erythromycin-Susceptible FQRSA

Table 18: Distribution (%) of MIC of Erythromycin on FQRSA isolates from both Humans and Animals

MIC	**Nasal FQRSA**		**Urinary FQRSA**		**Wound FQRSA**	**Pig**	**Cattle**	**Chicken**
Ug/ml	HTV n=40	PTS N=16	HTV N=19	PTS n=33	PTS N=28	AHP n=6	AHcat N=9	AHC n=5
0.063	1(3)	0(0)	0(0)	0(0)	0(0)	1(17)	2(22)	0(0)
0.125	6(15)	3(19)	2(11)	5(15)	6(21)	0(0)	1(11)	0(0)
0.25	2(5)	0(0)	2(11)	7(2)	0(0)	0(0)	0(0)	0(0)
0.5	2(5)	1(6)	2(11)	0(0)	3(11)	0(0)	0(0)	1(20)
1	2(5)	0(0)	1(5)	1(3)	1(4)	0(0)	0(0)	0(0)
2	0(0)	0(0)	2(11)	2(6)	0(0)	0(0)	1(11)	0(0)
4	1(3)	0(0)	0(0)	0(0)	5(18)	0(0)	1(11)	0(0)
8	4(10)	0(0)	2(11)	1(3)	2(7)	0(0)	0(0)	1(20)
16	3(8)	2(13)	0(0)	1(3)	5(18)	3(49)	0(0)	3(60)
32	4(10)	2(13)	2(11)	0(0)	1(4)	1(17)	0(0)	0(0)
64	7(18)	4(25)	3(16)	1(3)	4(14)	1(17)	4(44)	0(0)
128	3(8)	4(25)	2(11)	11(33)	1(4)	0(0)	0(0)	0(0)
256	5(13)	0(0)	1(5)	4(12)	0(0)	0(0)	0(0)	0(0)

Key :HTV = Healthy Volunteer, PTS = patient specimen, AHP= apparently healthy pig, AHcat = apparently healthy cattle. AHC = apparently healthy chicken.

Table 19: Frequency distribution (%) of MIC of ciprofloxacin on the FQRSA isolates from different source.

MIC	**Nasal FQRSA**		**Urinary FQRSA**		**Wound**	**Pig**	**Cattle**	**Chicken**
Ug/ml	n=40	N=16	n=19	N=33	n=28	n=6	n=9	n=5
	HVNF	PNF	HVUFs	PUFs	PWFs	AHP	Ahcat	AHC
4	15(37.5)	4(31.3)	5(26)	8(24.4)	8(29)	3(50.)	6(66.7)	2(40)
8	9(22.5)	2(12.5)	4(21.1)	2(6.1)	7(25)	2(3.3)	1(11.1)	1(40)
16	5(12.5)	6 (37.5)	0(0)	0(0)	4(14.3)	0(0)	1(11.1)	1(20)
32	5(12.5)	2(12.5)	2(10.5)	11(33.5)	9(32.1)	0(0)	1(11.1)	0(0)
64	4(10.0)	1(6.3)	2(10.5)	4(12.1)	0(0)	1(16.7)	0(0)	0(0)
128	2(5.0)	1(6.3)	6(31.6)	3(9.1)	0(0)	0(0)	0(0)	0(0)
256	0(0)	0(0)	0(0)	4(12.1)	0(0)	0(0)	0(0)	0(0)
512	0(0)	0(0)	0(0)	1(3.0)	0(0)	0(0)	0(0)	0(0)

Key: HVNF = Healthy Volunteer Nasal FQRSA., PNF = Patients Nasal FQRSA. HVUFs = Healthy Volunteer Urinary FQRSA, PUFs = patient urinary FQRSA. PWFs = Patient wound FQRSA., AHP= apparently healthy pig, AHcat = apparently healthy cattle. AHC = apparently healthy chicken.

The MIC value of gentamicin on FQREC isolates from both human and animals are shown in **Table 20**.

Different ranges and mean MIC values are clearly shown against the isolates specimen source. For FQREC isolates from humans urinary and faecal samples, the percentage susceptibility to gentamicin are 34 and 55% respectively. In animal, 65 and 75% of the pig and chicken FQRSA isolates were susceptible to gentamicin respectively. The resistant rates, mean MIC values and the MIC range of gentamicin on FQREC isolates are presented in the Table 20. The distribution (%) of MIC of gentamicin against FQREC isolates from both humans and animals are shown in Table 21. For urinary FQREC isolates, the range of MIC values of gentamicin was 0.5 ug/ml–128 ug/ml for both patients and asymptomatic healthy carriers. The largest number (greatest percentage) of FQREC isolates from patients and asymptomatic healthy carriers have the MIC values of 32 ug/ml and 1.0 ug/ml respectively. Even in chronic wound patients, the modal MIC value of gentamicin was 64 ug/ml. For animal FQREC isolates, there was even distribution of MIC values across a wide range from 0.5.

The frequency (%) of MIC values of ciprofloxacin against FQREC isolates from different specimen are distributed in **Table 22.** The MIC value of FQREC isolates from urine specimen of both healthy carriers and patients were concentrated more within the MIC range of 4-64 μg/ml and 64 μg/ml – 256 μg/ml repectively, with the respective MIC modal values of 16 μg/ml and 128 μg/ml. Like urinary FQREC isolates, the MIC values of ciprofloxacin on both healthy carriers and patients faecal isolates were widely distributed within 4 μg/ml through 512 μg/ml and 1024 μg/ml for healthy carriers and patients respectively ug/ml to 64 ug/ml.

Table 20: MIC of gentamicin against FQREC isolates from both humans and animals in the study area.

Subject	**Specimen**	**No of FQREC**	**Mean MIC ± SEM**	**MIC range**	**Number(%) G=res**
HV	urine	19	31.81± 14.05	0.-128	12(63)
PS	urine	71	34.03±6.30	0.5-128	47(66)
HV	stool	38	8.58 ± 2.71	0.12-64	17(45)
PS	stool	93	27.53 ± 4.89	0.125-128	47(51)
PWS	wound swab	15	27.53 ± 4.89	0.25-128	8(53)
Pig	pig specimen	14	14.40 ± 3.84	0.25-64	5(36)
Cattle	stool, nasal, meat,	0	0	0	0
Chicken	meat and skin swab	20	15.50 ±5.68	0.125-64	5(25)

Key:

HV = healthy volunteer specimen.
PS = patient specimen,
G. Res = gentamacin- resistance FQREC

Table 21: Distribution (%) of MIC of gentamicin against FQREC isolates from both humans and animals.

MIC (ug/ml)	Urinary HVUS N=19	FQREC PSU N = 71	Faecal HVSS N=38	FQREC PSS N=93	Wound PWS N=15	Pig AHP N=14	Chicken AHC N=20
0.125	0(0)	0(0)	2(5)	2(2)	0(0)	0(0)	0(0)
0.25	0(0)	0(0)	1(3)	4(4)	1(7)	2(14)	2(10)
0.5	1(5)	3(4)	9(24)	9(10)	2(13)	1(7)	2(10)
1.0	5(26)	2(3)	1(3)	3(3)	3(20)	0(0)	2(10)
2.0	0(0)	10(14)	1(3)	7(8)	0(0)	1(7)	3(15)
4.0	1(5)	5(7)	2(5)	5(5)	0(0)	1(7)	3(15)
8.0	1(5)	5(7)	5(13)	12(13)	1(7)	4(29)	3(15)
16.0	4(21)	11(15)	10(26)	14(14)	0(0)	3(21)	1(5)
32.0	2(11)	16(23)	4(10)	11(12)	2(13)	0(0)	1)5)
64.0	4(21)	13(18)	3(8)	17(18)	5(33)	2(14)	3(15)
128.0	1(5)	6(8)	0(0)	5(5)	1(7)	0(0)	0(0)

Key : HTV = Healthy Volunteer, PTS = Patient specimen

AHP = apparently health pig. AHC = apparently healthy chicken.

Cattle recorded zero prevalence of FQREC and thus not included here.

Table 22: Frequency occurrence (%) of MIC of ciprofloxacin on FQREC isolates from different specimen source.

MIC (ug/ml)	Urinary HTV N=19	FQREC PTS N=71	FECAL HTV N=38	FQREC PT-S N=93	WOUND PTS N=15	PIG AHP N=14	CHICKEN AHC N=20
4	4(21)	8(11)	7(18)	7(8)	1(7)	5(36)	12(60)
8	3(16)	8(11)	4(11)	12(13)	1(7)	3(21)	2(10)
16	6(32)	4(6)	6(16)	910)	1(7)	3(21)	1(5)
32	2(11)	8(11)	6(16)	11(12)	1(7)	2(14)	1(5)
64	2(11)	10(14)	398)	23(25)	5(33)	0(0)	3(15)
128	1(5)	16(23)	8(22)	14(15)	4(27)	1(7)	1(5)
512	0(0)	5(7)	4(11)	6(6)	0(0)	0(0)	0(0)
1024	0(0)	0(0)	0(0)	5(5)	0(0)	0(0)	0(0)

Key:

HTV = healthy Volunteer,
PTS = patient specimen
AHP = Apparently health pig.
AHC = Apparently health chicken.
Cattle recorded zero prevalence of FQREC and thus not included here.

The modal MIC values for healthy carriers and patients were 128 μg/ml and 64 μg/ml respectively. For wound swab isolates, the MIC values were within the range of 64 - 256 μg/ml . In animals, most of the FQREC isolates have their MIC values of ciprofloxacin within the range of 4-64 μg /ml with the modal ciprofloxacin value of 4 μg/ml for both the pig and chicken tested. More of high level ciprofloxacin resistance (MIC of 64 - 256 μg /ml) were recorded for FQREC isolates from patients compared to FQREC isolates from healthy carrier.

4.5 **Fluoroquinolone Resistance Genes.**

4.5.1. Screening for gyr A gene in both FQREC

PCR analysis showed that *gyrA* gene was found to be present in 58.1 % (157 of 270) of the test FQREC isolates in the study area as shown on the representative gel image of **Figure 31.** This result revealed the prevalence of *gyrA* gene in human FQREC isolates at the range of 52.6 to 63.4% depending on the source of the isolates (Table 23). In animals tested, the prevalence of *gyrA* gene in FQREC was 21.4 and 40.0% in pig and chicken respectively.

The percentage distributions of *gyrA* gene on animal and human FQREC isolates in relation to MIC of ciprofloxacin are shown in Tables 24 and 25 respectively. In pigs tested, all the isolates with *gyrA* gene have the MIC values of ciprofloxacin as 64 μg/ml, while in chicken the *gyrA* gene positive FQREC isolates have ciprofloxacin MIC range of 4 – 128 μg/ml (Table 24). In human isolates, the *gyrA* genes were concentrated within the MIC values of ciprofloxacin of 32–512 μg/ml (Table 25). The gyrA gene were found most in isolates from human patients with high level ciprofloxacin resistance (32 – 1024 μg/ml).

4.5.2 Prevalence and Distribution of qnr A gene in animasl and humans FQREC

In this study, a total of 270 FQREC isolates from different sources (humans and animals) were screened for *qnrA* gene by PCR and the prevalence of the gene is shown in Table 23. The representative gel images of the PCR analysis are shown in **Figures 32- 35.** The qnrA gene was present in 12.5% of all the FQREC isolates. In healthy volunteers, the urinary and faecal FQREC isolates from healthy volunteer have *qnrA* prevalence of 15.8 and 10.5% respectively. In patient, the prevalence of *qnrA* for urinary and faecal isolates were 16.9 and 22.6% respectively. In wound FQREC isolates, the prevalence of *qnrA* gene was 13%. The prevalence rate of *qnrA* among the FQREC isolates in children ≤ 5 years in Enugu state Nigeria was 12.5%. In animals, the prevalence are 7.1% (pig) and 10% (chicken) as shown in table. The prevalence of *qnrA* was lower in pig (7.1%) than in chicken (10%), this implies that there is a relationship between the incidence of *qnrA* gene and the source of isolates.

The percentage distributions of *qnrA* gene on animals and human FQREC isolates in relation to MIC of ciprofloxacin are shown in Tables 26 and 27 respectively. In all the animal FQREC isolates screened for *qnrA* gene, none had the MIC values of ciprofloxacin > 4 μg/ml. In FQREC isolates from both human patients and healthy volunteers, most of the *qnrA* + isolates were unevenly distributed across a wide range of MIC of ciprofloxacin (4-1024 μg/ml).

Table 23. Prevalence of *qnrA* and *gyr A* in humans and animals according to specimen source**.**

Isolates source	**No of FQREC**	**No (%) of *qnr A***	**No (%) of *gyr A***
HVUS	19	3(15.8)	10(52.6)
PSU	71	13(18.3)	46(64.8)
HVFS	38	4(10.5)	19(50.0)
PFS	93	20(21.5)	59(63.4)
WS	15	2(13.3)	12(63.4)
Pig	14	1(7.1)	3(21.4)
Cattle	0	0(0)	0(0)
Chicken	20	2(10.0)	8(40.0)

Key:

HVUS = Healthy Volunteer Urine specimen , PSU = Patient specimen of urine

HVFS = Healthy volunteer fecal specimen .PFS =patient fecal specimen

W/S =wound specimen, *qnr A*= Plasmid mediated quinolone resistant determinant

gyr A = Fluoroquinolone resistant gene

Table 24: Frequency (%) of *gryA*-mutation in FQREC in relation to MIC of ciprofloxacin in Animals

MIC (ug/ml)	**PIG**		**CHICKEN**	
	F	***gyr A*+(%)**	**F**	***gyr A*+(%)**
4	**5**	**0(0)**	**12**	**2(17)**
8	3	0(0)	2	0(0)
16	3	0(0)	1	1(100)
32	3	0(0)	1	1(100)
64	1	2(100)	3	3(100)
128	0	0(0)	1	1(100\)
256	0	0(0)	0	0(0)
512	0	0(0)	0	0(0)
1024	0	0(0)	0	0(0)

Key : gyrA + = isolates positive to gyrA gene

Table 25: Frequency of occurrence (%) *gyrA* gene in relation to MIC of ciprofloxacin in human

MIC (ug/ml)	**Healthy Volunteers**		**Patient**		
	Frequency of *gyrA* + (%) isolates from		Frequency of *gyrA* + (%) isolates from		
	URINE	STOOL	URINE	STOOL	WOUND
4	00	00	00	00	00
8	00	00	03	15	100
16	83	00	00	22	00
32	50	83	38	16	00
64	100	100	90	82	100
128	100	100	100	100	100
256	100	-	100	100	100
512	-	100	100	100	-
1024	-	-	-	100	-

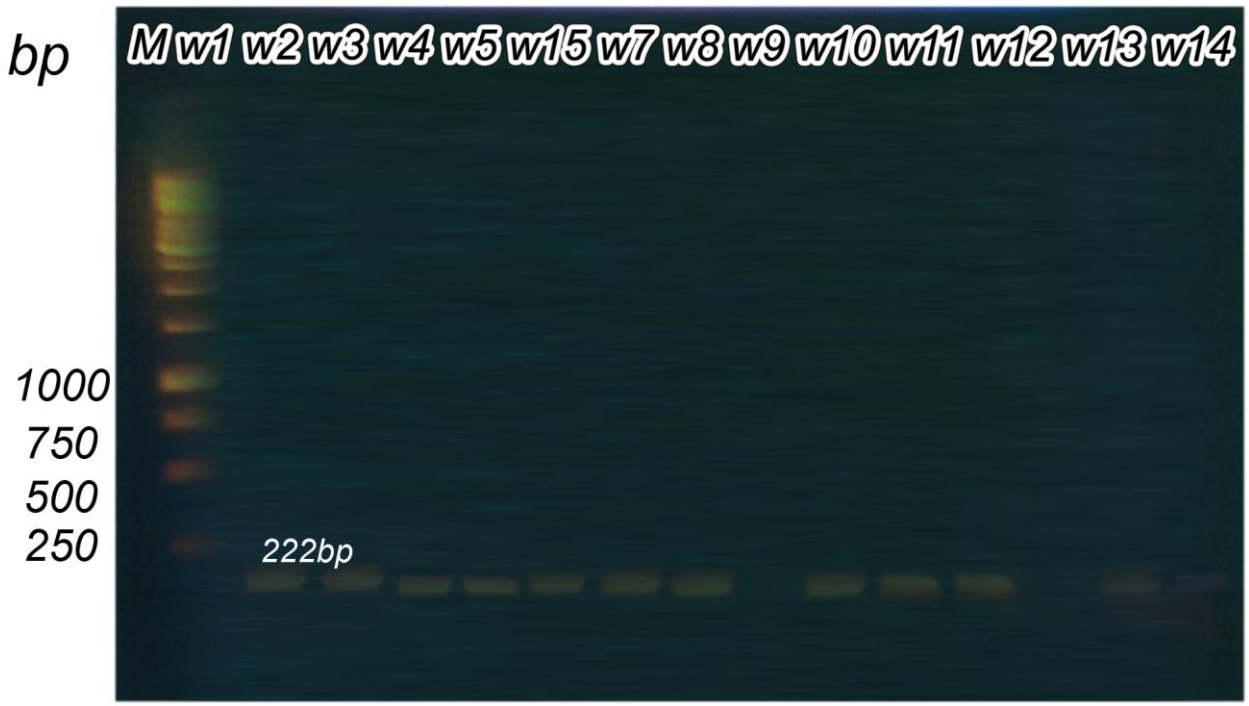

Figure 31 : A gel image of PCR amplification of QRDR of gyrA gene in FQREC isolated.

Key: Lane M = Ikb DNA marker;
Lane w1 = Positive control of gyrA gene
Lane w2-w6 = DNA isolated from FQREC isolates recovered from Wound swab.
Lane w7-w10 = DNA isolated from FQREC isolates recovered from Specimen of urine
Lane w11-w14= DNA isolated from FQREC isolates recovered from Animal specimens.
Lanes with band correspond to positive isolates while the remaining lanes correspond to negative isolates

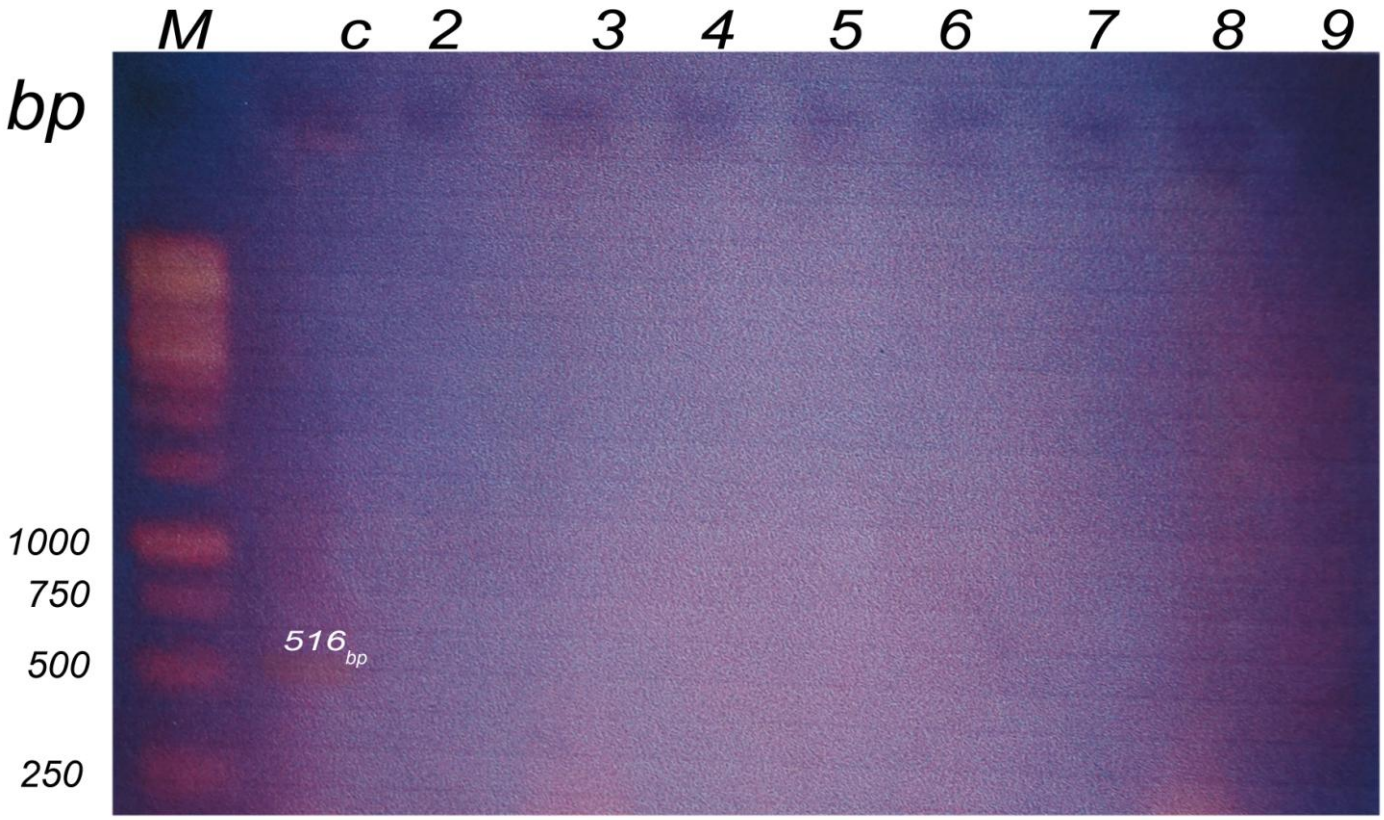

Figure 32: PCR amplification of *qnr A* gene in FQREC isolates.

Key: Lane M = 1kb DNA marker.
Lane C = DNA of *E.coli* qnrA-positive control
lanes 2- 9 = DNA of qnrA negative isolates from the healthy volunteer urine specimens

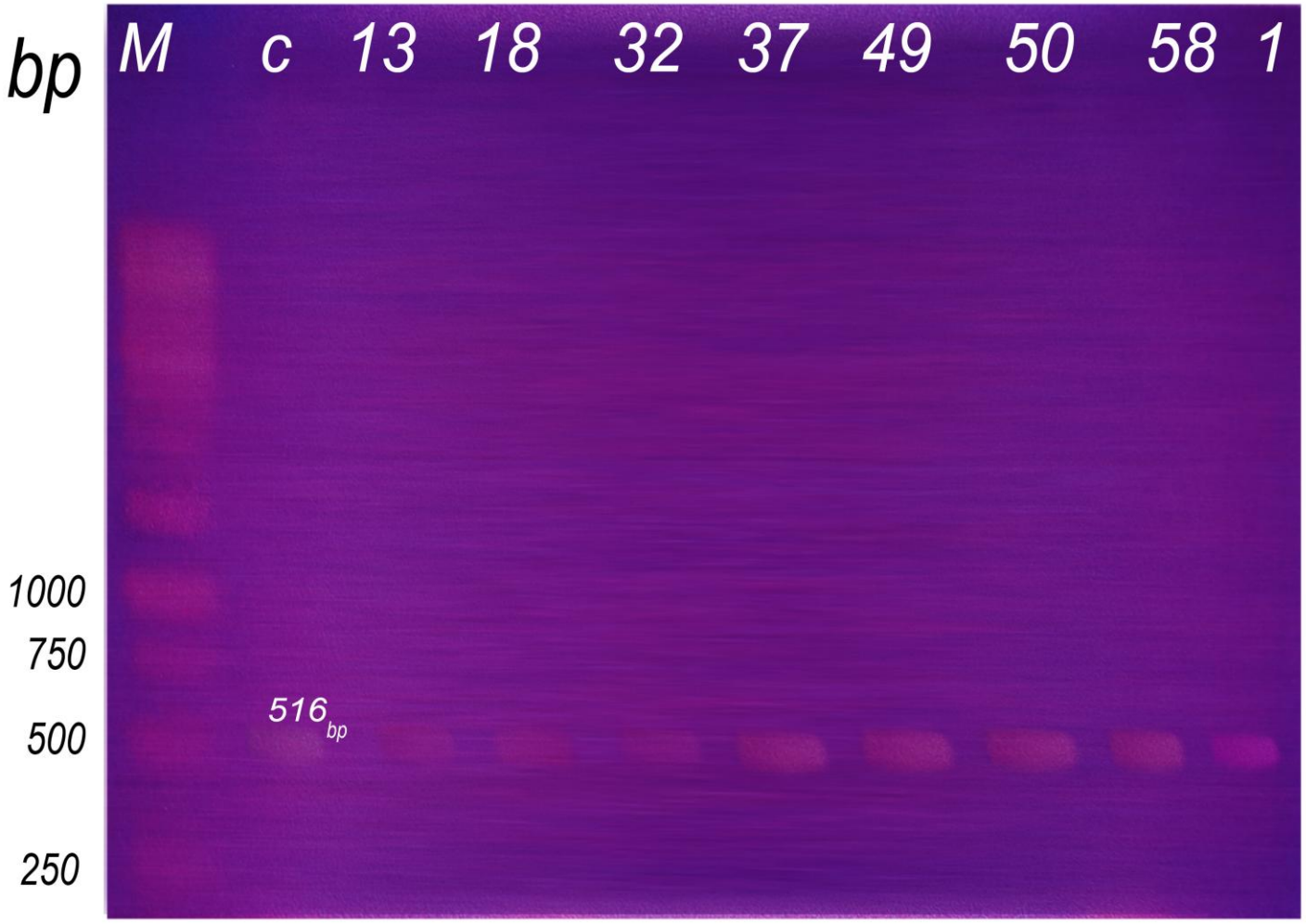

Figure 33: PCR amplification of qnr A gene in FQREC isolates.

Key: Lane M = 1kb DNA marker
Lane C = DNA of *E.coli* qnrA-positive control
lanes 1, 13 and 18 = FQREC isolates from the healthy volunteer urine specimen
Lanes 32,37, 49 and 50 = DNA from isolates recovered from healthy volunteer stool specimen and 58 from patient stool.
They are all FQREC qnrA –positive isolates

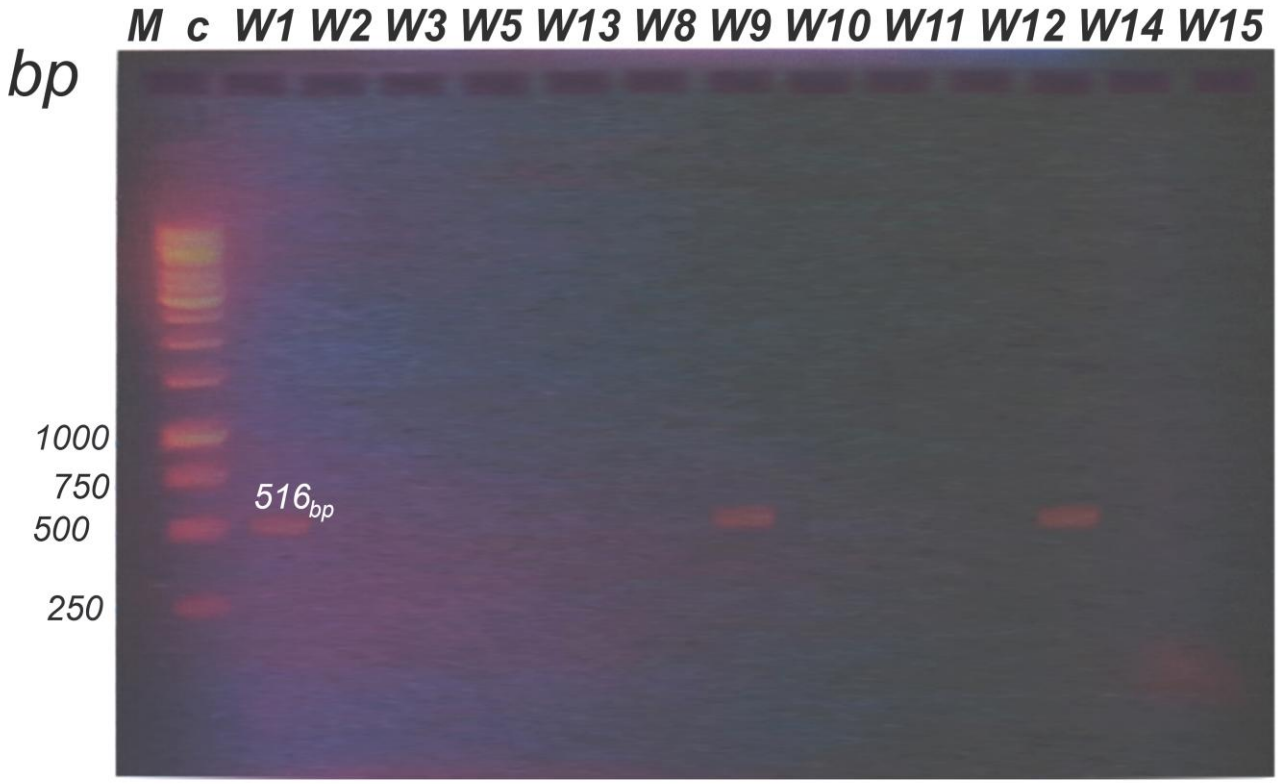

Figure 34: PCR amplification of qnr A gene in FQREC isolates

Key: Lane M = 1kb DNA marker
Lane C = DNA of *E.coli* qnrA-positive control
Lanes W8 and W12 = DNA of FQREC qnrA –positive isolates from wound swab
Lanes 1-7, 9-11, 14, 15 = DNA of qnrA negative isolates from wound swab.

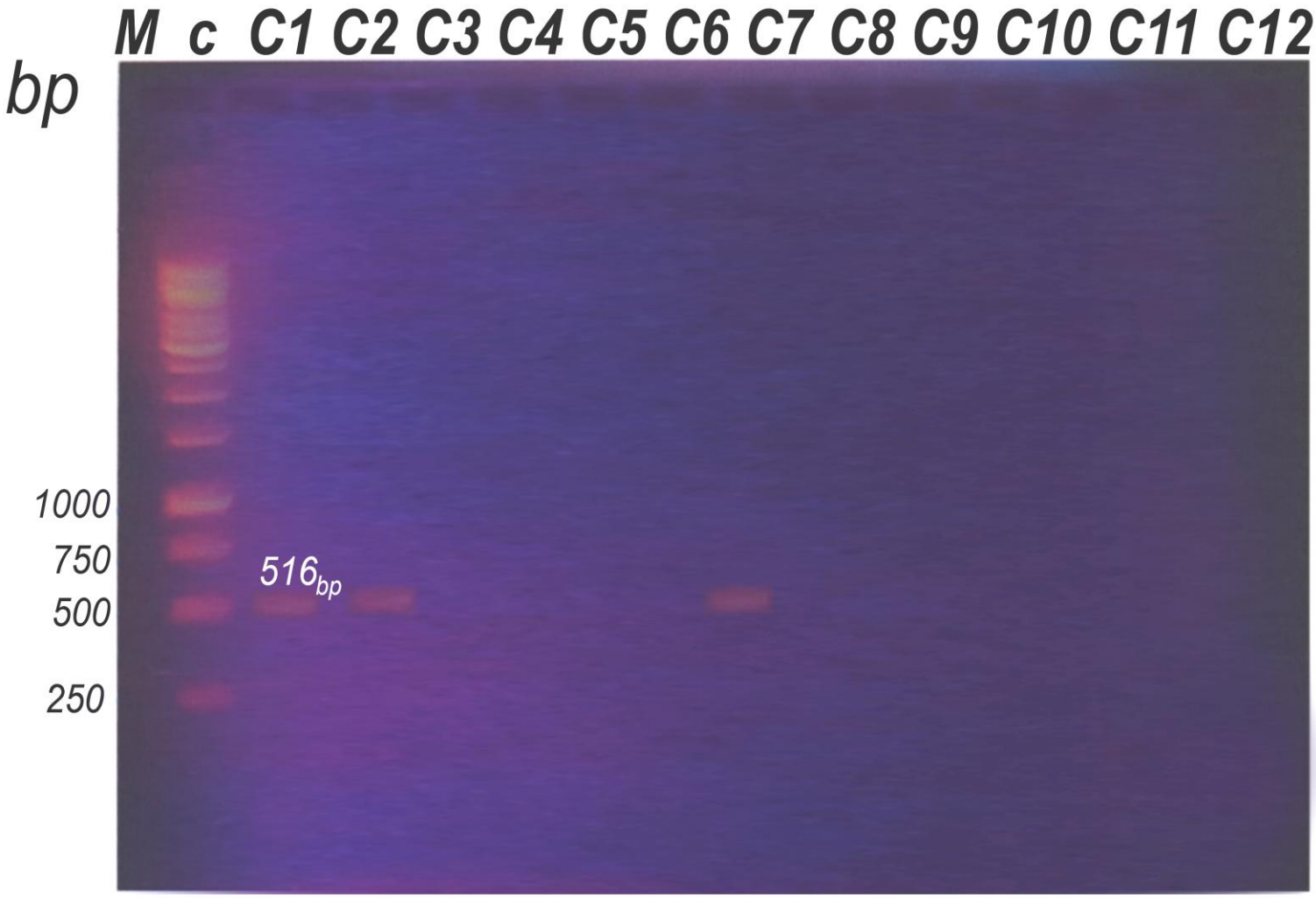

Figure 35 : PCR amplification of qnr A gene in FQREC isolates from animal

Key: Lane M – 1kb DNA marker
Lane -- DNA of *E.coli* qnrA-positive control
Lanes C2 and C6 -- DNA of FQREC qnrA –positive isolate from chicken
Lanes 1, 3-5, 7-12--- DNA of FQREC qnrA negative isolates from chicken.

Table 26: Percentage distribution of *gyrA* gene on animal FQREC in relation to MIC of Ciprofloxacin

MIC	**PIG**		**CHICKEN**	
(ug/ml)	**Fq**	**gyr A+(%)**	**Fq**	**gyr A+(%)**
4	5	1(20)	12	2(16.7)
8	3	0(0)	2	0(0)
16	3	0(0)	1	0(0)
32	2	0(0)	1	0(0)
64	0	0(0)	3	0(0)
128	1	0(0)	1	0(0)
256	0	0(0)	0	0(0)
512	0	0(0)	0	0(0)
1024	0	0(0)	0	0(0)

Key:

qnrA+ = FQREC that are *qnr* A gene positive
Fq = number of fluoroquinolone resistant E.coli isolates having the corresponding MIC.

Table 27 : Percentage distribution of *qnr A* gene on human FQREC isolates in relation to MIC of Ciprofloxacin

MIC (ug/ml	Healthy Volunteers				Patients Specimen					
	UFQREC		F FQREC		U FQREC		F FQREC		W FQREC	
	Fq	qnrA+ (%)	fq	qnrA (%)	fq	qnrA+ (%)	fq	qnrA+ (%)	fq	qnrA+ (%)
4	4	1(25)	7	1(14)	8	3(38)	7	4(57)	1	1(100)
8	3	0(0)	4	(25)	8	3(38)	12	4(33)	1	0(0)
16	6	0(0)	6	0(0))	4	1(25)	9	2(22)	1	0(0)
32	2	1(50)	6	0(0)	8	1(13)	11	2(9)	1	1(100)
64	2	1(50)	3	0(0)	10	2(20)	23	3(13)	5	0(0)
128	1	0(0)	8	2(25)	16	1(6)	14	0(0)	4	0(0)
256	1	0(0)	0	0(0)	12	1(8)	6	2(33)	2	0(0)
512	0	0(0)	4	0(0)	5	0(0)	6	1(17)	0	0(0)
1024	0	0(0)	0	0(0)	0	0(0)	5	2(40)	0	0(0)
Total	19	3(15.8)	38	4(10.5)	71	12(16.9)	93	21(22.6)	15	2(13.0)

Key: fq = number of fluoroquinolone resistant *E. coli* isolates having the corresponding MIC

UFQREC = urinary fluoroquinolone resistant *E. coli* isolates

F FQREC = faecal fluoroquinolone resistant *E. coli* isolates

W FQREC = Wound fluoroquinolone resistant *E. coli* isolates

qnrA+ = FQREC isolates that are qnr a positive

4.5.3. **Detection and Prevalence of efflux pump mediated fluoroquinolone resistance gene-*Nor A* gene in FQRSA from human and animals.**

The *NorA* gene was present in 14 (25%) of the 56 FQRSA isolates from human nasal swab specimens; out of which 20% were from nasal swab of healthy volunteer and 37.5% were from nasal swab of patient subjects who have been on antibiotics (Table 28). The prevalence of *NorA* in human FQRSA urinary isolates were 26.3 and 36.4% for healthy volunteers and individual patients respectively. The prevalence was highest (71.4%) in FQRSA isolates from human wound swabs. In animal subjects the respective prevalence's of *NorA* gene in chicken, cattle and pigs were 0, 22.2, and 33.3% respectively. In this study, the *NorA* gene was present with both low-level and high level ciprofloxacin (MIC 4-512) resistant *S.aureus* isolates from both human and animal. The representative gel images of the PCR analysis are shown in **Figures 36-37.**

Table 28 Prevalence of Nor A gene among FQRSA isolates from different specimen sources

Specimen source	No of FQRSA	Prevalence (%)	MIC range of Cpx affected
HVns	40	8(20)	4-128
Pns	16	6(37.5)	4-32
Hvus	19	5(26.3)	32-128
PSU	33	12(36.4)	4-512
PWS	28	20	71.4
Pig specimen	6	2(33.3)	8-64
Cattle specimen	9	2(22.2)	8-32
Chicken specimen	5	0(0)	0

Key ; Cpx = Ciprofloxacin,

() = percentage prevalence

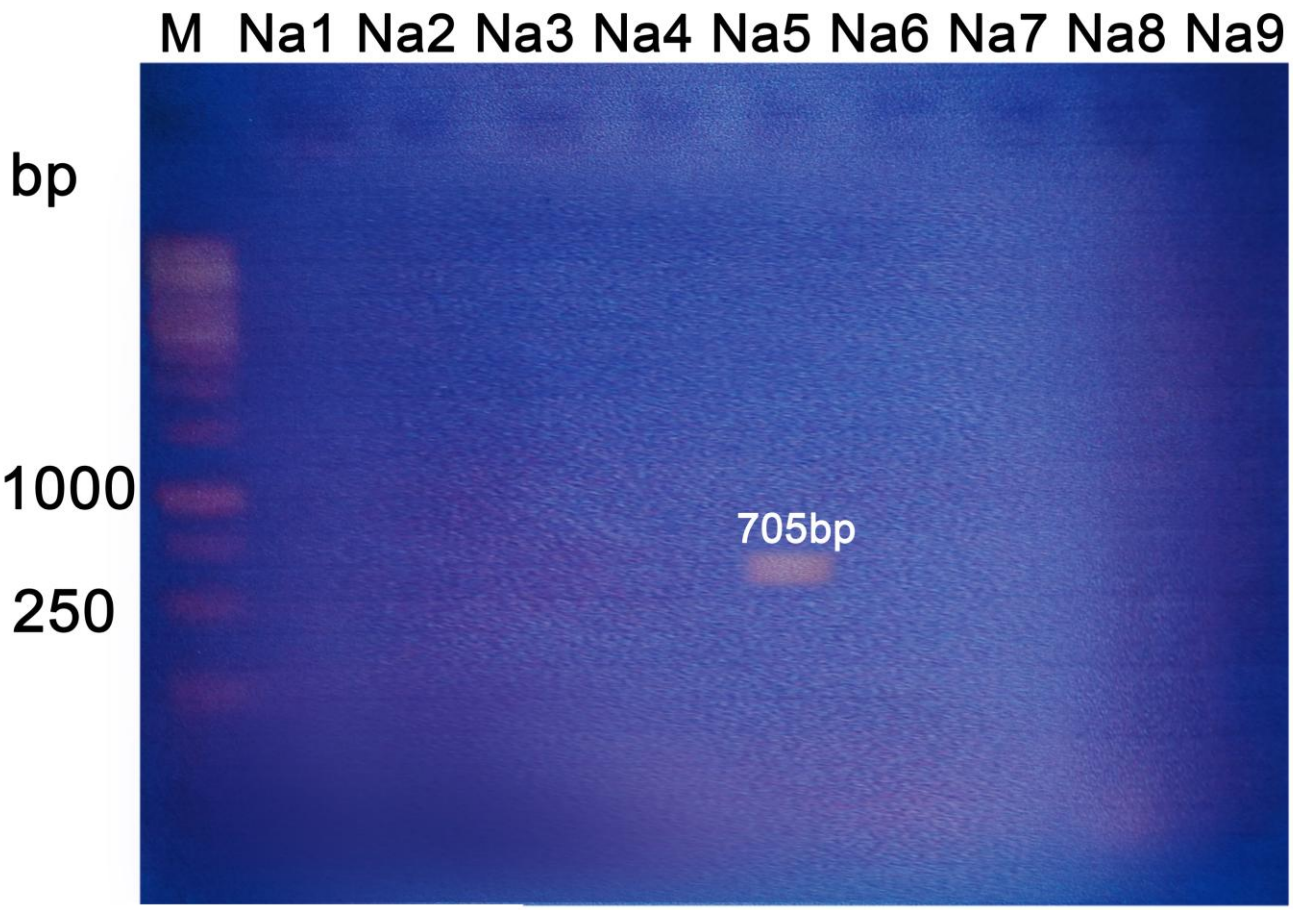

Fig 36: PCR amplification of *Nor A* gene in FQRSA isolates from nasal swab specimen of human healthy volunteers subjects.

Key: Lane M = 1kb DNA marker

Lane Na5 = DNA of *NorA* –positive FQRSA isolate from nasal swab specimen of human healthy volunteers subjects.

Lanes Na1- Na4, Na6-Na9 = DNA of *NorA* negative FQRSA isolates from nasal swab specimen of human healthy volunteers subjects.

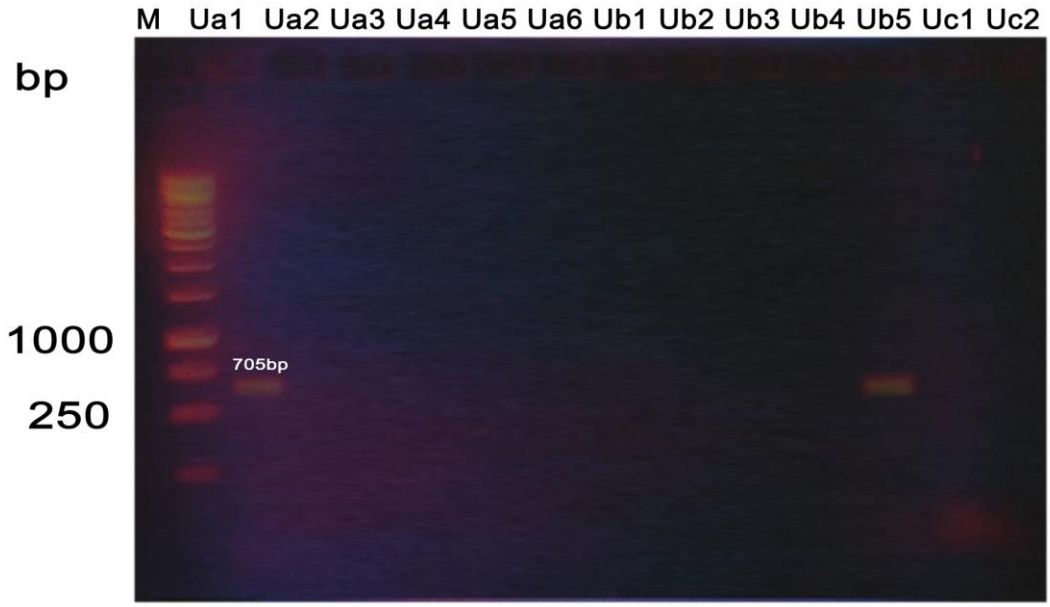

Fig 37; PCR amplification of *Nor* A gene in FQRSA isolates

Key: Lane M = 1kb DNA marker

Lane ub5 = DNA of *NorA* –positive isolates from urine specimen of patients.

Lanes ua1-ua6 = DNA of *NorA* negative FQRSA isolates from urine specimen of healthy volunteers

Lanes ub1-ub4 = DNA of *NorA* negative FQRSA isolates from urine specimen of Patients.

Lanes uc1 and uc2= DNA of *NorA* negative FQRSA isolates from cattle

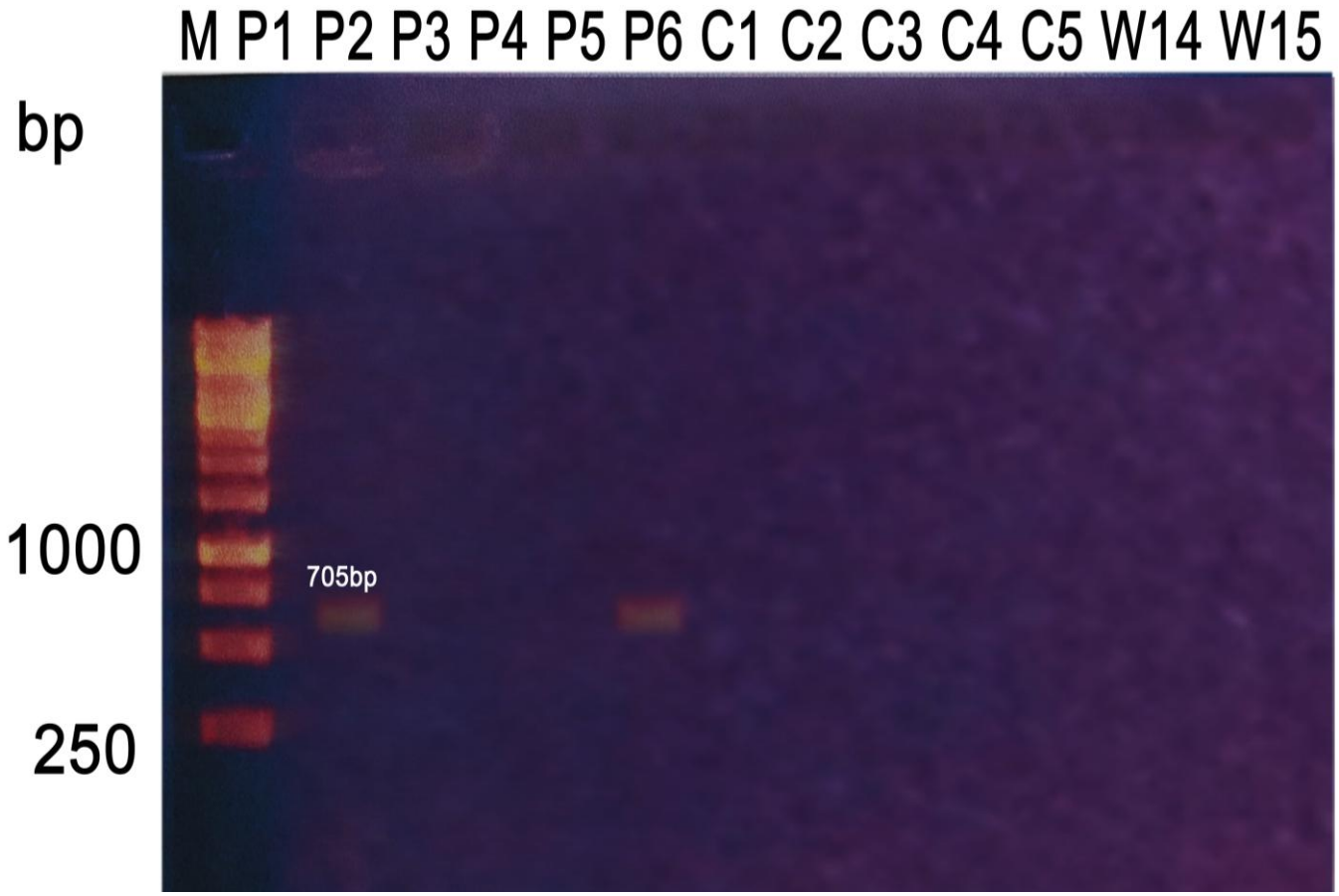

Fig 38: PCR amplification of Nor A gene in FQRSA isolates from pig, chicken, and wound swab specimens.

Key: Lane M = 1kb DNA marker

Lanes P1-P6= DNA of FQRSA isolates from pig.

LanesC1-C5= DNA of FQRSA isolates from Chicken.

Lanes p1 and p5 correspond to NorA –positive isolates whereas the other lanes correspond to negative to isolates

4.5.4. **PLASMID PROFILE**

4.5.4.1. **Fluoroquinolone-resistant S. aureus**

One hundred and sixteen (74.4 %) FQRSA isolates out of 156 isolates from both human and animal were found to habour a total of 172 plasmids with molecular sizes ranging from 0.5 to 23.1KB. Out of these, 144 plasmids were detected in human FQRSA and 28 plasmids in animal FQRSA isolates. Most of the plasmids were shared among the human and animal isolates. Of all the plasmids detected, the 23.2KB was the modal plasmid with the prevalence of 66.7% in human and 67.9% in animal isolates respectively. All the isolates bearing this plasmid also harboured one or more smaller plasmids, and they were resistant to six or more antibiotics including gentamicin and ceftriaxone (aminoglycoside and cephalosporin respectively). In general, eight different plasmid profiles were observed with 6.6, 9.2 and 23.1KB occurring in almost all the health districts in both human and animal FQREC and FQRSA isolates. Tables 29 and 30 show the respective distribution of plasmids in FQRSA isolates according to specimen source and location . .

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. Like the antibiotics resistance pattern of human FQRSA isolates, 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 these FQRSA isolates occurred at a considerable low rate 2.7-5.9% . The human FQRSA isolates from other districts were more diverse in terms of plasmid distribution. Though six plasmid profiles were observed in the FQRSA isolates 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 human FQRSA isolates. 2.0KB plasmid was present in FQRSA isolates from Nsukka, Udi and Agbani health districts. Of the 28 plasmids detected in the animal FQRSA isolates. 10 different profiles were recorded, 3 profiles in pigs, 5 in cattles and 2 in chickens respectively. The 2.0, 4.4, 6.6, 9.2 and 23.1KB plasmids were detected across the animal hosts. Similar to the results from human FQRSA isolates, the 23.1KB plasmid was most frequent in the three animals sampled; 67% in pig, 64.3% in cattle, and 67% in chicken respectively. Figures 39-48 show the detailed gel images of the plasmid profile of the test isolates used in the study.

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Table 29: The distribution of Plasmids in FQRSA according to specimen source in both humans & animals

Plasmid Size	**Frequency of Distribution according to specimen Source**							
(KB	HVnF N=38 (%)	PnF N=30 (%)	HVUF N=15 (%)	PUF N=38 (%)	PWS N=23 (%)	PIG N=8 (%)	Cat N=14 (%)	Chicken N=6 (%)
0.5	01(2.6)	01(33)	00(0)	01(2.6)	0(0)	0(0)	0(0)	0
1.0	00(0)	00(0)	00(0)	00(0)	0(0)	0(0)	0(0)	0
2.0	02(5.3)	02(6.6)	00(0)	01(2.6)	0(0)	0(0)	1(7.1)	0(0)
2.3	00(0)	01(3.3)	01(6.7)	01(2.6)	0(0)	0(0)	0(0)	0(0)
4.4	02(5.3)	02(6.6)	00(0)	01(2.6)	0(0)	1(12.5)	2(4.3)	0(0)
6.6	02(5.3)	01(3.3)	02(13.3)	02(5.3)	5(21.7)	0(0)	1(7.1)	2(33)
9.2	02(5.3)	09(30)	01(6.7)	03(7.9)	5(21.7)	1(12.5)	1(7.1)	0(0)
23.1	29(78.3)	14(46.7)	11(73.3)	29(76)	13(56.5)	6(75)	9(64.3)	4(67)

Key: HVNF = Healthy Voluneer Nasal FQRSA., PFN = Patients Nasal FQRSA.
HVUF = Healthy Volunteer Urinary FQRSA, PUF = Patient Urinary FQRSA
PWF = Patient Wound FQRSA.

Table 30: 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

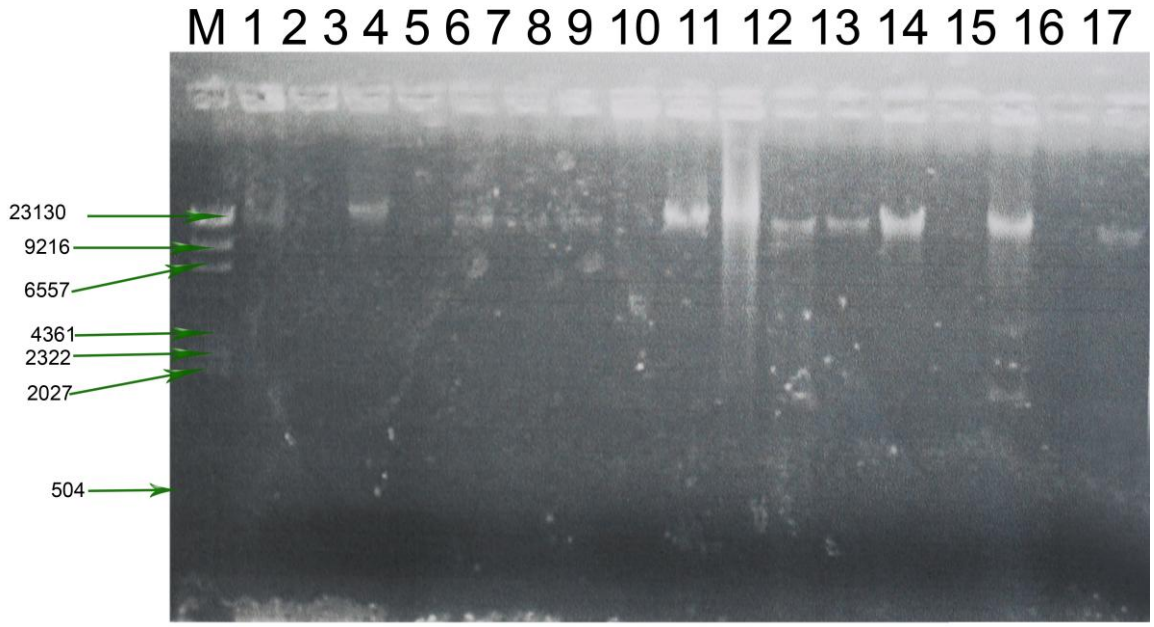

Figure 39 : The Image of gel electrophoresis of Plasmid DNA from FQRSA isolates tested.

Key: Lane M = HIND III DNA Marker (shown in appendix)
Lane 1-17=DNA of FQRSA isolates from nasal swab of healthy volunteers
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

bp

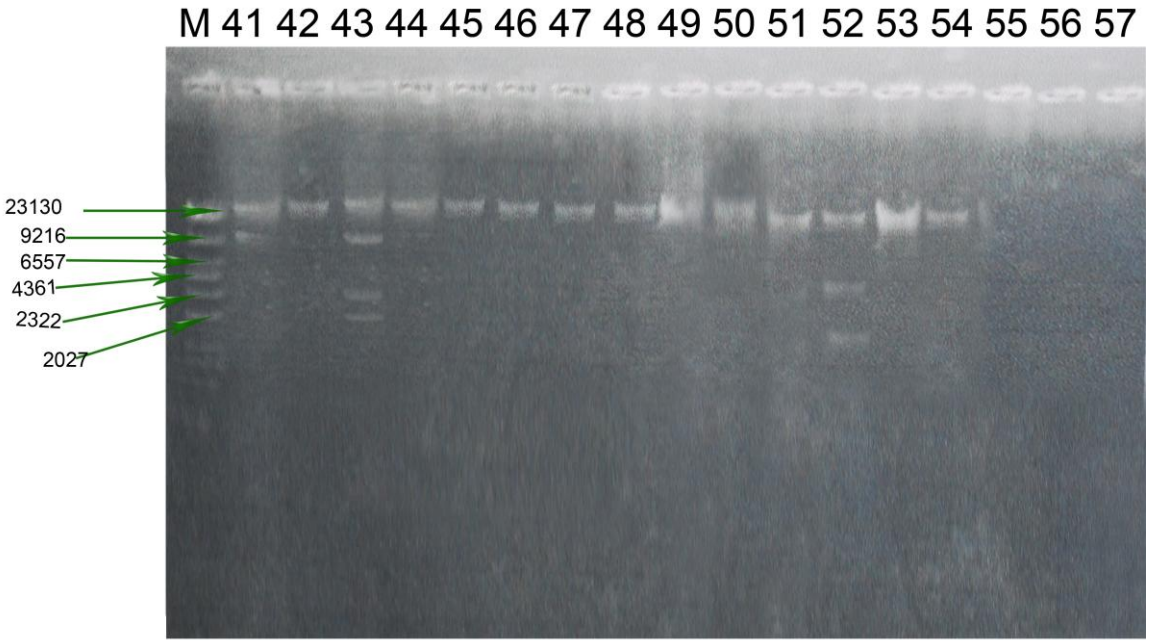

Figure 40 : The Image of gel electrophoresis of Plasmid DNA from the FQRSA isolates tested.

Key: Lane M = HIND III Marker.

Land 41- 57= FQRSA isolated from nasal swab specimens of patients.

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.

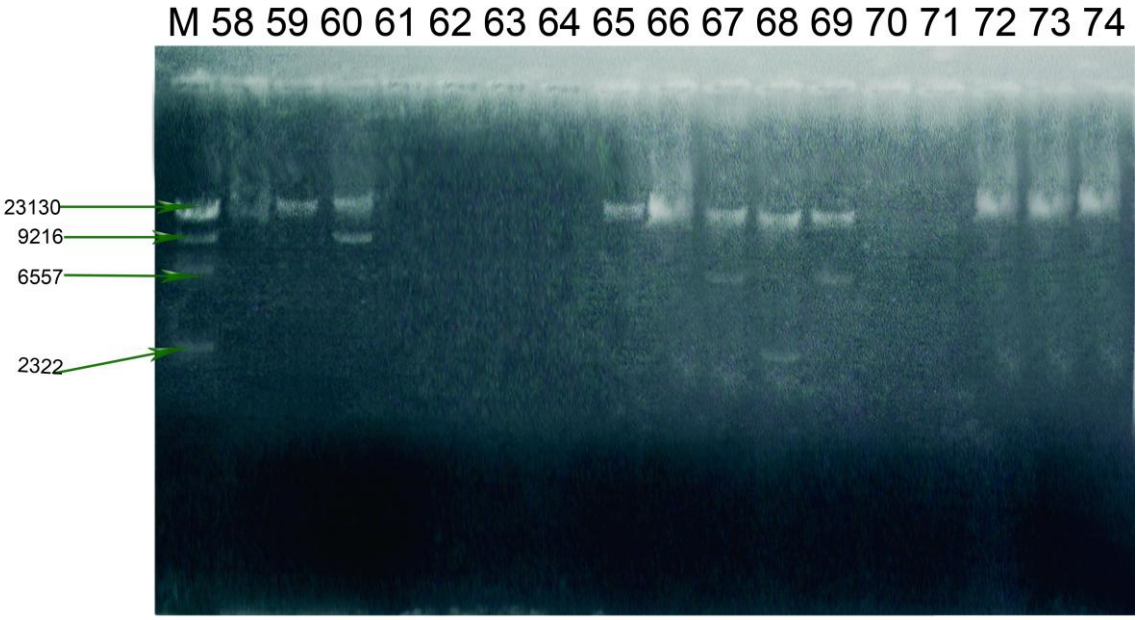

Figure 41: The Image of gel electrophoresis of Plasmid DNA from the FQRSA isolates tested. Apart from lanes 61, 62, 63, 64 and 70 and 71 without plasmid band, other lanes had at least one plasmid band of size 23.1kb.

Key: Lane M = HIND III Marker.
Lane 58-74= Plasmid DNA from the FQRSA isolated from urine specimen of healthy volunteers.

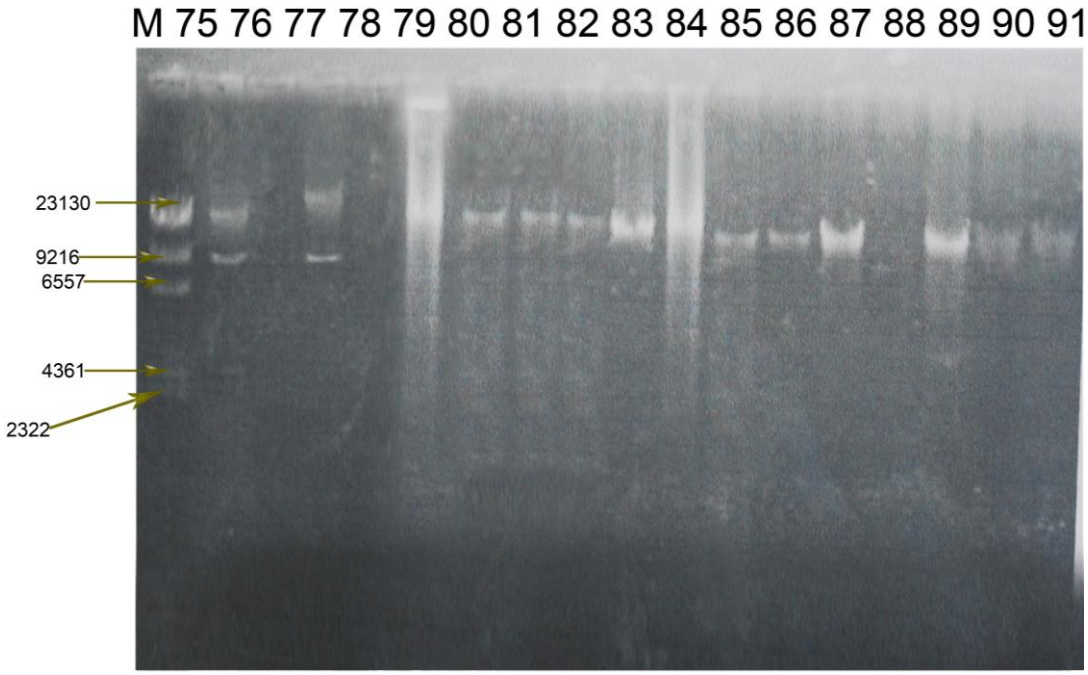

Figure 42: The Image of gel electrophoresis of Plasmid DNA of FQRSA isolates tested.

Key: Lane M is HIND III Marker.

Lane 75-91= Plasmid DNA from the FQRSA isolates from urine specimens of patients.

Apart from lanes 76, 78 and 88 without plasmid band, other lanes had at least one plasmid band of size 23.1kb.

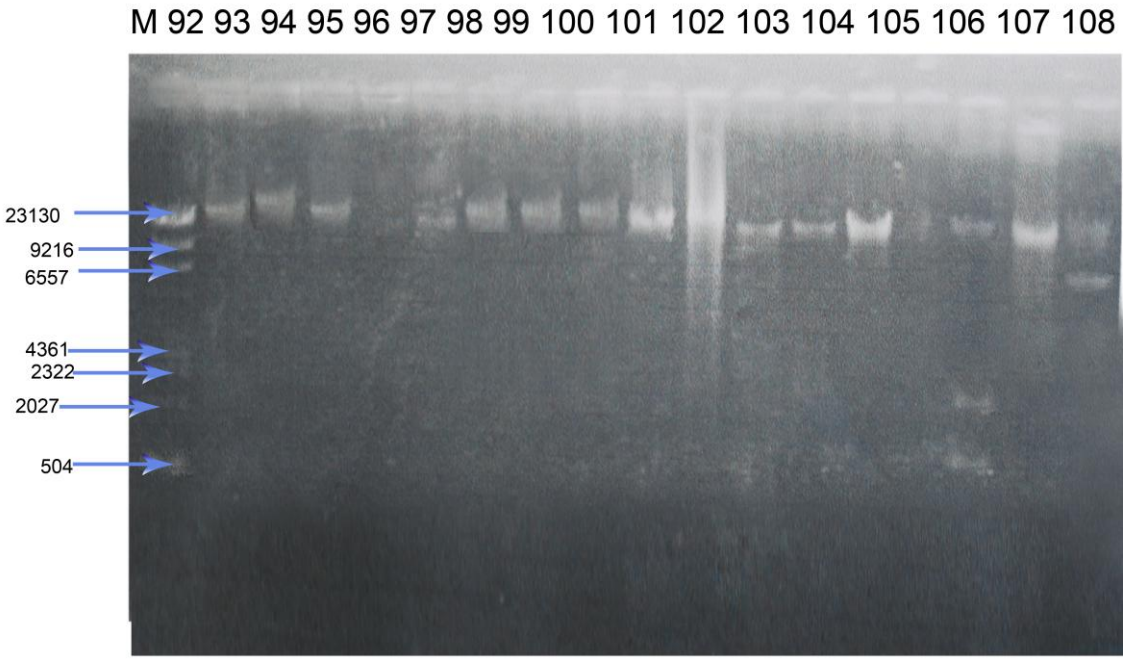

Figure 43 : The Image of gel electrophoresis of Plasmid DNA of FQRSA isolates tested.

Key: Lane M = HIND III Marker.

Lane 92-108 = Plasmid DNA from the second group of FQRSA isolates from urine specimens of patients

Apart from lanes 95 and 105 without plasmid band, other lanes had at least one plasmid band of size 23.1kb.

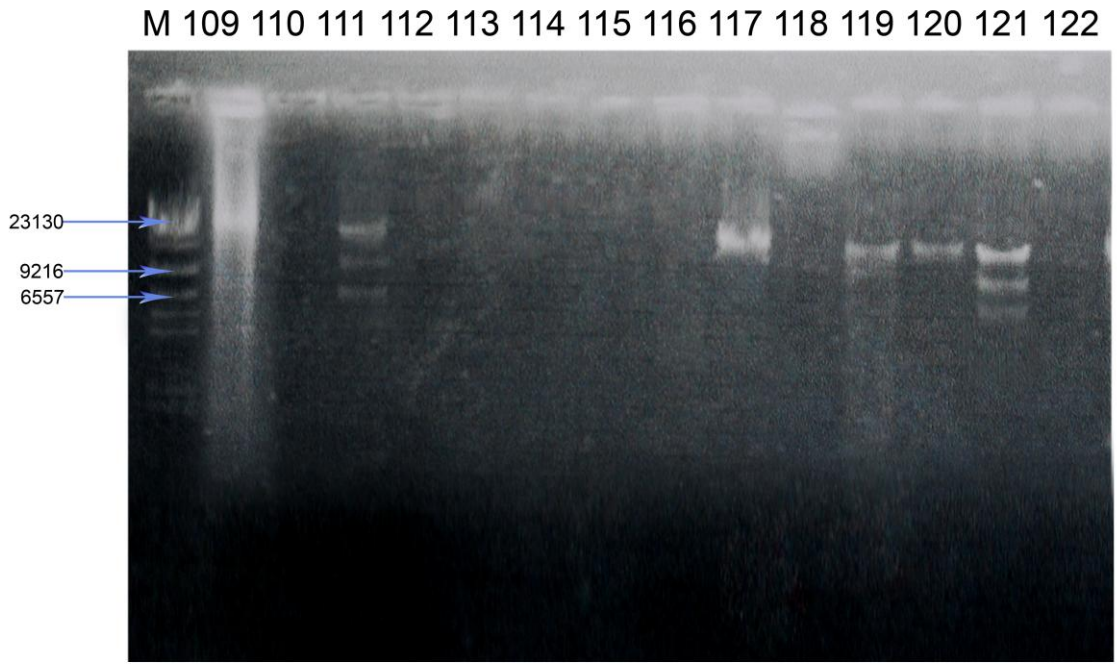

Figure 44: The Image of gel electrophoresis of Plasmid DNA of FQRSA isolates from wound swab specimens of patients.

Key: Lane M = HIND III Marker.

Lane 109- 122 = Plasmid DNA from the first group of FQRSA isolates from wound swab specimens of patients.

Apart from lanes 111 and 117, 119, 120 and 121 with at least one plasmid band, other lanes had no plasmid band.

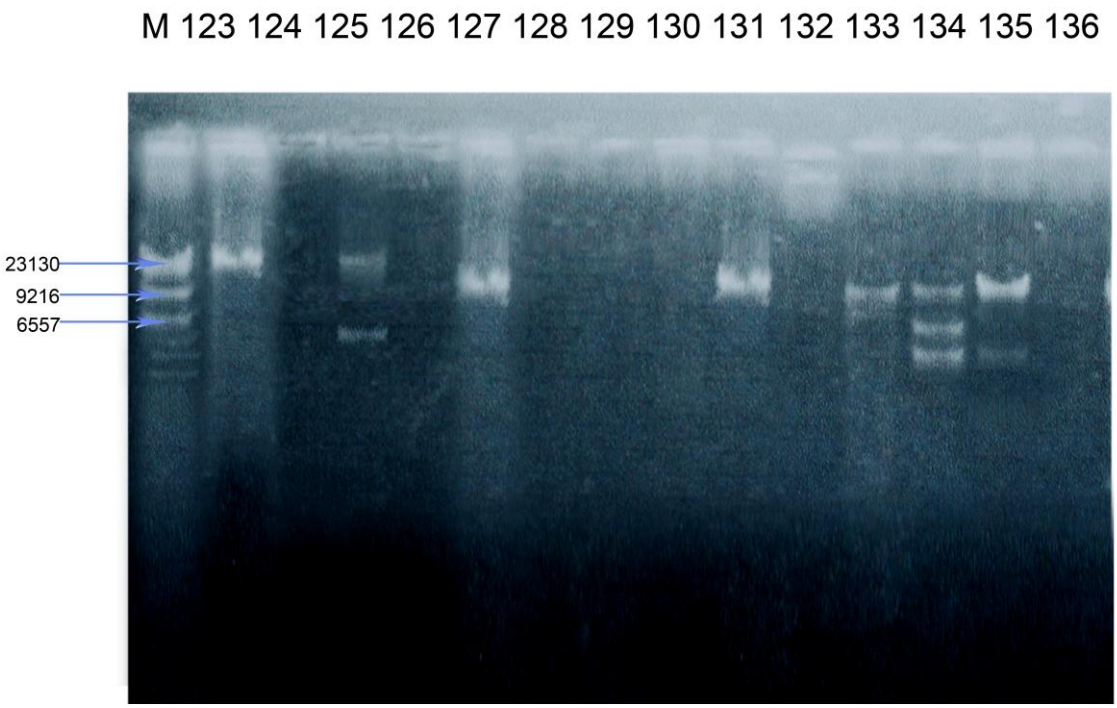

Figure 45: The Image of gel electrophoresis of Plasmid DNA from the second group of FQRSA isolates from wound swab of chronic wound patients.

Key: Lane M = HIND III Marker.
Lane 123- 136 = Plasmid DNA from FQRSA isolates from wound.
Apart from lanes 124,126, 128,129,130, 132 and 136 without plasmid band, other lanes had at least one plasmid band of size 23.1kb.

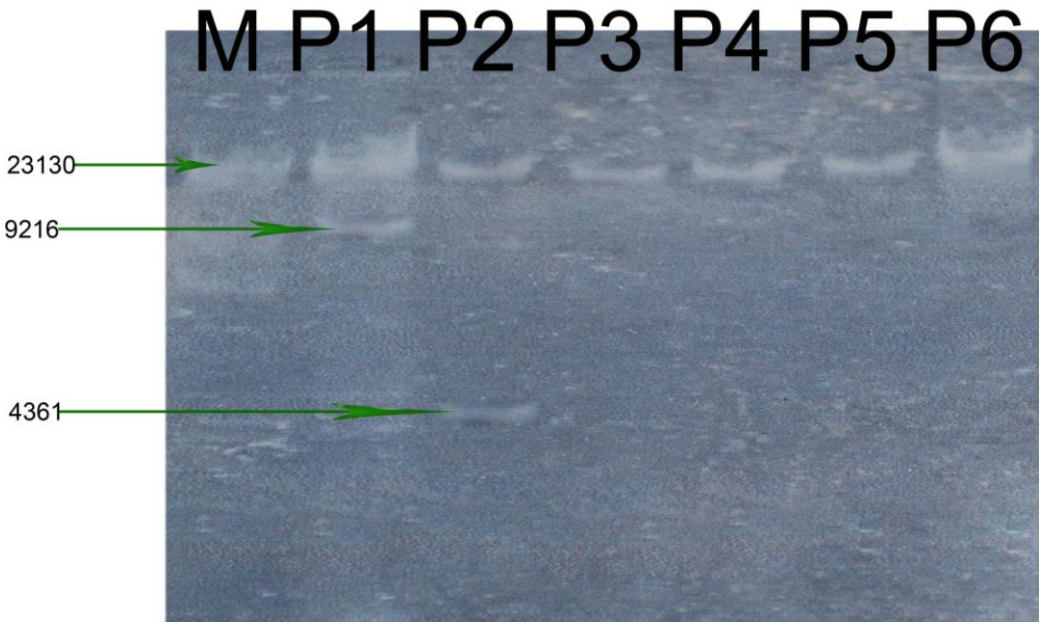

Figure 46 : The Image of gel electrophoresis of Plasmid DNA from FQRSA isolates from pig. All the lanes has at least one plasmid band of size 23.1kb.

Key: Lane M = HIND III Marker.
Lane P1-P6 = Plasmid DNA from FQRSA isolates from pig

bp

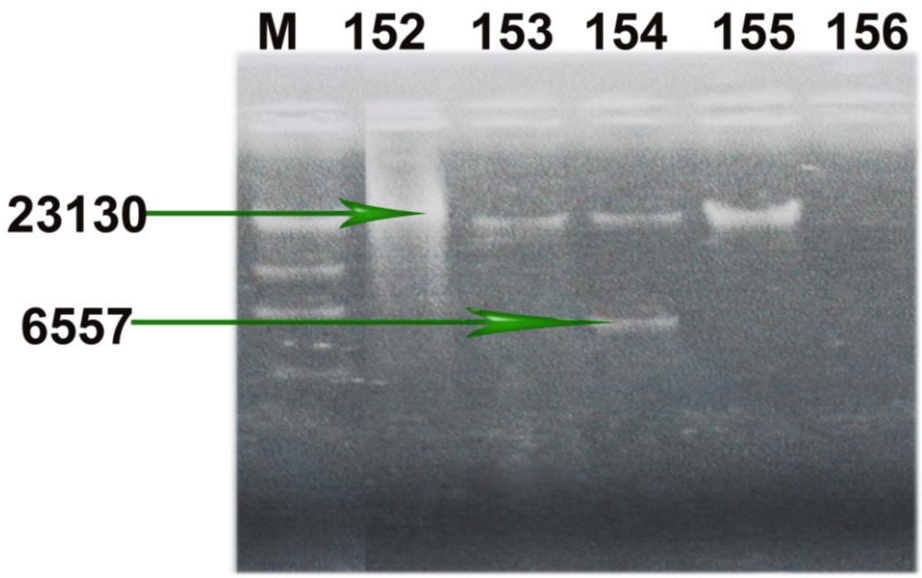

Figure 47: The Image of gel electrophoresis of Plasmid DNA from FQRSA isolates from cattle. All the lanes has at least one plasmid band of size 23.1kb.

Key: Lane M = HIND III Marker.
Lane 152-156 = Plasmid DNA from FQRSA isolates from cattle

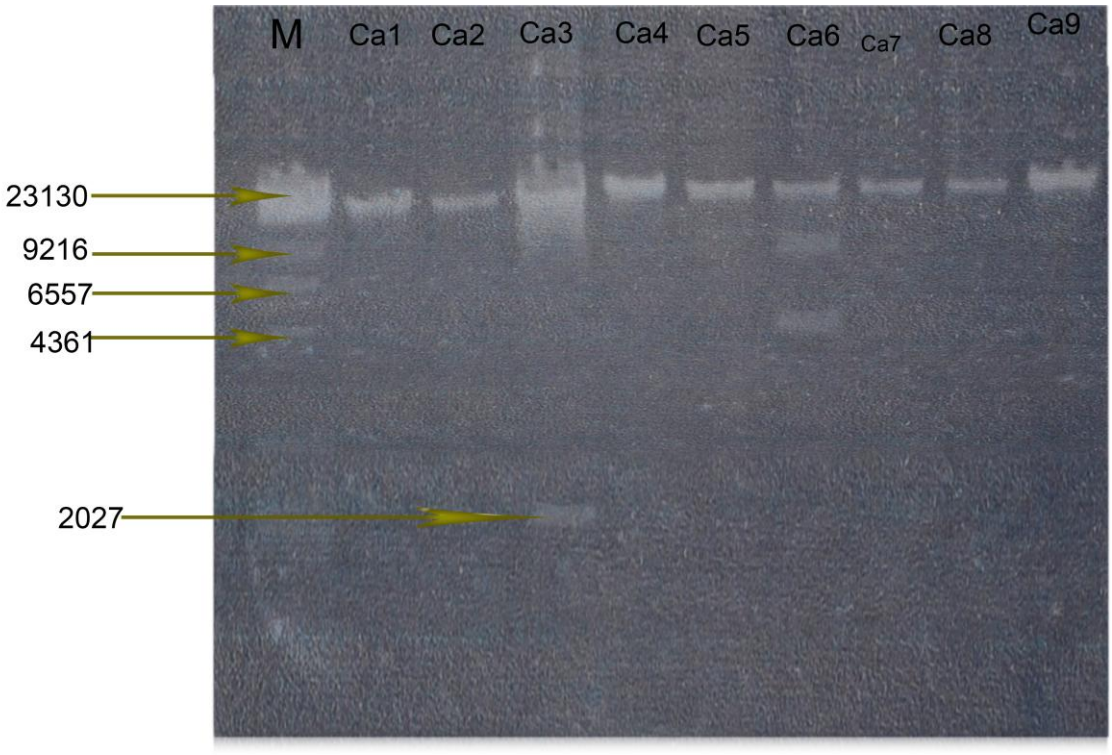

Figure 48 : The Image of gel electrophoresis of Plasmid DNA from the FQRSA isolates from chicken.

Key: Lane M = HIND III Marker.
Lane ca1-ca9 = Plasmid DNA from the FQRSA isolates from chicken. Apart from lane 156, other lanes had at least one plasmid band of size 23.1kb.

4.5.4.2. Plasmid profile of FQREC

A total of 223 plasmids were detected from both human and animals FQREC isolates used for the study. Out of this, 24 plasmid were detected from animals and a total of 199 were detected from humans FQREC isolates (Table 31). Some isolates harbored one or more plasmids.

The distribution of plasmids in human FQREC according to health district in the study area is shown in Table 31. In ascending order of their number, 14 plasmids were detected from each of Udi and Enugu Ezike, district 15 Plasmids from Ikem district, 33plasmids from Awgu and Nsukka districts, 42 plasmids from Agbani and 48 plasmids from Enugu-urban district. Similar to what was observed in the antibiotic resistance patter in both Agbani and Enugu-Urban districts, the plasmid distributions in the test isolates from the districts showed unique patterns. Five plasmid profile each was found in both Agbani of Enugu-urban districts, with the 23130 and 6557 bp plasmids being the most frequent (41.6 and 20.8%) respectively, followed by the 9216 bp and 4361 bp (18.8 and 16.7%) respectively, the remaining plasmid harbored by these isolates occurred at considerable low rate ($\leq 2.1\%$).

The FQREC isolate from other districts were more diverse in terms of plasmid distribution. Though, 5 plasmid profiles were detected in Enugu-Ezike districts, their distribution are different. In Enugu-Ezike districts, 2027 bp plasmid was detected to a level of 28.6% of the entire plasmids found in FQREC isolates in the region whereas 2027 bp plasmid was not detected in human FQREC isolate both in Nsukka and Udi districts. High level of plasmids of size 2322 bp and 504 bp were detected among FQREC isolates from Ikem districts to a level of 53.3 and 13.3% respectively whereas the plasmids of such magnitude were absent in Nsukka, Awgu districts Enugu-Urban and Agbani Districts.

The distribution of plasmids in FQREC according to specimen source in human and animals is shown inTable 32. Of the 199 plasmids detected in the FQREC isolates from different human specimens, 76 plasmids (38.2%) were detected from FQREC isolates from stool of patients. Sixty five plasmids (32.7%) were detected in FQREC from midstream urine specimen of patients, 10 plasmids (5.5%) from urinary FQREC isolates from healthy volunteers. Of the 24 plasmids detected in the FQREC animal isolates, 9 different profiles were recorded; 3 in pigs and 6 in chicken. The 23130, 6557 and 2027 bp plasmids were detected across the both healthy volunteers and patients. These plasmids also occurred in human FQREC isolates. Similar to the results from human isolates, the 23130 bp plasmid was most frequent in FQREC isolates from both animals; 57.1% in Pig and 52.9% in chicken. The 504 bp plasmid, which was detected in FQREC isolates from human (both patients and healthy volunteers) from all the districts except Enugu-urban and Agbani, was absent in both animals. The 2322 bp, 4361 pb and 9216 were present in FQREC isolates from chicken but, not detected in pigs. Of all the plasmids detected, 23130 bp plasmid was most frequently found; 49.2% in human and 54.2% in animal isolates. All the isolates bearing this plasmid also harbored one or more smaller plasmids, and they were resistant to six or more antibiotics including ceftriaxone, a third generation cephalosporin. The gel electrophoresisof plasmid DNA is in Figures 49-55

Table 31: The Distribution of Plasmids in FQREC isolates from humans according to health district in the study area.

Plasmid Size (bp)	Frequency of Distribution according to location/Districts						
	Enugu Urban N=48(%)	Nsukka N=33(%)	Euugu Ezike N=14(%)	Awgu N=33(%)	Agbani N=42(%)	Ikem N=15(%)	Udi N=14(%)
504	0(0)	1(3.0)	2(14.3)	1(3.0)	0	2(13.3)	1(7.1)
2027	0(0)	0(0)	4(28.6)	1(3.0)	0	1(6.7)	0(0)
2322	1(2.1)	0(0)	1(7.1)	0	2(4.8)	8(53.3)	2(14.3)
4361	8(16.7)	4(12.1)	0(0)	1(3.0)	6(14.3)	0	0
6557	10(20.8)	1(3.0)	1(7.1)	3(9.0)	5(11.9)	1(6.7)	2(14.3)
9216	9(18.8)	7(21.2)	0(0)	3(9.0)	10(23.8)	1(6.7)	2(14.3)
23130	20(41.6)	20(60.6)	6(42.9)	24(72.7)	19(45.2)	2(13.3)	7(50)

N= Number of Plasmids

Table 32: The distribution of Plasmids in FQREC isolates according to specimen source in human and animals

Plasmid Size (bp)	**Frequency of Distribution according to specimen Source**						
	HVUS	PSU	HVSS	PSS	PWS	AHP	AHC
	11(%)	65(%)	37(%)	10(%)	10(%)	7(%)	17(%)
504	0	3(4.6)	2(5.4)	1(1.3)	1(10)	0(0.0)	0(0)
2027	0	4(6.2)	0	2(2.6)	0	1(14.3)	2(11.8)
2322	4(36.4)	1(1.5)	3(8.1)	5(6.6)	1(10)	0(0)	1(5.9)
4361	1(9.1)	3(4.6)	6(16.2)	7(9.2)	2(20)	0(0)	2(11.8)
6557	1(9.1)	5(7.7)	4(10.8)	14.5)	2(20)	2(28.6)	1(5.9)
9216	2(18.2)	11(16.9)	8(21.6)	11(14.5)	0	0(0)	2(5.9)
23130	3(27.3)	38(58.5)	14(37.8)	39(51.3)	4(40)	4(57.1)	9(52.9)

KEY: HVUS = healthy Volunteer urine specimen
PSU = patient Specimen of Urine
HVSS = Healthy Volunteer Stool Specimen
PSS = patient specimen of stool
PWS = patient wound swab
AHP = apparently healthy pig
AHC = apparently healthy chicken

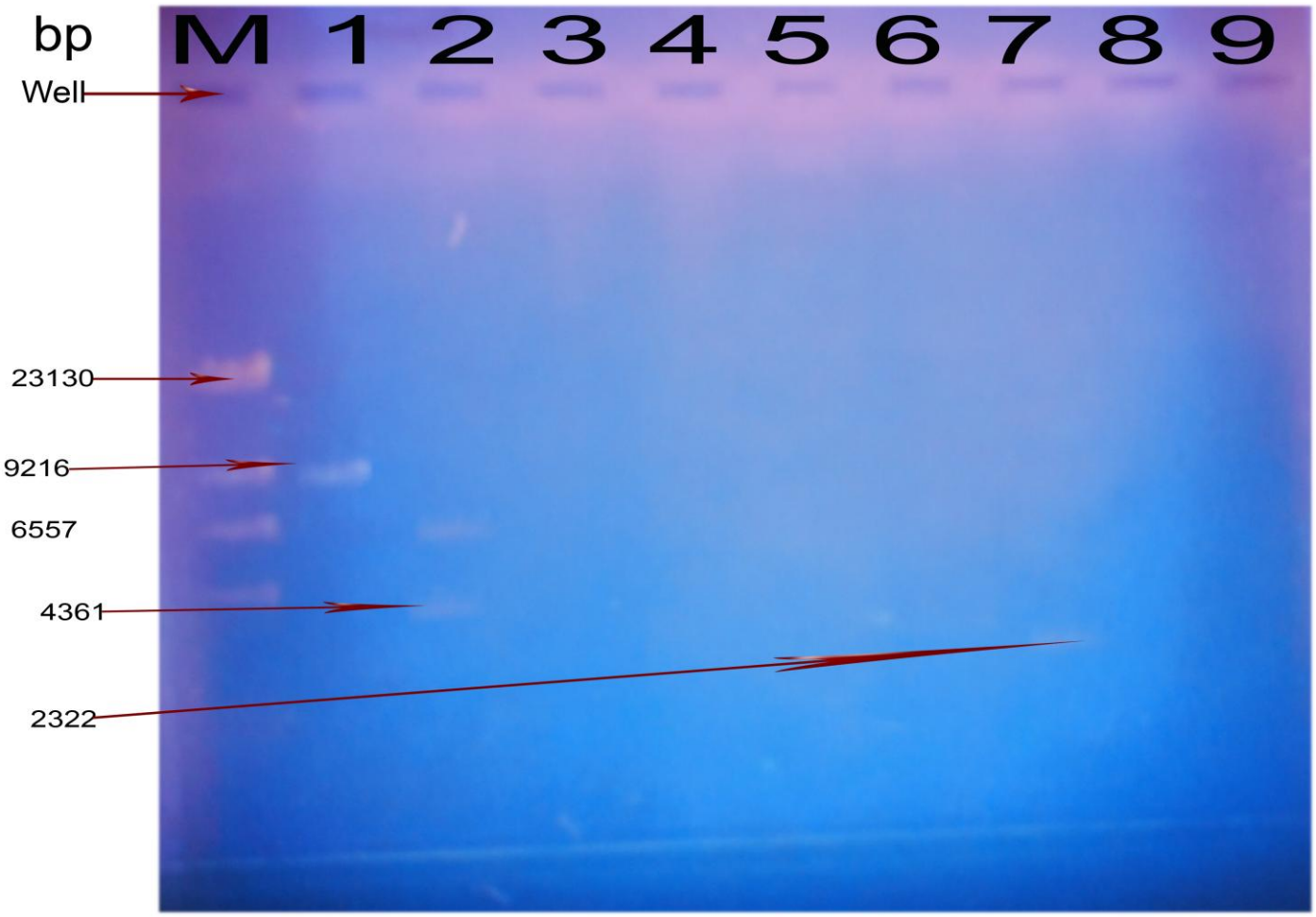

Figure 49: The Image of gel electrophoresis of Plasmid DNA from the FQREC isolates from the first group of healthy volunteer urine specimen.

Key: Lane M = HIND III Marker
Lanes 1- 9 = Plasmid DNA from the FQREC isolates from healthy volunteer urine specimen.

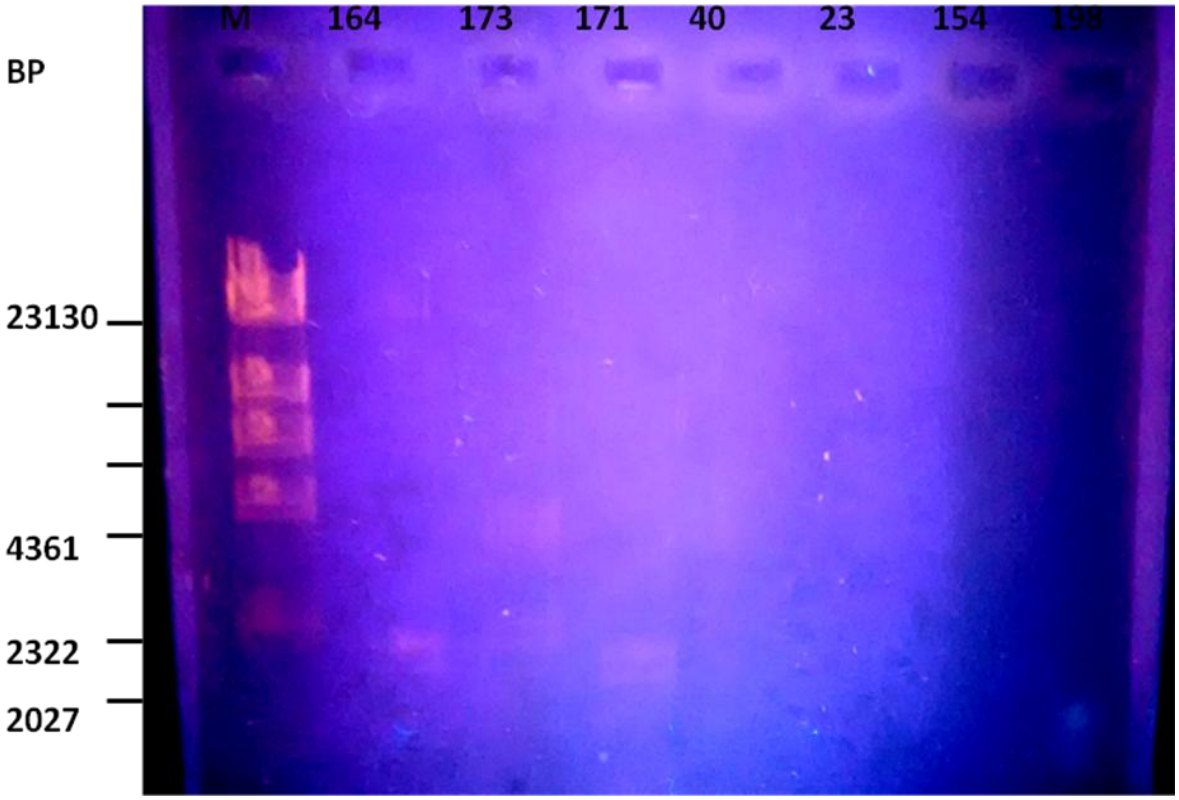

Figure 50: The Image of gel electrophoresis of Plasmid DNA from the FQREC isolates from healthy volunteer urine specimen. Lane 164 has one positive band of size 2001bp, In lane 173 the isolate has 2 positive bands of sizes 4031 bp and 2322 bp respectively, lane 171 shows plasmid bands corresponding to 2122 bp Lanes 40, 23, 154 198, had no plasmid bands

Key: Lane M = HIND III Marker.

Lane 164,173,171,40= Plasmid DNA from the FQREC isolates from urine specimen of healthy volunteer (age:6-18 yrs) urine specimen

Lane 23,154 and198 = Plasmid DNA from the FQREC isolates from urine specimen of healthy volunteer (age:19-60 yrs) urine specimen

bp

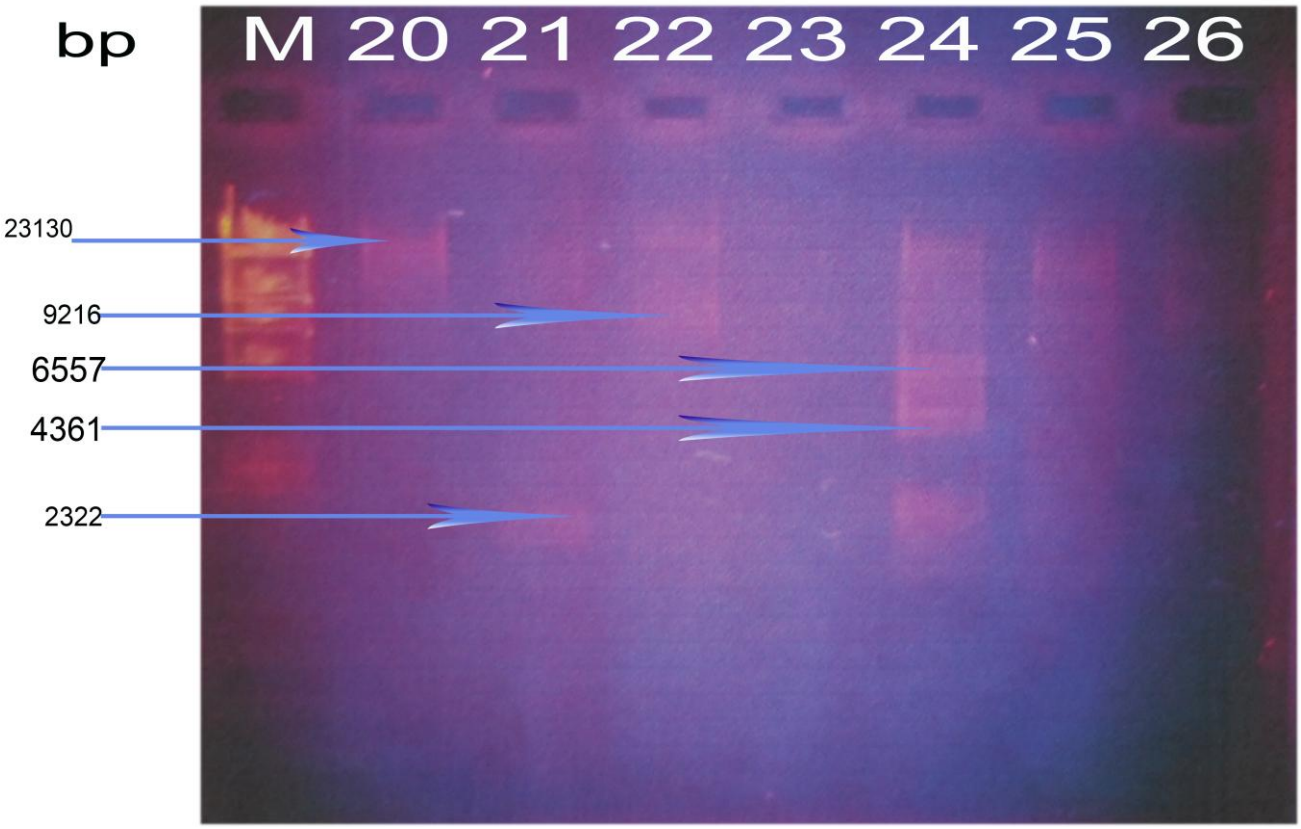

Figure 51: The Image of gel electrophoresis of Plasmid DNA from the FQREC isolates tested .

Key: Lane M = HIND III Marker

Lane 20- 26 = Plasmid DNA from the FQREC isolates from healthy volunteer stool specimen.

Apart from Lanes 23 and 26 that had no plasmid, other lanes possessed one or more plasmids.

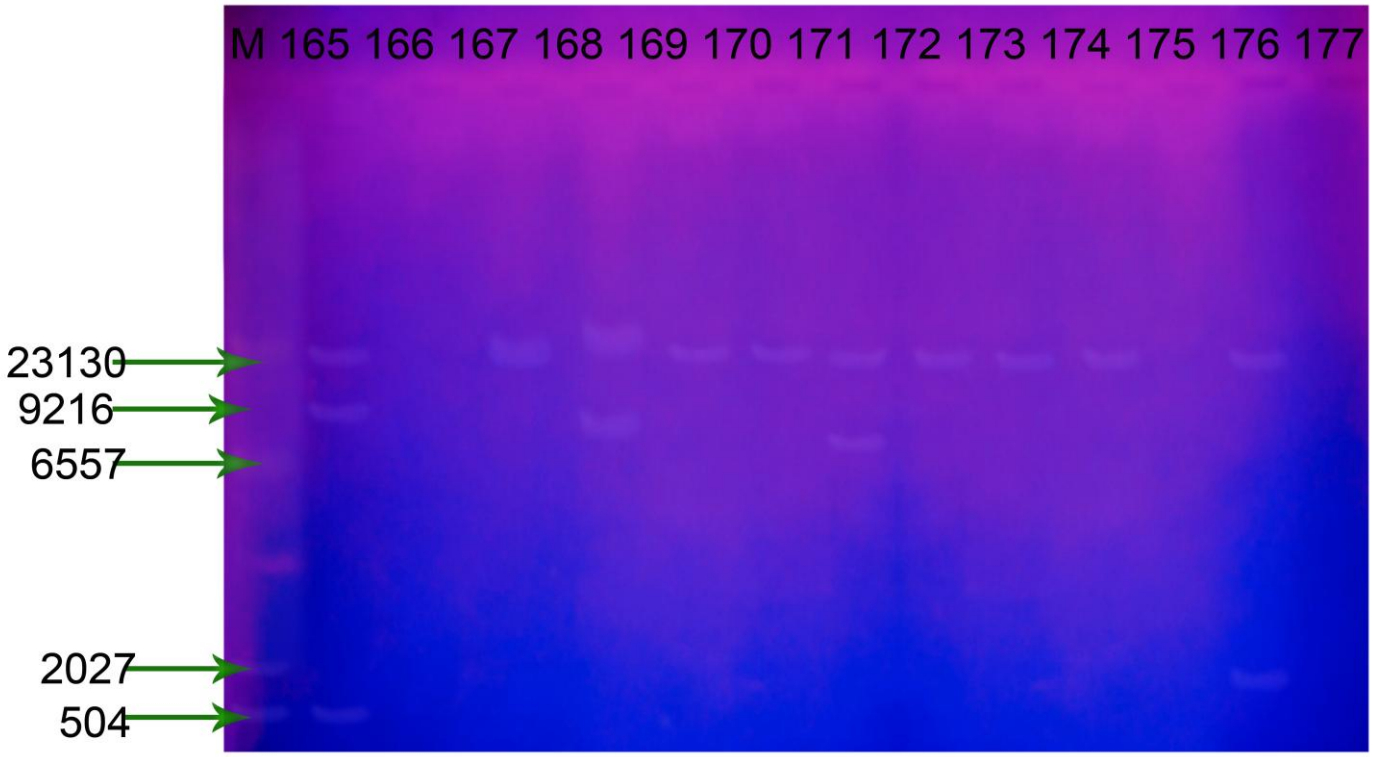

Figure 52 : The Image of gel electrophoresis of Plasmid DNA from the urinary FQREC isolates tested.

Key: Lane M = HIND III Marker

Lanes 165- 177 = Plasmid DNA from the FQREC isolates from the patients' urine specimen.

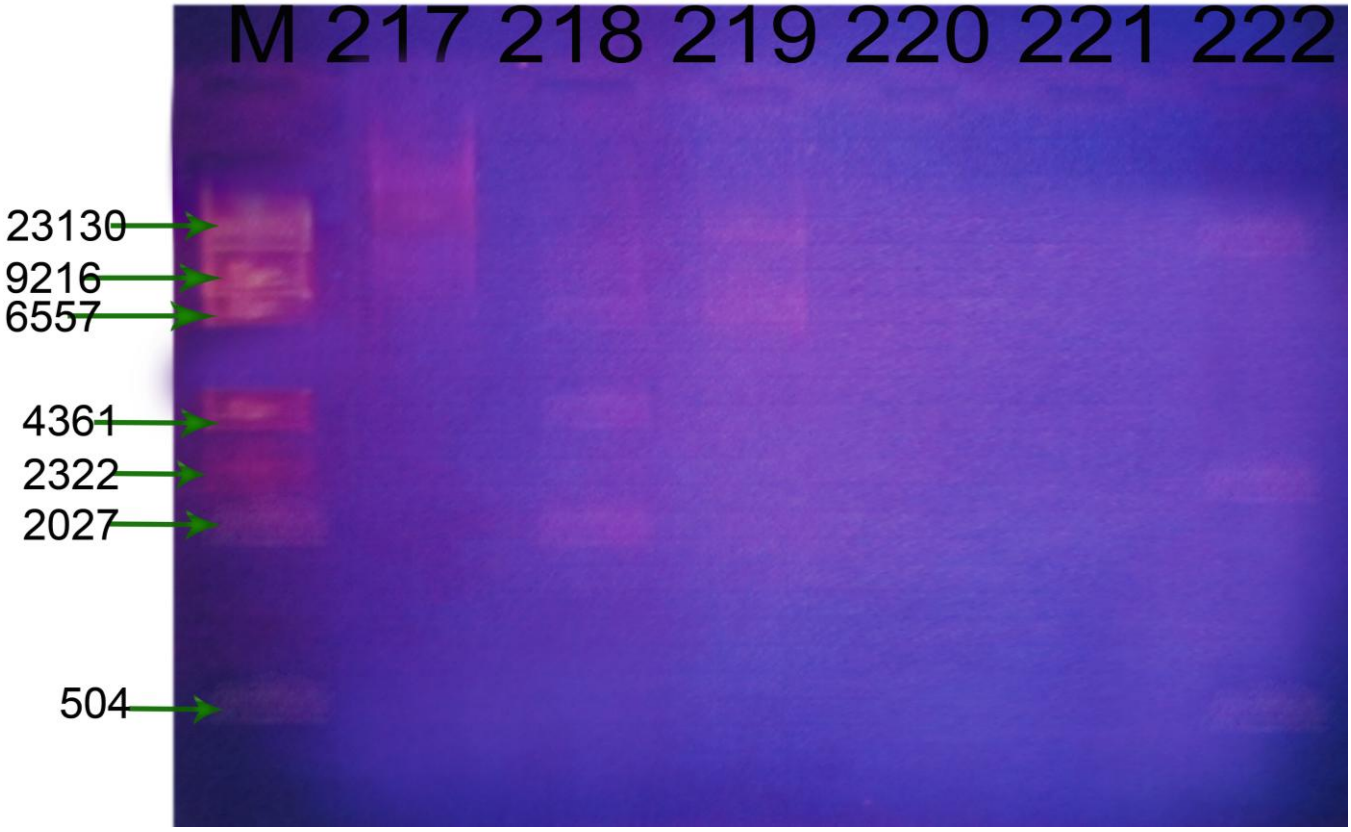

Figure 53: The Image of gel electrophoresis of Plasmid DNA from the wound FQREC isolates tested.

Key: Lane M = HIND III Marker

Lanes 217-222= Plasmid DNA from the FQREC isolates from wound swab specimen.

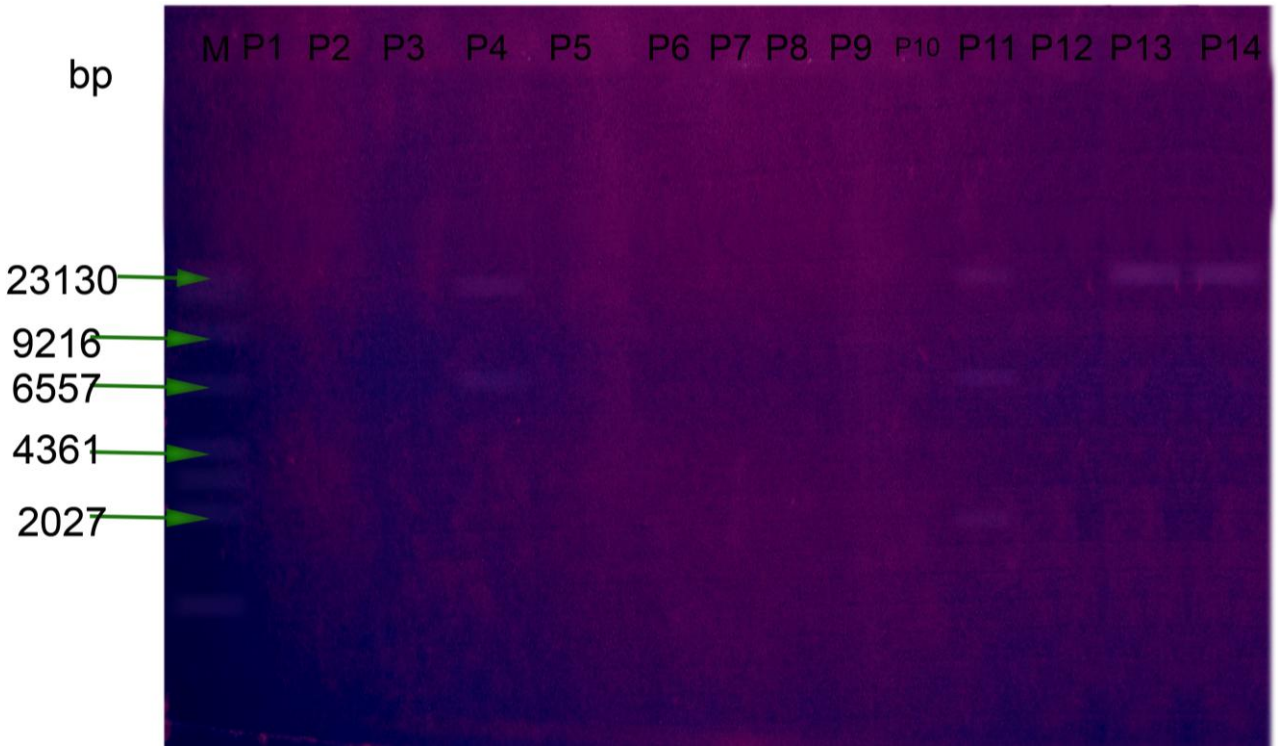

Figure 54: The Image of gel electrophoresis of Plasmid DNA from the Pig FQREC isolates tested.

Key : Lane M = HIND III Marker.
Lanes P1-P14 = Plasmid DNA from the FQREC isolates from pig in the study area.

Figure 55 : The Image of gel electrophoresis of Plasmid DNA from the FQREC isolates from chicken in the study area.

Key: Lane M = HIND III Marker
Lanes C11-C20 = DNA from the FQREC isolates from chicken

4. 5. 6. PLASMID CURING EXPERIMENT

In the plasmid curing experiment with acridine orange, the percentage of plasmid carrying FQRSA isolates cured are shown in Table 333. In FQRSA isolates from urine and nasal swabs of patients, 93.1 and 92.9% of the plasmids were cured respectively. In healthy volunteers, the percentage of plasmid cured in FQRSA isolates from human urine and nasal swab were 36.4 and 73.3% respectively. The FQRSA isolates from wound swab had 76.9% of the plasmid cured. From the results of the plasmid curing experiment in FQRSA isolates from human, it is evident that the greater percentage of fluoroquinolone resistance is plasmid –mediated.

In animals, similar results were obtained as all the plasmid carrying FQRSA isolates from cattle and chicken were cured (% plasmid cured =100%) while 66.7% of those of the pig isolates were cured. Similarly, in FQREC isolates 40- 80% of the plasmids were cured depending on the source of the isolates (Table 34). These results confirm the contribution of plasmid in mediating fluoroquinolone resistance in both FQREC and FQRSA isolates.

Table 33: Percentage of the Plasmid cured from the FQRSA according to Specimen source.

Specimen Plasmid	**No of isolates with Plasmid**	**Number (%) of cured**
HVNs	30	22(73.3)
PNs	14	13(92.9)
Hvus	11	4(36.4)
PSU	29	27(93.1)
PWS	13	10(76.9)
Pig	6	4(66.7)
Cattle	9	9(100)
Chicken	4	4(100)

Table 34 Percentage of the plasmid cured on the FQREC according to Specimen source.

Specimen Plasmid	**No of isolates with Plasmid**	**Number (%) of cured**
HVUS	10	4(40.0)
PSU	20	16(80)
HVFS	20	13(65.0)
PFS	31	23(74.2)
PWS	12	8(66.7)
Pig	7	3(42.9)
Chicken	10	7(70.0)

4.6 Result of Conjugation Experiment

Ten transconjugants were successfully obtained from the 35 *qnrA* – positive FQREC used as donors in conjugation experiments (Appendix 10). The MIC of ciprofloxacin for the ten transconjugants ranged from 0.5µg/ml to 2µg/ml and this is equivalent to 16 to 64 fold higher than that for the recipient bacteria *E. coli* BL21 (with GenBank accession number CP010816.1) with MIC of 0.03 µg/ml. All the transconjugants acquired decreased sensitivity to the fluoroquinolones tested: ciprofloxacin (MIC range 0.5 µg/ml), ofloxacin (MIC range 0.5 µg/ml to 2mg1ml), levofloxacin 4 µg/ml) and pefloxacin (0.25 µg/ml to 4 µg/ml); these MICs are 16 to 64 fold, 8 to 32 – fold, 2 to 64 – fold and 2 to 32 fold the MIC for the preconjugated recipient *E. coli* (0.03, 0.06, 0.06 and 0.13 µg/ml respectively). For non-fluoroquinolone antibiotics tested, some transconjugants did not show decreased susceptibility to some non fluoroquinolone antibiotics used i.e, gentamicin (MIC range: 1 to 4 µg/ml), amoxicillin (16 to 64 µg/ml) cefriaxone (0.5 to 4 µg/ml), and doxycyline (16 to 128 µg/ml); these MICs are 2- to – 8 fold, 2 – to 4 - fold, 2- to 32 – fold and 0- to 8- fold of the MIC of the preconjugated recipient *E. coli* (0.05 -16, 0.25 and 16 µg/ml respectively. In FQRSA conjugation experiments, PMQR were transferred from six (16.7%) isolates (Appendix 11). The range of minimum inhibitory concentrations of the test fluoroquinolones for these six transconjugants increased from 0.25 µg/ml to 2 µg/ml), which was 4- to 32 – fold that of the recipient *Staphylococcus cohnii subsp urealyticium* bacteria. All the transconjugants conferred decreased susceptibility to the fluoroquinolones tested; ciprofloxacin (MIC range 0.5 to 2 µg/ml), levofloxacin (0.25 to 1 µg/ml) ofloxacin (range 0.5 to 1 µg/ml) and pefloxacin (MIC range 0.25 to 1 µg/ml); these MICs are 8 to 32- fold, 4 – to 16 – fold, 8 to 16 fold and 4- to 16 fold the MIC for the preconjugated recipient bacteria, *S. cohnii subsp urealyticum*.

Similarly, for non-fluroquinolone antibiotics tested, all the transconjugants showed decreased susceptibility to the test antibiotics; gentamicin (MIC range 1 µg/ml), ceftriaxone (MIC range 2 to 8 µg/ml) and doxycycline (MIC range 16 to 64 µg/ml); these MICs are 2 – to 16 – fold, 4 to 16 fold, 8 to 32 – fold and 2 to 8 – fold the MIC of the preconjugated recipient bacteria- *S. cohnii subsp urealyticum* (0.5, 2, 0.25 and 8 µg/ml respectively).

4.7 EFFECT OF COMBINATION OF EFFLUX PUMP INHIBITOR – OMEPRAZOLE- AND CIPROFLOXACIN ON THE SUSCEPTIBILITY OF FQRSA AND FQREC

In a first step of screening, the disk diffusion method was used to identify the optimal concentration of omeprazole, and to detect any efflux pump inhibitory (EPI) activity of the tested agent. The FQRSA and FQREC isolates exhibited reduced inhibition zones towards fluoroquinolones, particularly ciprofloxacin, compared to fluoroquinolone susceptible isolates. At concentrations less than 32 µg/ml, omeprazole had no effect on ciprofloxacin activity, while at 128 µg/ml, the effect was clear as there was statistically significant difference ($P< 0.5$) in the IZD produced at different omeprazole concentrations. (Tables 35 and 36). Thus, the latter concentration was subsequently used for the agent (omeprazole) in order to maximize the chance of observing an effect. Actually, at 128 µg/ml, omeprazole increased the inhibition zones (IZD) of the fluoroquinolones, particularly ciprofloxacin against FQRSA while for FQREC, the effect on the IZD of the fluoroquinolones, particularly ciprofloxacin was evident at 64 µg/ml.

In a second step of screening, these data were confirmed by MIC determinations. The FQRSA and FQREC isolates tested exhibited ciprofloxacin MICs that were higher than those of fluoroquinolone susceptible isolates, and the presence of each omeprazole at 128 µg/ml resulted in a reduction in the ciprofloxacin MIC (2- to 16-fold) for FQRSA (Table 38) and for FQREC omeprazole at 64 µg/ml, the MIC values were increased for the most of the isolates (Table 37) .

Table 35: *In vitro* activities of fluoroquinolone in the presence of Omeprazole (efflux pump inhibitor) by agar diffusion method against FQREC

FQREC Source	Omeprazole conc (µg/ml)	IZD± SEM (mm) CPX	OFX	LEV	PEF
UFQREC	0	10±0.00[a]	4.0 ±2.0[a]	7.67±088[a]	0.00
	32	6.00±00[b]	2.00±2.00[b]	8.00±0.58[a]	0.00
	64	0.00±00	0.00±00	2.00±2.0	0.00
	128	0.00±00	0.00±00	2.00±20	0.00
FFQREC	0	9.00±0.58[a]	10.33±0.33[a]	10.33±0.33[a]	6.00±0.00[a]
	32	9.33±0.33[a]	10.00±0.00[a]	6.67±0.67[b]	6.33±0.33[a]
	64	7.67±0.33[b]	8.33±0.33[b]	6.00±0.00[b]	0.00±0.00[b]
	128	7.67±0.33[b]	8.67±0.33[b]	6.00±0.00[b]	0.00±0.00[b]
WFQREC	0	0.00±00	0.00±0.0	2.00±2.00	0.00±0.00
	32	0.00±00	0.00±0.0	0.00±0.0	0.00±0.00
	64	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.00
	128	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.00
PFQREC	0	10.67±0.67[a]	9.67±0.33[a]	8.33±0.33[a]	6.33±0.00[a]
	32	10.00±0.58[a]	9.67±0.33[a]	8.33±0.33[a]	2.00±2.00[b]
	64	6.00±0.00[b]	8.33±0.67[a]	8.00±00[a]	0.00±0.00
	128	6.00±0..00[b]	7.33±0.88[a]	8.00±00[a]	0.00±0.00
CH FQREC	0	7..33±0.88[a]	7.00±0.58[a]	6.67±0.67[a]	7.33±0.33[a]
	32	6.00±0.00[a]	2.00±2.00[a]	6.00±0.00[a]	0.00±0.00[b]
	64	0.00±0.00[b]	0.00±0.00	0.00±0.00	0.00±0.00
	128	0.00±0.00[b]	0.0±0.0	0.0±0.0	0.00±0.00

Different Superscripts in a row indicate significant differences between the groups ($p<0.05$).

Keys: a b and c = Levels of significance
UFQREC, Urinary FQREC, FFQREC = Faecal FQREC, WFQREC – Wound FQREC, PFQREC = pig FQREC, = chicken FQREC

Table 36: In vitro activities of fluoroquinolone in the presence of Omeprazole (efflux pump inhibitor) by agar diffusion method against FQRSA

FQRSA Source	Omeprazole conc (µg/ml)	IZD± SEM (mm) CPX	OFX	LEV	PEF
UFQRSA	0	8.67±0.33^{a}	10.00 ±0.00^{a}	7.67±0.33^{a}	4.00 ±2.00^{a}
	32	9.67±0.33^{a}	12.33±0.33^{a}	9.33±0.33^{a}	7.00 ±1.00^{a}
	64	15.67±0.33^{b}	16.67±0.88^{b}	13.67±1.45^{b}	11.00 ±2.33^{c}
	128	19.33±0.67^{c}	17.67±0.88^{c}	18.00±1.15^{c}	15.00 ±1.53^{d}
nFQRSA	0	4.00±2.00^{a}	7.33±0.67^{a}	10.00±1.00^{a}	2.00±2.00^{a}
	32	11.67±0.67^{b}	11.00±0.58^{a}	11.00±0.00^{s}	4.67±2.40^{a}
	64	14.67±0.67^{c}	15.33±1.45^{c}	14.67±1.33^{b}	10.00±0.00^{b}
	128	21.33±0.67^{d}	18.67±0.88^{d}	20.00±1.15^{c}	13.3±00^{c}
WFQRSA	0	4±2.0^{a}	2.00±2.00^{a}	6.33±0.33^{a}	0/00±0.00^{a}
	32	6.0±0.0^{a}	2.0±2.0^{a}	6.67±0.67^{a}	0.00±0.00^{a}
	64	9.0±0.58^{b}	5.0±2.60^{a}	6.67±0.67^{a}	0.00±0.00^{a}
	128	15.33±0.67^{c}	7.33±1.33^{a}	7.67±0.88^{a}	7.00±057^{b}
PFQRSA	0	10±0.00^{a}	11.33±0.67^{a}	9.67±0.33^{a}	6.33±0.33^{a}
	32	11.33±0.67^{a}	12.33±0.33^{a}	10.00±00.0^{a}	7.33±0.67^{a}
	64	15.33±0.33^{b}	15.67±0.33^{b}	13.00±0.00^{b}	7.66±0.88^{a}
	128	15.67±0..33^{b}	15.67±0.33^{b}	13.33±0.33^{a}	7.66±0.88^{a}
CH FQRSA	0	0.0±0.0	0.0±0.0	2.0±2.0	0.0±0.0
	32	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	64	2.0±0.0^{a}	0.0±0.0^{a}	4.0±2..0	0.0±0.0
	128	6.0±0.0^{b}	7.33±0.67^{b}	7.67±0.88^{b}	0.0±0.0
Cat FQRSA	0	967±0.33^{A}	4.0±2.0	8.67±0.33^{a}	4.0±2.0^{a}
	32	11.67±0.33^{b}	8.67±0.33^{b}	10.33±0.33^{b}	6.0±0.0^{a}
	64	13.67±0.33^{c}	12.33±0.33^{b}	13.33±0.67^{c}	7.0±0.58^{a}
	128	17.33±0.33^{d}	13/67±0.67^{b}	19.33±0.88^{b}	12.0±0.0^{b}

Different Superscripts in a row indicate significant differences between the groups ($p<0.05$). Keys: a b and c = Levels of significance .UFQREC, Urinary FQRSA, nFQRSA = Nasal FQRSA, WFQRSA – Wound FQRSA, PFQRSA = pig FQRSA, = CH FQRSA = chicken FQRSA. Cat FQRSA = Cattle FQRSA

Table 37 : MIC values of Ciprofloxacin alone and its combination with Omeprazole against FQREC.

	MIC ± SEM (ug/ml)	
Source	Ciprofloxacin	Cipro + omeprazole
HVUS	25.58 ± 9.10	46.86 ± 19.95
PSU	123.89 ± 22.50	165.89 ± 26.10
HVSS	66.89 ± 55.67	82.89 ±33.83
PSS	133.37 ± 30.40	177.43 ± 33.91
PWS	93.60 ± 20.36	136.00 ± 22.34
AHC	21.80 ± 6.31	35.40 ± 13.37
AHP	20.29 ± 8.68	31.14 ± 11.31

Key;

HVUS = Healthy Volunteer urine Specimen

PSU = Patient Specimen of Urine

HVSS = Healthy Volunteer Stool Specimen

PSS = Patient specimen of stool

PWS = Patient wound swab

AHP = Apparently healthy pig

AHC = apparently healthy chicken

Table 38 : MIC values, ug/ml of ciprofloxacin alone and in the presence of omeprazole against FQRSA

	MIC ug/ml	
Source	**Cipro**	**Cpx + ome**
HVns	26.92 ± 10.56	10.61 ± 4.88
PNS	17.25 ± 4.03	5.47 ± 1.52
HVUS	46.99 ± 13.30	14.48 ± 4.94
PSU	65.30 ± 19.1	20.92 ± 7.41
PWS	15.71 ± 2.27	30.16 ± 8.30
AHP	15.33 ± 9.77	6.67 ± 1.14
CAT	8.89 ± 3.18	3.19 ± 1.67
AHC	8.00 ± 2.19	1.80 ± 0.20

Key;

HVns = Healthy Volunteer nasal Specimen

PNS = Patient Nasal Swab

HVUS = Healthy Volunteer urine Specimen

PSU = Patient Specimen of Urine

PWS = Patient wound swab

AHP = Apparently healthy pig

AHC = apparently healthy chicken

CAT = apparently healthy cattle

4.8 Chemical composition of EO of Lemongrass and Coconut oil

GC-MS analysis of the essential oil from the leaves of *Cymbopogon citratus* spf. identified sixteen constituents representing 100% of the extracted oil (Table 39). The main constituents of the oil were found to be beta-citral (Neral) (30.20%), alpha-citral (geranial) (8.45%), Nerolic acid (8.25%) and Yomogi alcohol (10.76%). Other notable representative compounds were detected as Geranic acid (5.29%), lemonol (4.25%), Hemellitol, (3.87%), Shellsol 140 (3.8%) and Paraxylene (5.19%) while the remaining components occur at a very low level (<2%). Similarly, the chemical constituents of coconut oil as shown by GC-MS analysis (Table 40), revealed presence of nineteen (19) components of which Oleicacid (19.9%), paraxylene (11.47%), shellsol 140 (7.56%), Docosenoic acid (7.8%) and Hendecane (9.01%) were found to be major fractions. Other constituents (>3% abundance) are Oktanenen (4.59), cyclogeraniolane (4.49%), cyclohexylpropanol (3.34%), normanthane (5.41%), Cumol (3.04%) and stearic acid (4.42%). Lauric acid (1.22%) and the remaining components other occur as trace constituents.

Table 39: Composition of essential oil in *Cymbopogon citratus*

S/N	Essential oil composition	Percentage of components	RT (min)
1	Oktanen	1.91	3.693
2	Ethylcyclohexane	1.58	4.193
3	Para-xylene	4.19	4.677
4	shellsol 140	3.83	5.034
5	Hemellitol	3.87	6.573
6	Linalool	1.06	8.193
7	Citronellene	0.80	9.379
8	B-citral (Neral)	30.20	10.350
9	Lemonol (Nerol)	4.25	10.527
10	α-citral (Geranial)	8.45	10.748
11	Prenderol	1.65	10.995
12	Nerolic acid	8.25	11.05
13	Geranic acid	5.29	12.121
14	Yomogi alcohol	10.76	12.198
15	3,7- Dimethyl 2,6- octadienoic acid	2.29	22.746
16	2-Octene, 2-methyl-6-methylene	1.3	22.825

Table 40: Chemical Composition of Coconut oil

S/N	Coconut oil composition	Percentage of components	RT min)
1	Oktanen	4.59	3.692
2	Ethycyclohexane	3.96	4.191
3	Cyclogeraniolane	4.49	4.240
4	TrimethylCyclohexane	3.00	4.440
5	Para-xylene	11.47	4.676
6	Shellsol 140	7 .56	5.035
7	Ethy 4-methylcyclohexane	2.27	5.225
8	Cyclohexyl-propanol	3.34	5.437
9	Normanthane	5.41	5.570
10	Pseudocumene	2.51	6.175
11	Hendecane	9.01	6.571
12	Cumol	3.04	7.049
13	n-Decane	1.15	8.128
14	Lauric acid	1.22	15.043
15	Myristic acid	1.70	18.513
16	Palmitic acid	3.17	20.983
17	Oleic acid	19.9	21.814
18	Hydrofol acid(Stearic acid)	4.42	22.08
19	Docosenoic acid	7.8	22.753

4.9 Sensitivity of the test isolates to the two oils

The preliminary sensitivity test was done with both essential oil of *Cymbopogon citratus* and coconut oil against fluoroquinolone susceptible (FQS-) *S. aureus and E. coli* isolates in order to establish their antibacterial potentials , first with these isolates and then with the FQ- resistant isolates. The sensitivity results of the two oils are shown in Tables 41 and 42. For both the human and animal *S. aureus* isolates, the IZD values ranges from 10.33 mm to 38.99 mm while for *E.coi* isolates, the IZD ranges from 9.11 mm to 34.22 mm depending on the concentration of the EO used. These are high IZD values and the result showed that the essential oil of *Cymbopogon citratus* has a good antibacterial activity against both fluoroquinolone susceptible *S. aureus* and *E. coli* isolates. For the coconut oil tested, the IZD for *S. aureus* ranged from 0.0 mm – 9.66 mm while for *E. coli* isolates (Table 42), no activity was detected (zero IZD recorded) with all the concentrations used including the undiluted oil. This means that the antibacterial activity against the two isolates (*S. aureus* and *E.coli*) was poor.

The IZD $\pm$ SEM mm produced by the essential oil of *Cymbopogon citratus* (lemon grass) and ciprofloxacin against FQRSA and FQREC are shown in Tables 43 and 44 respectively. Promising IZDs were obtained with the EO concentrations of ≥ 6.25 mm against both FQRSA and FQREC. The MIC of this EO ranged from 0.010 – 0.048 % and 0.069 – 0.140 % against FQRSA and FQREC respectively while the MBC values ranged from 0.01 - 0.094 % and 0.11 – 0.160% against FQRSA and FQREC isolates respectively. The coconut oil did not show any antibacterial activities against both FQRSA and FQREC.

Table 41: IZD produced by the essential oil of *Cymbopogon citratus* (lemon grass) with Ciprofloxacin against FQS-*S. aureus* and *E.coli* isolates.

Specimen source	**IZD (mm)**				**Ciprofloxacin (5µg)**
	25	12.5	6.24	3.13	5
Human *S. aureus* isolates	34	22	18	10	30
Animal *S. aureus* isolates	38	30	26	19	40
Human *E.coli* isolates	30	26	20	9	33
Animal *E.coli* isolates	34	27	21	15	35

Table 42: IZD produced by the coconut oil and Ciprofloxacin against FQS-*S. aureus* and -*E.coli* isolates.

Specimen source	**IZD (mm)**				**Ciprofloxacin (5µg)**
	100	50	25	12.5	
Human *S. aureus* isolates	7	6	2	0	29
Animal *S. aureus* isolates	7	4	0	0	30
Human *E.coli* isolates	0	0	0	0	28
Animal *E.coli* isolates	0	0	0	0	29

Table 43: The IZD± SEM mm produced by the essential oil of *Cymbopogon citratus* (%) and Ciprofloxacin against FQRSA.

Specimen source	**IZD mm**				**Ciprofloxacin (5µg)**
	50	25	12.5	6.25	5
HVns	36.33±2.10	27.56 ± 2.31	19.33 ± 1.72	10.56 ± 1.88	6.11 ± 1.64
PNS	28.9 ± 2.78	15.82 ± 2.08	9.64 ± 1,22	4.64 ± 1.13	6.75 ± 1.46
HVUS	31.6 ± 2.29	16.40 ±1.17	9.00 ± 0.45	4.00 ± 1.67	6.20 ±2.65
PSU	27.78 ±2.05	18.43 ± 1.74	10.07 ± 1.16	3.79 ± 0.94	6.14 ± 1.36
PWS	33.17 ± 2.23	19.61 ± 1.59	10.11± 0.83	4.33 ± 0.76	5.56 ±1.16
AHP	30.00 ± 2.16	15.50 ± 1.44	9.0 ± 0.82	6.23 ± 0.50	11.25 ± 0.47
CAT	33.86 ± 4.28	20.86 ± 2.40	13.28 ±2.30	5.71 ± 2.24	6.14 ± 2.25
AHC	39.00 ± 2.12	24.5 ± 2.40	14.5 ± 1.7	7.50 ± 0.95	8.75 ± 1.10

Key;

HVns = Healthy Volunteer nasal Specimen

PNS = Patient Nasal Swab

HVUS = Healthy Volunteer urine Specimen

PSU = Patient Specimen of Urine

PWS = Patient wound swab

AHP = Apparently healthy pig

AHC = apparently healthy chicken

CAT = apparently healthy cattle

Table 44: IZD (mm ± SEM) produced by the essential oil of lemon grass (%) and Ciprofloxacin against FQREC.

Specimen source	**IZD (mm)**				**Ciprofloxacin (5µg)**
	50	25	12.5	6.25	5
HVUS	25.37 ±1.79	16.0 ± 1.30	9.10 ± 1.09	4.57 ±0.97	4.74 ± 1.21
PSU	25.36 ± 1.20	14.68 ± 0.93	6.88 ±0.85	2.84 ± 0.72	5.7 ± 0.95
HVSS	22.36 ± 1.64	11.00 ± 1.14	6.29 ± 0.98	2.886 ± 0.93	8.57 ±0.63
PSS	25.53 ± 1.78	14.93± 1.12	9.8 ± 0.80	4.67 ± 1.0	8.2± 1.0
PWS	23.80 ±1.77	14.20 ± 1.74	9.6 ± 0.85	4.0 ± 1.37	5.1 ± 1.6
AHP	27.6± 2.70	15.5 ±1.79	9.7 ±1.64	4.9 ± 1.3	5.1 ± 1.70
AHC	25.83 ± 3.22	14.67 ±0.88	7.8 ± 1.62	4.8 ±1.5	5.67 ± 1.9

Key; HVUS = Healthy Volunteer urine Specimen
PSU = Patient Specimen of Urine
HVSS = Healthy Volunteer Stool Specimen
PSS = Patient specimen of stool
PWS = Patient wound swab
AHP = Apparently healthy pig
AHC = apparently healthy chicken

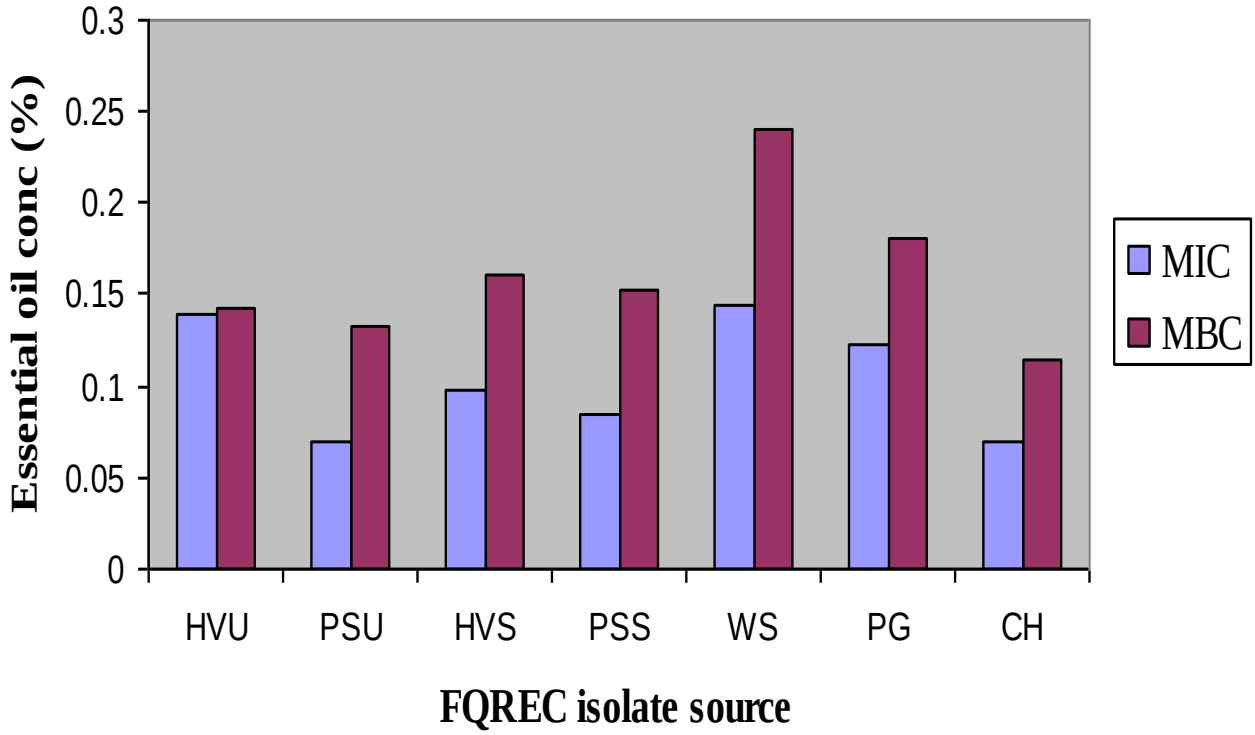

Fig 56: The mean MIC and MBC of essential oil of *Cymbopogon citratus* against fluoroquinolone resistant *E coli* (FQREC) isolates from both humans and animals.

Key; HVU= Healthy Volunteer urine Specimen ,PSU = Patient Specimen of Urine HVS = Healthy Volunteer Stool Specimen ,PSS = Patient specimen of stool, WS =Patient wound swab. AHP = Apparently healthy pig, CH = apparently healthy chicken

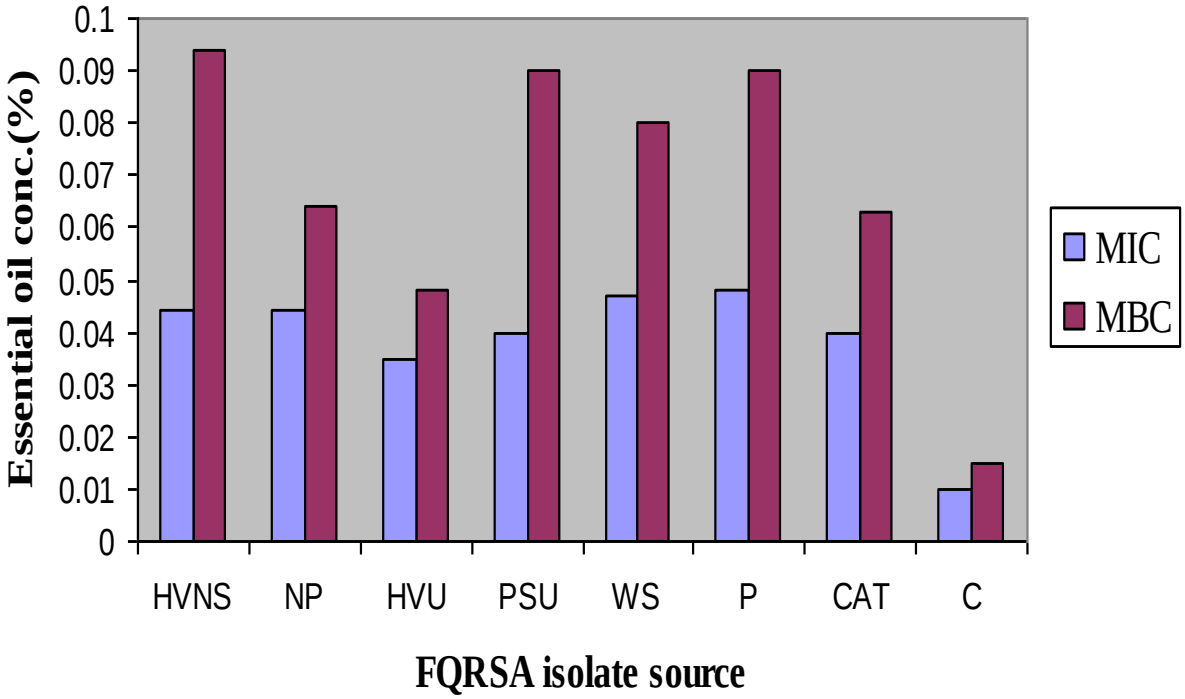

Fig 57: The mean MIC and MBC of essential oil of *Cymbopogon citratus* against FQRSA isolates from both humans and animals.

4.10 Combined effect of EO of lemongrass and ciprofloxacin against FQREC and FQRSA

The interactions of EO lemongrass and ciprofloxacin against FQREC and FQRSA were studied using checkaboard techniques and the results are shown **(Tables 46 and 47).** From the result, 53.9, 28.3 and 17.8% of the FQRSA isolates showed indifference, additive and synergistic effects respectively, when ciprofloxacin was combined with EO of lemongrass at different ratios. The synergistic and additive effects were more at ratios when the ciprofloxacin is more than the oil ie cpx: oil ratio of 9:1-6:4. There was no antagonistic effect recorded with the isolates with respect to the two agents combined together..

For FQREC isolates, 44.4, 35.5 and 20.6% of the isolates showed combined effect of additivity, synergism and indifference respectively when ciprofloxacin was combined with EO of lemon grass. Zero (0%) antagonism was also recorded with these FQREC (like FQRSA) isolates above.

The MIC studies confirmed that the coconut oil has no activity against FQRSA and FQREC isolates. Coconut did not show any inhibition of growth at concentration up to 5%. The results of the combined activity of the antibiotics with oil of *Cocos nucifera* are shown in **Tables 47 to 48.** At concentrations of 0.5 and 2%, the oil increased the MIC of ciprofloxacin against both FQREC and FQRSA isolates. The number of folds of increase in the MIC depends on the strain tested, but for FQREC, the range was between 4-32 times (for 0.5%) and 16-32 times (for 2%) respectively. For FQRSA, coconut oil at 0.5 and 2% increased the MIC of ciprofloxacin against FQRSA by 2-4 times and 4-8 times respectively. The combination of ciproflaxcin and

coconut oil resulted in statistically significant decrease in IZD ($P < 0.05$) at all the tested concentrations against FQREC and FQRSA. The results of the combined activity of ciprofloxacin and coconut oil against FQREC and FQRSA evaluated using thin overlay inoculum susceptibility disc (OLID) method are shown in Tables 48 and 49 respectively. In evaluation using this method, a 19% increase or more in IZD is usually taken as index of synergism, increment less than 19% increase in IZD produced additive effects while cases showing no variation in IZD had indifferent effects where as the decrease in IZD is taken antagonism. From the analysis, coconut oil exhibited antagonistic effect when combined with ciprofloxacin thereby further reducing the activity of ciprofloxacin.It is vital to note that there was a significant difference between the IZD at 0.5% and 2% concentration of oil when combined with ciprofloxacin. This implies that effect of oil is more at increased concentration of oil.

Table 45: Combined effects of Ciprofloxacin and lemon grass essential oil as determined by Checkerboard agar dilution against FQRSA (Fluoroquinolone resistant *S. aureus*).

		Number of isolates (%)			
Ratio		Synergism	Additive	Indifference	Antagonism
CPX	Oil	($\leq$0.5)^x	(>0.5-1)^x	(>1 – 2)^x	(>2)^x
10	0	-	-	-	-
9	1	0(0)	9(45.0)	11(55)	0(0)
8	2	1(5)	12(60)	7(35)	0(0)
7	3	4(20)	9(45.0)	7(35)	0(0)
6	4	2(10)	13(65)	5(25)	0(0)
5	5	4(20)	12(60)	4(20)	0(0)
4	6	5(25)	10(50)	5(25)	0(0)
3	7	6(60)	10(50)	4(20)	0(0)
2	8	4(20)	12(60)	4(20)	0(0)
1	9	6(30)	10(50)	4(20)	0(0)
0	10	-	-	-	-
Mean		17.8	53.9	28.3	0
Percentage (%)					

Key CPX = Ciprofloxacin
Oil = Essential oil from lemon grass
X fractional inhibitory Conc (FIC) Index

Table 46: Combined effects of ciprofloxacin and essential oil of lemongrass as determined by Checkerboard agar dilution against FQREC.

Combination ratio		Synergism (≤ 0.5)^x	Additive (> 0.5-1)^x	Indifference (>1 – 2)^x	Antagonism (>2)^x
CPX	Oil				
10	0				
9	1	3(15.0)	10 (50.0)	7(35.0)	0(0)
8	2	2(10.0)	11(55.0)	7(35.0)	0(0)
7	3	2(10.0)	10(50.0)	8(40.0)	0(0)
6	4	3(15.0)	9(45.0)	8(40.0)	0(0)
5	5	11(55.0)	7(35.0)	2(10.0)	0(0)
4	6	8(40.0)	10(50.0)	2(10.0)	0(0)
3	7	11(55)	9(45)	0(0)	0(0)
2	8	10(50)	8(40)	2(10)	0(0)
1	9	13(65)	6930)	1(5)	0(0)
0	10	-	-	-	-
Mean & Percentage		35%	44.4%	20.6%	0%

Key X = fractional inhibitory Concentration (FIC)

Table 47: MIC (mg/ml) of ciprofloxacin alone and in combination with oil of *Cocos nucifera* against FQREC.

FQREC Isolate	Ciprofloxacin Alone	Ciprofloxacin +0.5% oil	Ciprofloxacin+ 2% oil
1	32.0	512.0	512.0
2	8.0	256.0	256.0
3	4.0	128.0	128.0
4	32.0	128.0	512.0
5	4.0	32.0	64.0
6	32.0	128.0	256.0
7	16.0	64.0	128.0
8	8.0	64.0	128.0
9	8.0	128.0	256.0
10	8.0	256.0	256.0

Table 48: MIC (mg/ml) of ciprofloxacin alone and in combination with oil of *Cocos nucifera* against FQREC

FQREC Isolate	Ciprofloxacin Alone	Ciprofloxacin +0.5% oil	Ciprofloxacin+ 2% oil
1	32.0	64.0	128
2	16.0	64.0	128
3	32.0	64.0	64
4	8.0	32.0	32
5	16.0	32.0	128
6	16.0	32.0	32
7	16.0	32.0	64
8	32.0	64.0	128
9	8.0	16.0	32
10	4.0	8.0	8

Coconut oil showed no inhibition of the growth of both Staph and E.Coli at concentrations up to 5%.

Table 49: Combined activity (1ZD [MM] ±SEM) of Ciprofloxacin and Coconut oil against FQREC

Concentration (mg/ml)	Ciprofloxacin alone	Ciprofloxacin +0.5% oil	Ciprofloxacin +2% oil
25.0	16.7 ± 1.2^{a}	13.7 ± 0.3^{b} (-18.0)	9.7 ± 0.3^{c} (-41.9)
12.5	14.0 ± 0.6^{a}	11.0 ± 0.6^{b} (-18.0)	7.3 ± 0.3^{c} (-47.9)
6.25	9.00 ± 0.6^{a}	7.3 ± 0.3^{b} (-18.9)	5.3 ± 0.3^{c} (-41.1)
3.13	8.00 ± 0.0^{a}	5.3 ± 0.3^{b} (-33.8)	2.7 ± 1.3^{c} (-66.3)
1.56	6.33 ± 0.3^{a}	4.3 ± 0.3^{b} (-31.7)	1.3 ± 1.3^{c} (-79.4)
0.78	2.0 ± 2.0	0.0 ± 0.0 (-100)	0.0 + 0.0 (0.0)
0.39	0.0 +0.0	0.0 ± 0.0 (0.0)	0.0 + 0.0 (0.0)

Values in parentheses represent percentage decrease in IZD. Negative sign (-) indicates percentage decrease.

N.B : a,b and c represent levels of significance

Table 50 :Combined activity (IZD [MM±SEM]) of ciprofloxacin and coconut oil against FQRSA

Concentration (mg/ml)	**Ciprofloxacin Alone**	**Ciprofloxacin +0.5% oil**	**Ciprofloxacin +2% oil**
25.0	19.0 ± 0.6^{a}	11.7 ± 0.3^{b} (-38.4)	9.3 ± 0.3^{c} (-51.1)
12.5	15.7 ± 0.7^{a}	8.0 ± 0.6^{b} (-49.0)	6.0 ± 0.0^{c} (-61.8)
6.25	13.0 ± 0.0^{a}	5.3 ± 0.3^{b} (-59.2)	4.3 ± 0.3^{c} (-66.9)
3.13	8.0 ± 0.6^{a}	1.3 ± 1.3^{b} (-83.8)	$0.0 + 0.0^{c}$ (-100.0)
1.56	0.0	0.0	0.0
0.78	0.0	0.0	0.0

N.B : a,b and c represent levels of significance

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

5.1.1 Prevalent rate of *E coli* and *S. aureus* isolates.

Staphylococcus aureus is often found as a commensal associated with skin and mucus membranes of healthy carriers (Crossly, 1997). Asymptomatic carriage of *S. aureus* is especially common, particularly in the anterior part of the nasal cavity, where a prevalence rate of around 20% has been reported (Weidenmaier *et al*., 2012). In this study, *S. aureus* prevalence rates of 76.0% and 50.5% were obtained from human healthy carriers' anterior nares and urine specimens respectively. The isolation rates of *S. aureus* from patients nasal swab, wound swab and urine specimen were 45.5, 10.2 and 62.1% respectively.

In animals tested, the prevalence rates of *S. aureus* were 13.1% (pig), 9.5% (cattle) and 8.9% (chicken) respectively. In all these animals tested, the highest isolation percentage was obtained from specimen of meat/vendor's table pig (19.1%), cattle (15.1%) and chicken (14.9%) and the least percentage was obtained from skin swabs of pig (9.4%) and chicken (6.6%) and nasal swab of cattle (7.7%) respectively. Contamination of meat from the meat vendor's table and the meat sellers himself might have contributed to the increase in the isolation rate of *S. aureus* from the specimen.

Our findings on the prevalence of asymptomatic nasal carriage of *Staphylococcus aureus* in human which ranged from 31.7% - 76.0% (according to age of the healthy carriers) agree with the results of the work done by Weidenmaier *et al*., (2012). Similar data were obtained from *S. aureus* nasal colonization in the USA and UK which showed prevalence rates of 31.6% and 23% respectively (Graham *et*

al.,2006; Abudu *et al*., 2001). Our reports on the prevalence rates of *S. aureus* isolates from nasal cavity in children and adults are not in agreement with the results of the work done on Turkish children (17.3%) and Japanese adults (36%) (Soysal *et al*., 2006. Uemura *et al*., 2004). The reasons for these differences may be due to variations in isolation techniques, location, standard of living, period of isolation and antibiotic usage. The result of this work suggests that the isolation percentage of *S. aureus* from nare of healthy volunteer (76.0%) was greater than the isolation percentage of *S. aureus* from the patient nares (45.5%). The patients used for this study were recently or/and currently on antibiotics and these antibiotics might have reduced the population of occupant of the anterior nares in these patients. The isolation rate of *S. aureus* from chronic wound swab was 10.2%. The value is low when compared with the result of the work done by Badger-Emeka *et al.*, (2014). In our work we used patients with chronic wound who have been or currently on antibiotics. The isolation percentage may be higher if the patients were not on antibiotics. The differences observed in the prevalence rates between the specimen sources (nasal swab and urine) and the age ranges of the volunteers are statistically significant ($P < 0.05$). This means that there exists age related variation in the prevalent rate of *S. aureus* from asymptomatic healthy volunteers with the highest rate being found in the older subjects in the study area. An explanation to this disparity with respect to isolate from nares may be that as one ages, other things being equal (e.g in the absence of antimicrobial treatment), the total number of resident *S. aureus* occupant of the anterior nares as well as the transient occupant of the anterior nares increases. As per the specimen sources, the colonisation rate is significantly higher with the anterior nares than with the urogenital specimen. The high prevalence of nasal carriage of *S. aureus* got from this study further supports the fact that anterior

nares remains a principal reservoir of this organism and there is need to eliminate its virulent strains because of their participation in most severe community and hospital associated *S. aureus* infections in colonized individuals. The differences observed in the colonization rates between the groups of volunteers (pupils/students and villagers) and the sexes are not statistically significant (P <0.05). This shows that neither sex nor educational status is a risk factor for nasal colonization of *S. aureus* in the study area.

In this work, 1890 *E.coli* strains were isolated from non-duplicate samples of urine, stool and wound swab of human subjects. Out this total sum, 730 (57.9%) and 693 (82.50%) isolates were got from healthy volunteer and patient stool specimens respectively. For urine specimen, the isolation rates of *E.coli* in healthy volunteers and patient are 12.2 and 34.3% respectively. The isolation percentage of *E.coli* from chronic wound swab was 6.2%. The isolation percentage of *E.coli* in this study, was higher among patients than the healthy volunteer. The reason for this may be that the human urinary bladder is normally sterile and thus in asymptomatic healthy individuals the lower isolation rate of *E.coli* when compared to that of patients with urinary tract infection/cystitis is understandable.

In animals, the prevalent rate of *E.coli* in pig, cattle and chicken faeces specimens were 24.9, 19.4 and 23.7% respectively. These values were the greatest among the rates got from other specimen sources (nasal, skin and meat vendors table). This is understandable because *E. coli* is one of the most frequently encountered bacterial species in animal and human commensal intestinal flora (Rezvan, 2005).

Antibiotics susceptibility patterns and Prevalence of test FQREC and FQRSA Isolates.

The introduction of antibiotics was an important aspect of medical intervention that reduced both human and animal morbidity and mortality to appreciable low level. However, the intensive and inappropriate use of antibiotics (which was estimated in 2002 to be 100,000 – 200000 tonnes per annum (Andersson and Hughes, 2010), globally has increased the frequency of resistance among human and animal pathogens and threaten a loss of treatment options. In this study, we evaluated the antimicrobial susceptibility in both clinical and non clinical isolates of *Escherichia coli and Staphylococcus aureus* and compared them to commensal bacterial strains from some domestic animals. Because of the geographical sampling techniques used this surveillance provided a representative sample of the resistance trends in Enugu State of Nigeria.

For non fluoroquinolone antibiotics, all the animal and human *E. coli* isolates tested showed percentage resistance to gentamicin, ceftriaxone, amoxycillin, erythromycin and doxycycline in human, 24.0, 17.8, 84.7, 94.8 and 89.4% , in pig as 4.9, 2.9, 64.8, 73.0 and 63.5%, in cattle as 3.6, 1.8, 57, 90.5 and 64.9% and in chicken as 16.3, 10.9, 88.0, 96 and 87.1%) respectively(Tables 4 - 7). In our studies, high antibiotics resistance to erythromycin, amoxycilin and tetracycline were recorded among human and animal *E.coli* isolates. The reason for this may be as a result of inappropriate use of these antibiotcs in human as well as the use of them at subinhibitory concentration in animal feeds. Among these human and animal *E. coli* isolates, 89.4 and 83.8% from human, 63.5 and 86.3 % from pig, 64.9 and 83.3% from cattle, and 87.1 and 82.7% from chicken respectively were resistant to

tetracycline. The presence and high prevalence of tetracycline–resistant *E. coli* isolates from animals agree with findings of studies done elsewhere on antibiotic resistance in *E. coli* (Piddock, 2006; Van den Bogaard and Stobberingh 1999). In humans tetracyclines are common antibiotics got from patent drug vendors without formal prescription for the treatment of malaria, abdominal discomfort and other ailments by laypersons. Besides it is a commonly used first line antibiotics for human and animal domestic animals and is often used before the antibiotic sensitivity pattern of a pathogen has been determined.

The high resistance of *E. coli* to amoxycillin (84.7% in humans, 64.8% in pig, 57.0% in cattle and 88% in chicken) and erythromycin (85.9% in humans, 73% in pig, 90.5% in cattle and 96% in chicken), observed in this study is of public health concern. The widespread use, misuse and abuse of these two drugs account for high prevalent rate of *E. coli* resistance to them in human and animals in the study area. Aminoglycosides are frequently used for the treatment of animals. Among the aminoglycosides, only gentamicin and apramycin (the latter because of cross-resistance to gentamicin) are relevant for human therapy. Gentamicin and apramycin were introduced in veterinary therapy in the early 1980s in several European countries. Since 1980, resistance to gentamicin has increased among *E. coli* isolates from animals . In this study the overall rate of gentamicin resistance was higher in chicken (16.3%) than in cattle (3.6%) and pig (4.9 %) isolates. In humans, gentamicin is used (in combination with β-lactams) for treatment of severe infection, such as sepsis and endocarditis.

For non-fluoroquinolone antibiotics tested the percentage resistance of *S. aureus* isolates to gentamicin, ceftriaxone, amoxycillin, erythromycin and doxycycline were: in human 23.9, 18.3, 88.5, 27.7 and 84.4% ; pig 26, 16, 86, 24.7 and 86.3%; cattle

13.3, 10, 90, 19.2 and 83.3% ; and chicken 13.3, 5.3, 88, 16 and 82.3% respectively. The resistance rates of the *S. aureus* isolates (from human and animals tested) to gentamicin, ceftriaxone and erythromycin are low when compared with other antibiotics tested. This shows that each of these drugs (gentamicin, ceftriaxone and erythromycin) have a better activity against *S. aureus* than amoxycillin and doxycycline. In this study, the resistance rates observed in *S. aureus* isolates from these test animals are alarming with respect to the antibiotics tested. The resistance to gentamicin, ceftriaxone, amoxycillin, erythromycin and doxycycline is in conformity with previous observations that most isolates of *S. aureus* are resistant to a large number of commonly prescribed antibiotics (Olukoya *et al.*, 1995). These high rate of resistance against *S. aureus* isolates from animal indicates that these antibiotics have seriously been compromised and probably are currently of little value in the treatment of *S. aureus* infection in both human and animals. Like *E.coli* , the high resistance of *S. aureus* to amoxycillin (88.5% in humans, 64.8% in pig, 57.0% in cattle and 88% in chicken), observed in this study is of public health concern. This is also caused by the widespread use, misuse and abuse of this penicillin in human and animals in the study area. In humans amoxycillin is a common antibiotic that can be obtained from patent medicine vendors without formal prescription for the treatment of typhoid, respiratory, digestive tract, soft tissue infecton and other ailments by laypersons. Besides it is commonly used as first line antibiotics for human, animals and domestic animals and is often used before the antibiotic sensitivity pattern of a pathogen has been determined. Unlike *E. coli* isolates the resistance of *S. aureus* to erythromycin (26.7% in humans, 24.7% in pig, 19.2 % in cattle and 16.0 % in chicken) is low.

As noted above, the observed higher antibiotic resistance in both *E. coli* and *S. aureus* isolates from chicken than in other animals tested reflects the incessant use of

antibiotics in the poultry house/farms. This is understandable because antimicrobial use has been shown to be the most important selecting force in bacterial antibiotics resistance (Okeke *et al*, 1999). This point is clearly highlighted by the resistance results against the gentamicin and ceftriaxone. When compared with the antibiotics resistance pattern of the amoxicillin, erythromycin and tetracycline tested, the resistance rate of *E. coli* and *S. aureus* against gentamicin and ceftriaxone are low. The low resistance recorded against gentamicin here is in agreement with results of similar studies in Nigeria (Okoli *et al.*, 2002). The reason for low resistance rate is also related to the controlled use as there is no tablet dosage form for gentamicine and not all layperson can administer injections to their clients. They normally resort to available and common antibiotics like oral dosage forms of pencillins, macrolides and tetracyclines as the first line drugs for the treatment of ailments disturbing their clients. Our results are not in line with the results reported by Uwazuoke *et al* (2000) in *E. coli* isolates from poulty farm in Imo state. The case with the latter reports may be that these high gentamicin resistant *E. coli* isolates originated as direct human contaminants of the poultry feed ingredients through handling. In this study, the resistance rate of ceftriaxone against *E. coli* isolates from patients range from 14.4 to 26.9%. The highest percentage resistance to ceftriaxone (26.9 %) was noted with the *E. coli* isolates from chronic wound specimen. On average, more *E. coli* and *S. aureus* isolates (38.7 and 44.0%), from human patients were resistant to drugs tested than isolates from asymptomatic healthy carriers (30.9 and 29.7%) respectively. The reasons for this disparity may include inappropriate use of antibiotics by health workers, unskilled practitioners, laypersons, poor drug quality as well as unhygienic conditions leading to spread of resistant bacteria in hospitals and community (Okeke *et al.*, 1999)

Taking resistance according to specimen source in animals, there is strong evidence that the use of antimicrobial agents in poultry production can lead to the emergence and dissemination of resistant *E. coli and S. aureus* (Shroeder *et al*, 2002). These drug resistance can then be passed on to people through food or through direct contact with the poultry.

In this study, the resistance rates observed in *E. coli and S. aureus* isolates from poultry is alarming, 87.1 and 82.7% resistance to tetracycline, 96 and 16% resistance to erythromycin, 88 and 88% to amoxycilline, 16.3 and 13.3% to gentamicin, 10.9 and 5.3 % to ceftriaxone, 13.6-17.7% and 13.0 % resistance to fluoroquinolones respectively. These drug resistance rates against *E. coli* from chicken and *S. aureus* isolates indicate that these antibiotics have become seriously compromised and probably are currently of little value in the treatment of *E. coli* and *S. aureus* infections. These *E. coli* and *S. aureus* isolates may also constitute great reservoirs for genes encoding resistance against these antibiotics and foci for continual spread of resistance. The present data are similar to the report done elsewhere in *E. coli* isolates from a commercial poultry farm in Owerri, Imo state, Nigeria (Okoli *et al* 2005). It has been documented that soil dwelling bacteria could acquire resistance to naturally occurring antibiotics from environmental exposure, probably creating a reservoir of resistance factors generated outside host, humans and animals (Rysz and Alvarez, 2004). Some researchers have also demonstrated that farm environmental isolates showed reduced susceptibility (as measured by disc diffusion zone diameter) compared to faecal sample isolates to most agents studied (Sayah *et al*, 2005). They suggested that non-sampled sources such as farm workers and wildlife with access to the farm environment could be sources of resistance factors.

Based on the district or geographical locations, like the resistance pattern of *E.coli isolates*, the level of antibiotics resistance is more with *S. aureus* isolates from patients specimen than from the corresponding specimens obtained from healthy carriers. However ,there was no district in which both the healthy carriers and the patients *S. aureus* isolates would have the same level of antibiotics resistance as recorded with *E. coli isolates* and erythromycin. In Ikem, Udi, and Agbani districts the *S. aureus* isolates from healthy volunteers showed 100% sensitivity (0% resistance) to both gentamicin and ceftriaxone. In these districts, the rates of resistance of the test *S.. aureus* isolates to other antibiotics tested were high. Similarly, *S. aureus* isolates from patients from Ikem and Udi districts showed unique pattern of resistance to amoxycillin, erythromycin and doxycycline; 100% resistance to amoxycillin and doxycycline, and approximately 20% resistance to erythromycin. In all the districts in Enugu state, the test S. *aureus* isolates showed lower level of resistance to erythromycin than to amoxycillin and doxycycline. The resistance of patients *S. aureus* isolates to erythromycin are similar to that of fluoroquinolones in Awgu (42.9 and 42.9%) ,Enugu-Ezike (50 and 50%) and Enugu-Urban (33.3 and 33.3%) districts and is even better in Ikem district (40 and 20%) respectively. It has been shown that there is a tremendous variability in antimicrobial resistance patterns not only among pathogens causing various clinical infections but also in different geographical regions and over time (Hsueh *et al*.,2010). Evaluations of antibiotics susceptibility patterns of pathogens is therefore, important in the monitoring and detection of increase in resistance (Crandon *et al*., 2009; Njissen *et al.*, 2004). It is very helpful for clinicians in prescribing antimicrobial agents especially in cases where empiric treatment is employed. *E. coli and S. aureus* have been identified as a predominant pathogens for various bacterial infections especially urinary tract infections (Hooton *et al*., 2004; Gupta *et al*., 1999; Karlowsky *et al*.,2001; Karlowsky *et al*., 2003).

Fluoroquinolones have been proven to be highly effective broad spectrum agents especially against those infections caused by Gram-negative organisms (Karlowsky, 2001). Due to high resistance to cotrimoxazole and other antibiotics, fluoroquinolones became the first drug of choice for empiric treatment of urinary tract infections and other infections caused by Gram-negative bacteria and its widespread use has resulted in the development of resistance to them (Karaca *et al*., 2005).

Resistance to fluoroquinolones (FQ) in *Escherichia coli and Staphylococcus aureus* is an increasing health problem in Nigeria and other countries (Daini *et al*, 2006). The World Health Organization policy perspective on medicine indicates that even when drugs are made available, more than fifty percent are prescribed and dispensed inappropriately while 50% of patients fail to take the medicines correctly resulting in harmful consequences (WHO, 2002). One of the major consequences of such inappropriate use of antibiotics is the development of resistance strains of the hitherto susceptible organism, hence, in our study the prevalence of FQREC and FQRSA isolates from patients urine, stool, nasal swab or wound swab is higher than that of the FQREC and FQRSA isolates from the specimen of healthy volunteer subjects. Our finding is the first documentation of the prevalence of FQREC and FQRSA isolates from urine, nasal swab, stool and wound swab of both healthy volunteer subjects and patients in Enugu State. Our results agree with the finding of the work done in Indonesia in which fluoroquinolone resistant *E.coli* was prevalent in the fecal flora of 6% of patients at hospital admission and 23% of patients at discharges (Kuntaman *et al*., 2005). Three possible explanations for the high prevalence of FQ-resistant *E.coli* among patients that had been hospitalized for some days must be considered; transferable resistance, clonal spread and mutation-based selection of resistance fostered by the use of antimicrobial agents. Transferable plasmid mediated quinolone resistance has been described recently in *E.coli* from China (Wang *et a*l., 2003). Wang et al found that 6 (8%) of 78 ciprofloxacin resistant *E.coli* strains from a hospital in Shanghai contained qnr.

The prevalence of ciprofloxacin (20.8%) and levofloxacin (20.7%) were very similar to that of ofloxacin (20,5%) but differ from that of pefloxacin (21.5%). In both human and animals, the prevalence of pefloxacin resistant *E. coli* was highest when compared with other fluoroquinolones tested. Similar to our findings, lowest resistance rate of ofloxacin when compared with some other fluoroquinolone FQ-resistant *E. coli* was also reported elsewhere (Firdous *et al*., 2013). No fluoroquinolone resistance was noted with the *E. coli* isolates from the cattle, hence, the prevalence was zero. Taking the prevalence of fluoroquinolone resistant *E. coli* (FQREC) isolates by specimen source, the urine of asymptomatic healthy carriers and patients yielded isolates with respective prevalence of 12.3 and 24.9% and for stool specimen, the prevalence are 5.2 and 13.4% respectively. For chronic wound swab, the prevalence of FQREC is 57.7%; which is the highest of the prevalence of FQREC isolates in patients under study. The reason for this high fluoroquinolone resistance may be due to the fact that most of the chronic wound sampled have stayed for long time (>0.5 - 2 years) and the patients suffering from the wound infections must have used several antibiotics and topical preparation for the treatment. The prolonged use of antibiotics must have contributed to the increased drug resistance. *E. coli* isolates from urine showed resistance to fluoroquinolones more than *E. coli* isolates from stool. The increased prevalence of FQ- resistance in *E. coli* isolates from urine specimen of patients under study may be due to increased use of this drug in the treatment of UTI in both men and women, and prostatitis in men in the area. Though, the prevalence of FQREC isolates from stool specimen is lowest, when compared with FQREC isolates from urine and wound swab, this level of resistance is still high and may still be due to inappropriate use of antibiotics in patients in the area. For non-fluoroquinolones in patients, the *E. coli* isolates from wound swab showed least resistance to gentamicin, amoxycillin, erythromycin and doxycycline, followed by urine isolates and the stool isolates showed the highest resistance. The high susceptibility of wound *E. coli* isolates to gentamicin, amoxycillin, erythromycin and doxycycline when compared to isolates

from other source may be due to the fact that these drugs are not always prescribed for the treatment of wound infections in the area. These drugs especially tetracyclines and amoxycillin are frequently and /or inappropriately used by road-side drug vendors and lay persons for the treatment of abdominal discomfort and watery diarrhoea thereby inducing more resistance to stool isolates.

On average the mean percentage resistance of *E. coli* isolates from human to all the antibiotics tested were 38.7 and 31.4 % for patients and asymptomatic carriers respectively. In *E. coli* isolates from animal, the mean percentage resistance to drug was highest with the chicken (39.5%), followed by pig (30%) and the least was cattle (24.2%). Apart from the *E. coli* isolates from the chicken with average percentage resistance of 39.5%, more of the human isolates were resistant to drugs than animal isolates. The average prevalence of antibiotic resistance in apparently healthy chicken is greater than that of *E. coli* isolates from human patients. The reason for this situation in chicken may be connected with the daily use of antibiotics in these animals (more than in pigs and cattles) for prevention of diseases and in feed as growth promoters. To the best of our knowledge, this is the first report in Enugu State showing that the prevalence of antibiotic resistance is more in chicken than in humans (and most emphatically in human patients who have been on antibiotics). This finding is worrisome and therefore should be of great concern to public health in Nigeria and Enugu State in particular.

The prevalence of fluoroquinolone resistance among *S. aureus* isolates from human and animals tested are: human (22.6, 22.5, 21.6 and 24.8 %), pig (4.1, 3.4, 2.7 and 4.8 %), cattle (7.5, 7.5, 5.8 and 13.3%) and chicken (13.3, 13.3, 13.3, and 13.3%) for ciprofloxacin, ofloxacin, levofloxacin and pefloxacin respectively. The present data show that the prevalence of FQ resistance *S. aureus* isolates from human ranged from 21.6- 24.8%, while in animal, the range is 3.4- 13.3% depending on the source of the isolate and the structure of the fluoroquinolone under study. Therefore, more human isolates were resistant to the test

fluoroquinolone drugs than animal isolates. Like the resistance pattern in *S. aureus* isolates from human, the prevalence of levofloxacin resistant *S. aureus* in animals recorded the least resistant rate among all the fluoroquinolones tested while that of pefloxacin resistant *S. aureus* was highest. This also confirms the *in-vitro* antistaphylococcal superiority of levofloxacin over pefloxacin, ciprofloxacin and ofloxacin in the study area. The high resistance rate of pefloxacin over ciprofloxacin and ofloxacin against urinary *S. aureus* has been reported in Abuja, Nigeria (Onuanuga *et al*.,2005).

The high fluoroquinolone resistant rate found in this study could be due to the inappropriate use of this drug in animal husbandry in Enugu State, Nigeria. Other researchers in other parts of the world have indicated that fluoroquinolone resistance in bacteria isolates is increasing (Kariuki *et al*.,2007; Karlowsky *et al*., 2006; Yamane *et al*.,2008).

Comparatively, the prevalence of FQRSA isolates from non-duplicate samples of urine from asymptomatic healthy carriers and patients were 19.8 and 31.7 % respectively. Among *S. aureus* isolates from nasal specimen, the prevalences of FQRSA from asymptomatic healthy carriers and patients were 11.2 and 22.3% respectively. For chronic wound swab, the prevalence of FQRSA was 65.1%; which is the highest of the prevalence of FQRSA isolates in patients under study. The difference in the prevalent rate among fluoroquinolone resistant *E.coli* and *S. aureus* is caused by the difference in drug structure. The MICs of fluoroquinolone against organisms differ due to the effect of drug structure (Wang *et al*., 2008; Nordmann and Poirel, 2005; Ruiz 2003; Lindgren *et al*., 2003).

Based on the sex of the test subject (apparently healthy and human patients), 39% of FQREC isolates were gotten from male subject while 61% were gotten from female subject under study. The explanation may be related to the following reasons gathered from the questionaire; 1. that the female subjects abuse/ misuse antibiotics more than male counterpart for

treatment/prevention of urogenital infections in the study area. 2. Most female subjects use herbal remedies (which may contain natural antibiotics) over a long period of time as their belief is that these herbs cure infections better than orthodox antibiotics in the study area. 3. The female subjects dominate most of these rural districts in the study area than the male counterpart (who are always in the cities for white colar jobs) and patent medical vendors are the first 'health officers' to be consulted whenever they are sick and then polypharmacy involving many antibiotics is always the order of the day. Unlike FQREC, FQRSA 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*. (2008) who reported that resistance to quinolones was higher isolates from men, perhaps because of the association between UTIs and prostatitis, where quinolones are widely used. Like the general antibiotics tested in animals, the prevalence of fluoroquinolone resistant *E.coli and S. aureus* isolates from chicken was greater than that of isolates from pig or cattle isolates. One of the causes of this is that the use of fluoroquinolone is more in chicken than in the pig or cattle in the study area. In addition, human handling of chicken is more and more frequent (as they are more friendly) than with pigs and cattle and in so doing, resistant strain /gene may be transferred from human to animals. Moreso, the cattle mainly feed on grasses and the use of fluoroquinolones is minimal. The prevalence of FQ resistance *E. coli* from pig is higher than that of cattle isolates and this reflects the high rate of antibiotics use in pigs feed as well as for treatment of their ailments. However, in cattle, the prevalence of FQ resistant *S. aureus* is higher than that of pig . The reason for this may be related to the fact that the human contact and handling of cattle is more and more frequent (as they are more friendly) than with pigs and in so doing, resistant strain /gene may be transferred more frequently from human to cattle.

The high fluoroquinolone resistance found in this study could be due to the inappropriate use of this drug in Enugu State, Nigeria. Other researchers in other parts of the world have indicated that fluoroquinolone resistance in *E. coli* is increasing (Kariuki *et al*.,2007;

Karlowsky *et al*., 2006; Yamane *et al*.,2008). Our findings agree with the study done in China and Pakistan where the frequencies of the ciprofloxacin resistance among *E. coli* isolated from UTIs were 59.4 and 36.5% respectively. On the other hand, our findings are in contrast to studies conducted in the United state with lower ciprofloxacin resistant rate against uropathogenic *E. coli* (Moreno *et al.*, 2006). The reasons for this discrepancy abound, including the situation of drug use in Enugu state where people take antimicrobial drugs without a prescription, differences in animal husbandry and over the counter use of fluoroquinolones in human and veterinary medicine, as well as prolonged consumption of herbal drugs and environmental conditions. A significant relationship between fluoroquinolone use and resistance to these antibiotics has been documented (Goettsch *et al*., 2000).

Based on the age of human subjects, FQ-resistance to the test isolates increased with increase in age. 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 of UTI, prostatitis and other diseases in old age may contribute to high FQ- resistance in older subject (19-60) than in younger ones (6-18 years). In children 0-5 years, many drugs can be used as substitutes to FQ for the treatment of different infections and as a result, many clinicians do not support the use FQ in such children. This limited use of FQ in children can be the reason for lower FQ resistance in them than in adults. For antibiotic resistance pattern of *S. aureus* isolates from urine specimen of healthy volunteers according to age, the level of FQ resistance is higher with the isolates from individuals within the age range 19-60 years old than with the isolates from individuals within the range 0-18 years old. A different pattern of antibiotics resistance was obtained with non-FQ antibiotics and urinary *S. aureus* isolates. The highest level of resistance was recorded with urinary *S. aureus* isolates from individuals within the age range 6-18 years. These asymptomatic healthy carriers comprised of individual in late primary and mainly post primary schools. These students are

mainly living and staying together in student dormitory where they use toilet and other facilities in common. This public use of toilet, urinals, kitchen and others, can facilitate the transmission of bacteria from one point or person to another.

In animals tested, it was obvious that the prevalence of antibiotic resistance in both *S. aureus* and *E. coli* isolates was high. In most of the antibiotics tested the prevalence of drug resistance was least with the isolates from nasal specimen when compared with other isolates. The reason for this is unclear but may be related to the nature of the anatomical region. This may also be due to the limited number of microbial population (and thus limited acquisition of resistance) in the nasal cavity when compared to large population of micro-organisms on the animal skin, intestinal tract and vendor's tables. The prevalence of fluoroquinolone resistant *S. aureus* isolates from the skin swab of pig is higher than that of the meat/vendor's table but the case is reverse for cattle and chicken *S. aureus* isolates. The reason may be related to the lifestyle of pig and its relation with the environment. Acquisition of drug resistance may be facilitated by its lifestyle of living in the mud and dirty environment as this style may potentiate the conjugal transfer of resistant genes from soil or environmental organism to pig-skin resident bacteria and the extent or frequency of transmission of resistance may be greater than that seen between the meat sellers and / or their tables and the skin *S. aureus* isolates.

It is evident among the animals that the *E. coli* isolates from chicken exhibited the highest level of antibiotics resistance when compared with the pig and cattle isolates except for the gentamicin. In cattle, zero prevalence was recorded for the fluoroquinolone resistant *E. coli* whereas the fluoroquinolones resistant *E. coli* isolates were less in pig than in chicken. For animal *S. aureus* isolates, the prevalence of fluoroquinolones resistant S. *aureus* isolates was highest in chicken and lowest in pig. The reason for this is not clear but may be connected with the fact that S.*aureus* isolates thrives, multiply and acquire resistance better under the dry

environment and habitat of chicken than the muddy and wet environment of pig. The spread of bacteria pathogen goes with the spread of resistant genes from one bacterium to another.

5.1.3 Multiple antibiotic Resistance index (MARI).

Multi-drug resistance (MDR) was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, Extensively drugs resistant (XDR) bacteria are non-susceptible to at least one agent in all but two or fewer antimicrobial categories {ie bacterial isolates remain suscepible to only one or two categories} and pandrug resistant (P) bacteria are resistant to all agents in all antimicrobial categories. To confirm the multi-drug resistance properties of these test isolates, the percentage of FQRSA and FQREC isolates that are resistant to test antibiotics were calculated **.** The level of multi-drug resistance shown by the isolates in this study is of great concern **.** Apart from the FQRSA isolates from the nasal swab of healthy carriers with the modal value of MARI as 0.67, (corresponding to 36% of the isolates), the greatest proportions of FQRSA isolates from other sources (healthy volunteer urine, patient urine, patient nasal swab, wound swab, pig specimen, specimens of cattle and chicken) have their modal value of MARI as unity (1.0). Based on the antibiotics used, all these FQRSA were multi-drug resistant isolates. For FQREC isolates the greatest proportions of the isolates from patients' urine, stool, wound swab and pig specimen have MARI value of one (1.0). For the fact that a limited number of antibitotics were tested, we cannot be certain if most of our FQRSA and FQREC isolates fall into category of Extensively Drugs Resistant (XDR) or PanDrug Resistant (PDR), but most probably, some of the FQRSA and FQREC isolates are in the category of XDR bacteria especially those with MARI of one. In healthy volunteer urine specimens, all the FQRSA (100%) isolates are resistant to doxycycline and amoxycillin, approximately 64% were resistant to gentamicin, ceftriaxone and erythromycin. These observations confirm the postulation that healthy members of the community are reservoir of antimicrobial resistant bacteria (Lamikanra *et al*., 1996). The society is presently characterised with inappropriate

prescription, unethical dispensing and indiscriminate use of antibiotics. The rate at which most antibiotics are losing the battle against resistant organisms should be of immense concern to the health professionals and calls for effective measures (including trainings) to promote rational use of antibiotics and thereby prolong their life expectancy.

5.1.4 MIC (ug/ml) of Erythromycin and Ciprofloxacin against FQRSA isolates from both Humans and Animal.

In a technique to determine the MIC of erythromycin against FQRSA isolates, the results showed that appreciable number of the isolates from human urine and nasal swabs as well as specimens from pig and chicken were susceptible to the agent. The FQRSA isolates from human wound swab and skin / meat swabs from cattle exhibited high level of sensitivity against erythromycin. Though greater percentage of FQRSA isolates are also resistant to erythromycin, such large number of erythromycin -susceptible FQRSA are important because the drug in question can be used in handling infections caused by these multidrug resistant organisms. These erythromycin- susceptible FQRSA isolates have their modal MIC value as 0.125 μg /ml. Those FQRSA isolates within the intermediate susceptibility to erythromycin (MIC value 1- 4 μg /ml) are few and are about 2% of all human FQRSA tested. The low level MIC which is the mode shows that those susceptible ones are highly sensitive to FQRSA isolates and this therefore, has therapeutic implications. It is therefore, advisable that antibacterial sensitivity tests be conducted on all patients *S. aureus* before commencing treatment and erythromycin be included in the panel of the drug discs to be used.

Though high level ciprofloxacin resistance (MIC of 32-128 μg /ml) were recorded for nasal swab FQRSA isolates, the percentage of isolates having this high level is low (6-12.5%). Greater percentage of the nasal swab FQRSA isolates (12.5-37.5%) had low level of ciprofloxacin resistance (MIC of 4-16 μg/ml). This result suggests the presence of several mechanisms of FQ-resistance in the nasal FQRSA isolates. For those isolates with high level FQ-resistance,

combination of mutation at the target site or efflux-pump mediated mechanism and plasmid mediated mechanism are likely to be involved. For low level resistance, the mechanism of resistance is most likely to be either plasimid mediated or efftux pump mediated only. For urinary FQRSA isolates, more of the high level ciprofloxacin resistance isolates are found, than the low level ciprofloxacin resistance, albeit, both level are evenly represented in the distribution. This suggests the combination of several mechanisms of FQ-resistance in *S. aureus* urine isolates. MIC values of ciprofloxacin up to 256 and 512mg/ml were found in patient's urinary FQRSA isolates. This x-rays the contribution of antimicrobials in raising MIC value especially in patients who inappropriately use antibiotics.

5.1.5 MIC of Gentamicin and Ciprofloxacin against FQREC isolates from both humans and animals in the study area .

For humans urinary and faecal FQREC isolates, the percentage susceptibility to gentamicin were found to be 34 and 55% respectively. In animal, 65 and 75% of the pig and chicken FQRSA isolates were susceptible to gentamicin respectively. For urinary FQREC isolates, the range of MIC values of gentamicin is 0.5 ug/ml–128 ug/ml for both patients and asymptomatic healthy carriers. Even in chronic wound patients, the modal MIC value of gentamicin is 64 ug/ml. For faecal FQREC, the modal MIC values for gentamicin are 16 ug/ml and 64 ug/ml for healthy carriers and patients respectively. More of the patients isolates exhibited high level gentamicin resistance than the isolates from healthy carriers. This confirms the contribution of antibiotics as selection pressure for antibiotic resistance. The MIC value of ciprofloxacin against FQREC isolates shows that these values are widely distributed within 4 µg/ml through 512 µg/ml and 1024 µg/ml according to the specimen source of the isolates. This implies that several mechanisms are involved in ciprofloxacin resistance. More of high level ciprofloxacin resistance (MIC of 64 - 256 µg /ml) were recorded for FQREC isolates from patients urine than for healthy carrier urine isolates. This result also suggests the presence of

several mechanisms of FQ-resistance in patients urinary FQREC isolates which may include mutation at the quinolone resistant determining region (QRDR) of the *gyr A* gene, efflux-pump mediated mechanism and plasmid mediated mechanism. In animal, low level ciprofloxacin resistance isolates predominates and there was no MIC >128 μg /ml found in all the animal FQREC isolates. For low level resistance, the mechanism of resistance is most likely to be either single point mutation at the QRDR of the *gyr A* gene, plasmid mediated or efftux pump mediated only.

5.1.6 Fluoroquinolone resistant genes in the test isolates

The presence of *gyrA* gene in FQREC isolates was screened for by PCR techniques and the results revealed the prevalence of *gyrA* gene in human FQREC isolates at the range of 52.6-63.4 % depending on the source of the isolates. In animals tested, the prevalence of *gyrA* gene in FQREC were 21.4 and 40.0% in pig and chicken respectively. It means that the prevalence in human is greater than in animals tested. In human isolates, the *gyrA* genes were concentrated in isolates wih ciprofloxacin MIC of 32 – 512 μg/ml. In human patients isolates the *gyrA gene* was found in those isolates that have ciprofloxacin MIC of 32 – 1024 μg/ml. In pigs tested, all the isolates with *gyrA* gene have the MIC values of iprofloxacin as 64 μg/ml only while in chicken the *gyrA* gene positive FQREC isolates were found within the ciprofloxacin MIC range of 4–128 μg/ml. In both animals tested, other mechanisms of fluoruquinolone resistance abound especially with pig isolates.

The *qnr* genes encode proteins that protect DNA gyrase and topoismesase iv from inhibition by quinolones (Tran, 2005), and have recently been identified worldwide. The plasmid-mediated quinolone resistance associated with qnr (now named qnrA1) in *Klebsiella pneumoniae* was firstly found from the United States in 1998 (Martinez-Martinez et al., 1998). In our study, we found out that *qnrA* gene was present in 12.5% of all the FQREC isolates. In healthy volunteers,

the urinary and faecal FQREC isolates have *qnrA* prevalences of 15.8 and 10.5% respectively. In patient subject, the prevalences of *qnrA* in urinary and faecal isolates were 16.9 and 22.6% respectively. These results agree with the report of Nordmann and piorel (2005) who showed high prevalence among enteric isolates of quinolone resistance *(qnr*) genes. This study showed that the prevalence of plasmid-mediated quinolone resistance due to the *qnrA* gene among FQREC isolats from both human and animals in Enugu State, Eastern Nigeria is high and that it is much higher than that reported in other areas, such as China (Yang et al., 2008) and United stated (Robicsek et al., 2006). Based on the source of the isolates from the human subjects, the *qnrA* gene prevalence in healthy volunteers is lower than that in patients who have been on antibiotics which is also in agreement with the result of antibiotics pattern of both subjects in which the isolate from patients are more resistant to antibiotics than the isolates from healthy volunteers. In wound FQREC isolates, the prevalence of *qnrA* gene was 13%.

The prevalence of *qnrA* gene in bacteria isolates from human may range from <1 to > 50% (Wang *et al*., 2008; Jeong *et al.,* 2005; Kim *et al*., 2009) depending on the selection criteria and period of study for isolates. This range agrees with the findings of this work in which the the prevalence of qnrA gene in FQREC in human ranged from 10.5-21.5 according to isolate source and patient age. In this study, the prevalence of *qnrA* appeared to be lower in human FQREC isolates from healthy volunteer urine and stool specimens than in FQREC isolates from patients who were on antibiotics. The reason for this is not clear but may suggest the role of antimicrobials in increasing the prevalence of plasmid mediated resistance determinant. This study is the first report on the presence of *qnrA* gene in FQREC isolates from apparently healthy volunteers; ages 0 -5 years , 6- 18 years and 19 – 60 years as well as patients 6 – 18years and 19 – 60 years in Enugu state, Nigeria. The *qnrA* gene was detected in urinary FQREC isolates (1 of 3 FQREC isolates from urine specimen) and the gene was not detected in 5 faecal FQREC isolates used. The prevalence of *qnrA* in urinary FQREC isolates is higher than the prevalence

of *qnrA* detected among ciprofloxacin-resistant *E.coli* isolates in Shanghai, China and paediatric patients in China wherein there were 8 and 7.5% of FQREC isolates respectively (Wang *et al.*, 2003; Wang *et al.*, 2008). It is important to note that the *qnrA* gene was detected from FQREC isolate from a healthy volunteer child younger than one year of age. The prevalence of *qnrA* among the FQREC isolates in children ≤ 5 years in Enugu State Nigeria is 12.5%. The transferability of fluoroquinolone resistance due to *qnrA* gene among FQREC strains shows that plasmid-mediated fluoroquinolone resistance have been spread or may spread in nursery school children in Nigeria.

In the study area (Enugu State), fluoroquinolones are currently in use on both children and adult. Presumably, the source of the *qnrA* gene might either be directly connected to the selective pressure created by the fluoroquinolones used in children or related to horizontal transmission from adults or other reservoir. It has been documented that *qnrA* gene come from environmental Gram-negative bacterial species, such as Shewanella algae, the progenitor of the *qnrA* gene (Poiral *et al*., 2005). This shows that the aquatic environment is an important reservoir of novel fluoroquinolone-resistant determinant (Wang *et al*., 2008). Exposure to lower concentration of quinolones increases the chance for selection of resistance as these compounds are used in aquaculture (Poiral *et al*., 2006).

In animals studied, the prevalence of *qnrA* being lower in pig (7.1%) than in chicken (10%) implies that there is a relationship between the incidence of *qnrA* gene and the source of test isolates. From the results of this study, it is obvious that the *qnrA* gene prevalence is higher in human FQREC isolates than in animal FQREC isolates. This is in line with the antibiotics resistance pattern in which human isolates were found to be more resistant to test antibiotic than the animal isolate. This finding may be related to the extensive use of broad-spectrum agents in human medicine (Heuer *et al.*, 2005). Several reports had shown a low prevalence (0-0.6%) of the plasmid mediated quinolone resistance gene in food-producing

animals (Cavaco *et al*., 2007, Cavaco *et al.*, 2008, Cerquetti *et al*.,2009). Though, the prevalence of *qnr A* gene in cattle FQREC isolates was found to be zero percent which agree with some reports (Cavaco *et al.*, 2008,), our results did not demonstrate such a very low prevalence of *qnrA* in healthy pig and chicken. The finding of our study that some *qnrA* negative isolates were also resistant to ciprofloxacin signifies that other resistance mechanism and other *qnr* genes may be involved.

5.1.7. **Prevalence and distribution of efflux pump mediated fluoroquinolone resistance gene-*Nor A* gene- in FQRSA from human and animals.**

NorA is a membrane–associated multidrug efflux protein that can decrease susceptibility to fluoroquinolones in *Staphylococcus aureus* (Kaatz and Seo, 1995; Kaatz and Seo, 1997; Kaatz *et al.*, 1991; Kaatz *et al.*, 1993). It has been found to transport both fluoroquinolone and nonquinolone compounds (Yoshida *et al.*, 1990; Neyfakhm *et al*.,1993). *NorA* has been compared to a number of other drug efflux systems such as *Tet A, Bmr* and the mammalian multi-drug efflux tramsporter P-glyco protein (Pgp), but the greatest degree of homology (44%) has been found between *NorA* and *Bmr* (Neyfakhm *et al.*,1993; Kaatz *et al*.,1993; Neyfakhm 1992). *NorA* protein is the product of the *NorA* gene, and confers a baseline low level of intrinsic resistance to fluoroquinolones and other structurally unrelated compounds considered toxic to the bacteria cell such as chloramphenicol, ethidium bromide, rhodamine and puromycin (Neyfakhm *et al*.,1993; Kaatz *et al*.,1993; Neyfakhm 1992). The over expression of *NorA* in clinical isolates has been observed, and this is as a result of a mutation in the *NorA* promoter that resulted in the inability of the regulator protein to bind to the promoter (Ng *et al*., 1994). Some Hydrophilic fluoroquinolones are pumped out of the cells more efficiently than hydrophobic agents, but the reasons mechanism are yet to be elucidated (Kaatz *et al.*,1993).

In this study, the *NorA* gene was present in 14 (25%) of the 56 FQRSA isolates from human nasal swab specimens; out of which 20% were from nasal swab of healthy volunteer and 37.5% were from nasal swab of patient subjects who have been on antibiotics. The prevalence of *NorA* in human FQRSA urinary isolates were 26.3 and 36.4% for apparently healthy volunteers and individual patients respectively. The prevalence was highest (71.4%) in human FQRSA isolates from wound swabs. In animal subjects the respective prevalence's of *NorA* gene in chicken, cattle and pigs were 0, 22.2 and 33.3%. In the present study, the *NorA* gene was found in both low level and high level ciprofloxacin MIC (4-512 ug/ml) resistant *S. aureus* isolates from both human and animal. The development of resistance can occur when a bacterium is constantly exposed to an antibacterial agent. *S. aureus* exposed to increasing concentrations of ethidium bromide developed higher levels of resistance to fluoroquinolones and biocides compared to the parent strain, and this increased resistance was due to a several–fold increase in the expression of the *NorA* efflux gene, which in turn was to a 70 bp deletion in the *Nor A* promoter region (Couto *et al*., 2008). This explains why, in our findings, the prevalence of *NorA* was higher in patients who have been on antibiotics than the healthy volunteers subject and even highest in FQRSA isolates from chronic wound specimens. The wound swabs used were collected from chronic wound patients who have used many antimicrobial agents for wound dressing and treatment.

5.1.8 Plasmid Profile.

Plasmids are the major mechanism for the spread of antimicrobial resistant genes in bacterial populations (Shames *et al,* 2009). Multiple resistance genes are haboured on resistance plasmids, some of which are conjugative (Lloyd *et al*.,2007). The resistant plasmids could be attributed to indiscriminate use of antibiotics in the hospital and to the over the counter (OTC) availability of antibiotics. In this study plasmid profile analysis of the FQREC isolates by agarose gel electrophoretic techniques showed a total of 223 different plasmid bands occurring

in various combinations in FQREC isolates. The size of these bands ranged from 504 to 23130 bp and most of the plasmids were shared among the human and animal isolates. Of all the plasmids detected, 23130 bp plasmid was most frequently found; 49.2% in human and 54.2% in animal isolates respectively. This implies that plasmid-mediated FQ-resistance is more in animal than in humans. The reason may be because of incorporation of antibiotics at subinhibitory concentration in animals feeds. All the isolates bearing 23130 bp plasmid also harbored one or more smaller plasmids, and they were resistant to six or more antibiotics including ceftriaxone, a third generation cephalosporin and gentamicin. Similarly, all the FQRSA isolates bearing 23.1 KB plasmid also harboured one or more smaller plasmids, and they were resistant to six or more antibiotics including gentamicin and ceftriaxone (aminoglycoside and cephalosporin respectively). In general, eight (8) different plasmid profiles were observed with 6.6, 9.2 and 23.1KB occurring in almost all the health districts in both human and animal FQREC and FQRSA isolates. In previous study, the plasmid number ranged from 1-5 with size range 2.9- 66 kb (Al Bahry *et al.*,2006). Another study showed that the range of plasmid number was from 1-7. The study conducted in Bangladesh showed that the plasmid number from *E. coli* isolates ranged from 1-5 and size ranging from 0.5–40kb (Alam *et al*.,2010). The plasmid analyses study conducted on uropathogenic *E. coli* isolated from children showed the average copy number of 5.5 (ranging from 1–10) with plasmid size from 1–33kb (Farshad *et al*.,2012). The slight variation in results may be due to difference in origin of isolation of *E. coli*, geographical distribution of the bacteria and exposure to different antimicrobials. The difference in plasmid size might be due to the fact that the spread of resistance genes is evolutionary process which requires lot of energy. In order to carry small sized plasmid, less energy is required than large sized plasmids. This shows that there exists correlation between plasmid size and number to that of antibiogram of the isolates. In *E. coli* strains, antibiotics resistance increases as a function of time and their exposure to many agents such as chemicals, biocides, antibiotics, etc. In this

study, the greater percentage of the plasmids fall between 6557 and 23130 bp, this is similar to previous reports (Uchechi and Erinma, 2007; Adeleke *et al.*, 2010; Tula *et al.*, 2013; Akinjogunla and Enabulele, 2010).

The molecular weight of some of the plasmids observed in the study falls into the category of small multicopy plasmids that carry single resistance (Bery *et al*; 1998). These plasmids can be described as mobilizable resistant plasmids which are relatively small (often less than 10kb in size) encoding only a handful of genes including resistance genes (Esimone *et al*., 2010). The resistant plasmids observed in this study could be attributed to indiscriminate use of antibiotics in the hospital and to the over the counter (OTC) availability of antibiotics. In the present study, all the FQREC isolates with multiple antibiotics resistance index (MARI) of ≥ 0.9 habored 23130 plasmids while some other isolates with MARI of < 0.9 have the same plasmid and other plasmids of lesser magnitude. In FQRSA isolates the plasmids were cured to the range of 36.4-100% depending on the source of the isolates. Similarly, in FQREC isolates the plasmids were cured to the range of 40-80% depending on the source of the isolates thus, confirming the contribution of plasmid in mediating fluoroquinolone resistance in both FQREC and FQRSA isolates.

5.1.9 **Conjugation Experiment**

In this study, ten transconjugants were successfully obtained from the 35 qnrA – positive FQREC used as donors in conjugation experiments. In FQRSA conjugation experiments, PMQR were transferred from six (16.7%) isolates.The decrease in susceptibility of the most transconjugants to the non-fluoroquinolone show that other resistant markers must have been co-transferred from the FQREC to the recipient *E. coli* among FQREC isolates. Conjugation experiments showed that the *qnrA* positive FQREC isolate was able to transfer the *qnrA* gene to the transconjugant. Other studies have recorded that possibility of transferability, though, not all

qnr-positive isolates were able to transfer quinolone resistance (Jonas *et al*., 2005; Jeong *et al*., 2005). The lower resistance to ciprofloxacin in this transconjugant than its donor strain may imply the presence of additional chromosomal resistance mutation. Similarly, with the FQRSA isolates, the decrease in the susceptibility of the transconjugants to all the antibiotics tested compared to the pre-conjugated recipient *S. cohnii subsp urealyticum* as shown in the increase in MICs of test antibiotics to the transconjugants confirmed the transfer of PMQR markers to the recipient cells (for fluroquinolones) and other plasmid medicated resistance determinants to non-fluorquinolone antibiotics tested.

The FQRSA and FQREC isolates tested exhibited ciprofloxacin MICs that were higher than those of fluoroquinolone susceptible isolates, and the presence of omeprazole at 128 μg/ml resulted in a reduction in the ciprofloxacin MIC (2- to 16-fold) for FQRSA and for FQREC with omeprazole at 64 μg/ml, the MIC values were increased for the most of the isolates.

5.1.10 Antibacterial potential of essential oil of lemongrass and coconut oil alone, and in combination with ciprofloxacin against FQRSA and FQREC.

Natural substances which have some medicinal properties are usually source of chemical compounds with pharmacological and antimicrobial activities. Human beings have always sought different parts or extracts from these plants, not only for food but also for therapeutic purposes. These plant parts are used to manufacture products with high added value -essential oils, extracts, resins, etc.- that are presented as complex mixtures. Essential oils are among the great source of bioactive molecules (Dias *et al*., 2012). Essential oils of *Cymbopogon citratus* have been reported by many workers to contain many compounds and to have antibacterial activity against a diverse range of organisms comprising Gram-positive and Gram-negative organism, yeast and fungi (Shigeharu *et al*.,2001; Cimanga *et al*., 2002; Nguefack *et al*., 2004;

Pereira *et al.*, 2004). Our analyis of the essential oil of *Cymbopogon citratus* by GC-MS revealed 16 constituents. It is important to note that other researchers have recorded more than 16 constituents, and different chemical compounds found in the essential oil of *Cymopogon citratus* (Torres and Ragadio, 1996; Saleem *et al.*, 2003; Amit and Anushere, 2010). The reason for this disparity may be as a result of geographical, seasonal and edaphic variations. However, several reports (like our results) confirmed α-citral and β-citral as the major components (Amit and Anushere, 2010, Saleem *et al.*, 2003). The Yomogi alcohol also known as 3,3,6-trimethyl-1, 4 – heptadien-6-0l [C_{10} $H_{18}O$] which is the second most abundant constituents as found in our analysis was not detected by other researchers.

The results of the sensitivity tests of EO of lemongrass against both the FQ-susceptible *S. aureus* and *E.coli* isolates as well as FQRSA and FQREC shows that the susceptibility of *S. aureus* to EO of lemongrass is higher than that of *E.coli* isolates. Similar observations were made by the results of the work done elsewhere (Naik *et al.*,2010, Torris *et al*.,2012). There is no significant difference among the FQRSA isolates from different specimen sources (urine, nasal swab and wound swabs). For FQREC isolates, there was also no significant difference among the isolates from different specimen. The result of the MIC values of this EO against the test isolates showed that the potentials of this oil is more appreciated when the sensitivity is done using MIC techniques due to the problem of solubility of this EO. The MBC values of the oil on FQREC and FQRSA were significantly higher than that of the MIC values. This shows that the EO is both bacteriostatic and bacteriocidal in nature, but the later effect is normally seen at higher concentration. The high antibacterial activity of this EO could be correlated to the presence of high level of geranial (α-Citral) and neral (β-Citral) as its components. Citral is a mixture of two isomers, geranial and neral, which are acylic and α,β– unsaturated monoterpene aldehyde and possessed significant antimicrobial activity.

The antibacterial activity of *Cocos nulifera* (coconut) oil on both FQ-susceptible *S. aureus* and *E. coli* was poor. With both FQREC and FQRSA isolates, no activity was found. Poor or lack of activity of this oil against the test isolates may be attributed to the nature of the chemical compounds found in this oil. The study, as confirmed by the GG-MS analysis, showed that the components of the oil with high percentage abundance are not antibacterial in nature (para-xylene (11.47%), Cis – Oleic acid (19.9%), Shellsol 40 (7.5%), Hendecane (9.01%), and hydrofol (7.8%) whereas the component (Lauric acid) which has been shown to exhibit some levels of activity against some bacteria (Kabara *et al.*, 1972; Bergessson *et al,* 2002) occurred in small proportion (1.22%) in the analysis. Some *in vivo* and *in vitro* studies have shown that lauric acid from coconut oil have antibacterial activity against *S. aureus, Helicobacter pylori, Neisseria spp Chlamydia trachomatis and Clostridium difficile* (Bergasson *et al*., 1998, Bergesson *et al*, 1999, Abraham and Verallo, 2001,; Rouse *et al*., 2005). Little or no report on the antibacterial potential of coconut oil against Gram negative bacteria especially *E. coli* isolates was found.

5.1.11 *In-vitro* interaction of the two oils and Ciprofloxacin.

In this study, ciprofloxacin and essential oil of lemongrass showed combined effect of additivity, synergism and indifference when ciprofloxacin was combined with EO of lemongrass. Zero (0%) antagonism was recorded with these isolates. This is a promising interaction studies as these agents do not affect the activity of each other. The combined effects of additivity and synergism evaluated in this study encourage the combined use of ciprofloxacin and food or medicinal agents containing essential oil of lemongrass. Moreso, the synergistic effect which occurred in many of the combination ratios against the FQREC and FQRSA has a lot of therapeutic implications in the treatment of infections caused by FQ-resistant *E. coli* and *S. aureus*. Further purification should, therefore, be done to isolate the active ingredients which may serve as the lead structure for the development of new drugs that will be beneficial to man.

It can be inferred that EO of lemongrass enhance the activity of ciprofixacin against many FQRSA strains and this has therapeutic implications.

In the present research, the MIC studies confirmed that the coconut oil has no activity against FQRSA and FQREC isolates. Coconut did not show any inhibition of growth at concentration up to 5%. At concentrations of 0.5 and 2%, the oil increased the MIC of ciprofloxacin against both FQREC and FQRSA isolates. In the interaction studies using thin overlay inoculum susceptibility Disc (OLID), a 19% increase or more in IZD is usually taken as index of synergism, increment less than 19% increase in IZD produced additive effects while cases showing no variation in IZD had indifferent effects where as the decrease in IZD is taken as antagonism. From the analysis, coconut oil exhibited antagonistic effect when combined with ciprofloxacin thereby further reducing the activity of ciprofloxcin. It is vital to note that there was a significant difference between the IZD at 0.5 and 2% concentration of oil when combined with ciprofloxacin. This implies that the effect of oil is more at increased concentration of oil. Fluoroquinolone antibiotics especially ciprofloxacin are frequently consumed with food and coconut oils are used in preparing some food as well as some herbal medicinal agents. The concomitant intake of ciprofloxacin with food containing coconut oil or intake of coconut milk or kernel after taking ciprofloxacin should be discouraged.

5.2 CONCLUSION.

This study showed that the prevalence of FQRSA and FQREC and their resistant genes are high in Enugu State. The resistant traits or determinants are present in both hospital and community *E. coli* and *S. aureus* isolates. Moreso, these fluoroquinolone resistant determinants are distributed across hosts in different environments rather than being host or environment-specific. These FQ- resistant *E. coli* and *S. aureus* are all multi-drug resistant bacteria and some of them exhibited such high levels of antibiotic resistance to be qualified as Extensively Drugs Resistant (XDR) or PanDrug Resistant (PDR) isolates. A significant public health concern is that multi-drug resistant commensal *E. coli* and *S. aureus* strains may constitute a potential reservoir of resistance genes that could be transfered to pathogenic bacteria. The findings of our study provide strong evidence to corroborate studies that suggest the existence of a reservoir of antibiotic resistance genes. The high prevalence of fluoroquinolone resistant *E. coli* and *S. aureus* observed in this study suggests that there is an urgent need for public health education on the issue of antibiotic use in human and veterinary medicine in the study area. Human habits observed in Enugu State to enhance fluoroquinolone resistance include: prolonged antibiotic use, abuse and misuse of antibiotics, prolonged intake of herbal remedies, and use of antibiotics in animals feed and for therapeutic purpose.

Infections with fluoroquinolone-resistant *E. coli* and *S.aureus* limit the options available to treat infectious disease of animals and humans. Plant source of drugs in the form of essential oil of *Cymbopogon citratus* has been found in this study to inhibit the growth of FQ- resistant *E. coli* and *S. aureus*. Further, the result showed that the sensitivity of FQREC and FQRSA increases in the presence of ciprofloxacin and *Cymbopogon citratus* essential oil combination. Moreover, concomitant intake of ciprofloxacin with coconut oil reduces the activity of ciprofloxacin against FQREC AND FQRSA. Finally, we hope that these findings will make a positive impact in public health within Nigeria in general and Enugu State in particular,

especially in the area of drug prescription and strong search for alternative medicine. Our results will bring needed attention to how drugs are used in both humans and veterinary medicine and subsequently guide therapy for infections caused by FQREC and FQRSA in Enugu State.

5.3 CONTRIBUTION TO KNOWLEDGE

-Firstly, many fluoroquinolone- resistant *E. coli* and *S. aureus* isolated from humans and farm animals in Enugu State are habouring FQ-resistant genes(*gyrA* , *qnr A* and *Nor A* genes) and plasmids.

-Secondly, these resistant genes and plasmids mediate fluoroquinolone resistance.

-Thirdly**,** combined use of omeprazole and ciprofloxacin increases the sensitivity of *S. aureus* and decreases the susceptibility of *E. coli,* to fluoroquinolones

-Moreover**,** the sensitivity of FQREC and FQRSA increases in the presence of ciprofloxacin and *Cymbopogon citratus* essential oil combination.

-Furthermore, concomitant intake of ciprofloxacin with coconut oil reduces the activity of ciprofloxacin against FQREC AND FQRSA.

5.4 RECOMMENDATION

From our findings, it is important that the following activities be carried out by individuals and/or Government as a way to remedy the situations caused by FQ resistant *E. coli* and *S.aureus*

- Government should enforce an already existing law that discourages inappropriate use of antibiotics especially through patent medicine vendors.
- Public health Education on the use of antibiotics and herbal remedies should be encouraged and promoted.
- Incorporation of antibiotics in animal feed for growth promotion should be discouraged. Indiscriminate use of antibiotics especially in sub-therapeutic dose in animals should be stopped.
- Isolation and purification of active components of the essential oil of *Cymbopogon citratus* should be done to find a solution to problems caused by FQ-resistant *E. coli* and *S.aureus.*

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Appendix

APPENDIX 1

Escherichia coli O157:H16 strain Santai,GenBank: CP007592.1
GGRSTWYSATGCRAGTCGACGGTAACAGRAARCMGCTTGCTKYTTYGCTGACGAGTGGCGGA
CGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTA
ATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCC
CAGATGGGATTAGCTTGTTGGTGGGGTAACGGCTCACCWAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTC
GGGTTGTAAAGTACTTTCAKGGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACG
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GCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAA
TCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGT
AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGG
CCCCCTGGACGAAGACTGACGCTCAGMTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGG
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GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTM
CCTGGTCTTGACATCCACAGAASTTTTCCAGAGATGGAWWKGGTGCCCTTCGGGAAACTGTG
AGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCTGTGAATGATGGGTTTAAGTCCGCAACGA
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>26Y_27-F_F07_16
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GATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
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CCTTACCTGGTCTTGACATCCACAGAASTTTCCAGAGATGRAWAKGGTGCCTTCGGAACTGT
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>27Y_27-F_G07_19 ***Escherichia coli*** GenBank: KJ477001.1
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>29Y_27F_B01_04 ***Escherichia coli*** GenBank: JN162446.1
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>34Y_27-F_C09_09 *Escherichia coli* strain E195-4 GenBank: KJ477006.1

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AGTGCCTTCGGTAAGCTCRCAACAGATCTGACAGTCTGCTKTAGTYGTCGATGCATGTGAAAT
GTGAGTCTAGTGCCGACTGCACGCTAGCTGAACGATGTTGCATCGATCAGCTCGTCACTGCCA
GTACACACGAGGTTAAC

>37Y_27-F_F09_18 ***Escherichia coli strain*** CCFM8339 GenBank: KJ803896.1
CGGACTACACATGCAGTCGAACGGTAACAGRAAGCAGCTTGCTGCTTYGCTGACGAGTGGCGG
ACSGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCT
AATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGC
CCAGATGGGATTAGCTWGTWGGTGGGGTAACGGCTCACCWAGGCGACGATCCCTAGCTGGT
CTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCC
TTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTG
ACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG
CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTG
AAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGG
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CGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTC
CGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGAG
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAATGTTGGGTTAAGTCCCGCAACGAGC
GCAACCCTTATCCTTTGGTTGCCAGCGGTCCGACTGCACTCAAGGAGACTKGCCAGTGATAAA
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TTAG

>38Y_1492-R_G10_19 *Escherichia coli* GenBank: CP007393.1
TGAGTTSSTTAGCGYCCCYAAGTAAGCTACYYACTTCTTTTGCACCCACTCCCATGGTGTGCGG
GCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGGCATTCTGATCCACGATTACTAGCGAT
TCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCG
CTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTGGTCG
TAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTT
TGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACC
CAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCACRGTTCCCGAAGGCA
CCATCTCTGAAASTTCYGTGGATGTCAAGACCAGGTAAGGTTCTTCGCGTTGCATCGAATTAA
ACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCG
TACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTC
CAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTT

TCGCACCTGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTC
TACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCTTGCCAGTAT
CAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAAACCGCCTGCGTGC
GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGCACG
GAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATGAGCAAAGGTATTAACTTTACTCCCTT
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>39Y_27-F_H09_24 ***Escherichia coli*** GenBank:CP009166.1
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TWGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCA
CACTGGAACTGAGACACGGTCCARACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT
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TCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCAGAASAAR
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TGACGCTCARGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
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CGGAAGTTTTCAGAKATGAGAATGTGCCTTTCGGGAACCGTGAGACAGTGCTGCATGGCTGT
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>40Y_27-F_A09_03 ***Escherichia coli*** GenBank: CP002729.1
CAGTATCATGCAGTCGAACGGTAACCTTTCCSCTTGCTKGCTCGCTGACAGGGGACGGSTGAS
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CCAAGACCAAAGAGGGGGACCTTCAGACCTCTTGCGATCGATGTGCCCATATGGGATTARCTG
TKGGTGGGGTAACGGCTCACCTGGCRACGATCCCTARCTGGCTGAGAGGATGACCSCCACACT
GGAACTGASACACGGGCCAGACTCCTACGGGAGGCACTCCTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCCATGCCGCGTGTATGAASAAGGCCTTCGGGTTGTAAAGTACTTTCAGC
GGGGASGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTACCCGCASAAGAAGCACCGG
CTAACTCCGTGCCMGCAGCCGCGGTAATACGSAGGGTGCAAGCGTTAATCGKAATTACTGGG
CGTAAAGCGCATGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGSAAC
TGCATYTSATACTGGCRAGCTTGAGTCTCGKAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG
AAATGCGTAGAKATCTGGAKGAATACCGRTGGCGAAGGCSGCCCCCTGGARARCACTGACGCT
CASGYGCRAARCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGATCCACGCCSTARACGAT
GTCSACTTGSAGGTTGTGCCCTTGAGGCGTTGGATTCCKGASCTCCYKCGTTATTCAACCGCTC
GKGAGTACSTCCGCMTGGTTAAYACTACGTGAGTKRCGGSACCCCGCACWWGMSGGGGGAG
CATGTGATTYARTTKCARTGYGTCGCKAAAYCYTTACCTCGCTTRAGAYTYCTACGRRAGTCT
TTKACAGASATKAGACCTGYGYCTTTMCGAGRACCCGTGMSACWTSGTGCTGCAATGCTGTC
GTCGSTCGTGTYGGTSARATGYTSGGKTTAGTCMCAKSAATCGAGYGCAATCCTCATCGTTGC
TRCYACSYGATTCAKACCTGGCACTCCATTGAKACTGACCATCTGCACTGAGCAYTGACYGGA
ATGAATTGAAGC

APPENDIX 2 : MIC (mg/ml) of ciprofloxacin alone and in combination with oil Cocos nucifera against FQREC

Strain	Ciprofloxacin alone	Ciprofloxacin + 1v/v% coconut oil	Ciproflocin + 2% coconut
1	32	512	512
2	4	256	256
3	4	128	128
4	32	128	512
5	4	32	64
6	32	128	256
7	16	64	128
8	8	64	128
9	4	128	256
10	4	256	256

APPENDIX 3 (mg/ml) of ciprofloxacin alone and in combination with oil of Cocos nucifera against FQREC

Strain	Ciprofloxacin alone	Ciprofloxacin + 1 % coconut oil	Ciproflocin + 2% coconut
1	32	64	128
2	16	64	64
3	32	32	64
4	8	32	32
5	16	32	64
6	16	16	32
7	16	32	64
8	32	32	128
9	8	8	32
10	4	4	8

Appendix 4: Combined activity (IZD) (MM) ± SEM of ciprofloxacin coconut oil against FQRSA

Concentration (mg/ml)	Ciprofloxacin alone (Triplicate Analysis)			Ciprofloxacin + 1% oil			Ciprofloxacin + 2% oil		
32.0	22	21	22	15	13	15	13	12	12
16.0	20	20	19	14	13	14	10	10	12
8.0	16	`5	17	14	13	13	10	9	8
4.0	14	13	15	12	11	10	8	7	7
2.0	10	8	9	8	8	8	6	6	6
1.0	8	8	8	6	7	6	0	6	0
0.5	6	7	6	0	6	0	0	0	0
0.25	0	6	0	0	0	0	0	0	0

Appendix 5 ; Combined activity (IZD) (MM) ± SEM of ciprofloxacin coconut oil against FQRSA

Concentration (mg/ml)	Ciprofloxacin alone (Triplicate Analysis)			Ciprofloxacin + 1% oil			Ciprofloxacin + 2% oil		
32.0	40	36	37	10	6	11	8	8	6
16.0	35	33	35	8	6	9	6	6	6
8.0	20	19	18	6	0	6	0	0	6
4.0	15	17	16	6	0	0	0	0	0
2.0	13	14	14	0	0	0	0	0	0
1.0	6	8	7	0	0	0	0	0	0
0.5	0	6	0	0	0	0	0	0	0
0.25	0	0	0	0	0	0	0	0	0

Appendix 6 MIC and MBC values (MIC ± SEM)mm of Lemongrass essential oil against FQRSA

Source	**MIC**	**MBC**
HVns	0.044 ± 0.02	0.094 ± 0.03
PNS	0.044 ± 0.01	0.064± 0.02
HVUS	0.035± 0.01	0.048 ± 0.01
PSU	0.040 ± 0.01	0.09 ± 0.02
PWS	0.047 ±0.01	0.08 ± 0.02
AHP	0.048 ± 0.02	0.09 ± 0.04
CAT	0.040 ± 0.02	0.063 ± 0.056
AHC	0.01 ± 0.002	0.01 ± 0.002

Key;

HVns = Healthy Volunteer nasal Specimen

PNS = Patient Nasal Swab

HVUS = Healthy Volunteer urine Specimen

PSU = Patient Specimen of Urine

PWS = Patient wound swab

AHP = Apparently healthy pig

AHC = apparently healthy chicken

CAT = apparently healthy cattle

Appendix 7 : MIC and MBC values of Lemon grass essential oil against FQREC

Source	**MIC**	**MBC**
HVns	0.044 ± 0.02	0.094 ± 0.03
PNS	0.044 ± 0.01	0.064± 0.02
HVUS	0.035± 0.01	0.048 ± 0.01
PSU	0.040 ± 0.01	0.09 ± 0.02
PWS	0.047 ±0.01	0.08 ± 0.02
AHP	0.048 ± 0.02	0.09 ± 0.04
CAT	0.040 ± 0.02	0.063 ± 0.056
AHC	0.01 ± 0.002	0.01 ± 0.002

KEY;

HVUS = Healthy Volunteer urine Specimen

PSU = Patient Specimen of Urine

HVSS = Healthy Volunteer Stool Specimen

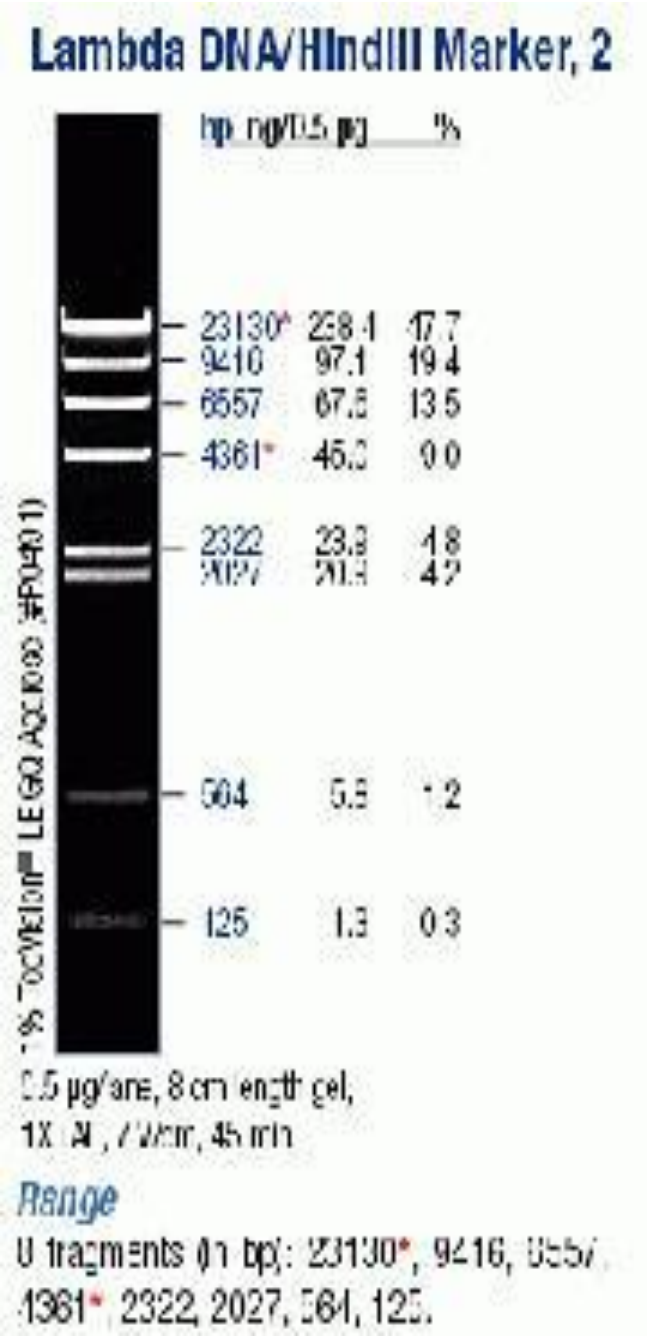
PSS = Patient specimen of stool

PWS = Patient wound swab

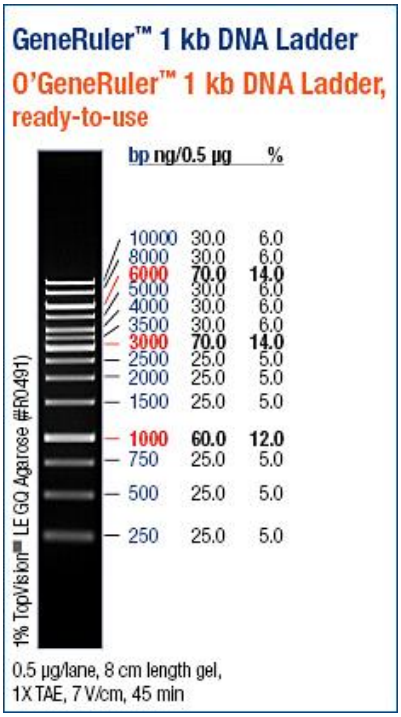
AHP = Apparently healthy pig

AHC = apparently healthy chicken

Appendix 9



Appendix 8



Appendix 10: Evaluating the susceptibility of PMQR *E. coli* donor and their transconjugants to some commonly used antibiotics

Isolate	Lab Code	Minimum inhibitory concentration mg/L							
		CPX	Lev	Ofx	Pef	GN	Amx	CT	Doxy
EC1	Pu_{70}	8	8	32	32	4	8	16	64
EC2	Hv_{16}	64	128	128	128	64	32	128	128
TC EC2	TCE_2	2	2	4	4	2	32	4	16
EC3	HS_{38}	64	32	64	128	2	64	16	256
EC4	HS_{16}	64	128	128	>256	128	>256	64	256
EC5	HS_{20}	128	256	>256	>256	>256	256	128	>256
TCEC5	TC_5	1.0	0.5	0.5	0.5	1	64	0.5	16
EC6	HS_{42}	64	32	64	64	4	128	8	>256
EC7	HS_{44}	32	32	64	128	32	>256	28	>256
EC8	DX_3	16	4	32	16	8	64	16	64
EC9	DX_5	64	16	32	32	8	32	16	64
TC EC9	TC_9	1.0	0.5	1.0	1.0	1	64	8	64
EC10	DX_{20}	32	16	32	32	64	128	64	256
EC11	DX_{26}	64	16	32	32	64	128	2	64
EC12	DX_{31}	64	32	64	128	32	128	2	64
TC EC12	TC_{12}	0.5	1	1	0.5	1	64	0	32
EC13	DX_{36}	4	4	8	8	64	64	2	64
TC E13	TC_{13}	0.5	0.5	0.5	0.5	1	64	2	64
EC14	DX_{40}	128	128	128	256	>256	>256	64	>256
EC15	DX_{85}	64	32	64	64	128	>256	64	>256
EC16	DX_{86}	64	32	64	64	4	>256	128	128
EC17	DX_{99}	32	16	16	32	32	64	2	256
EC18	DY_{33}	64	32	64	64	128	64	32	128
TC EC18	TC_{18}	0.5	0.5	0.125	0.5	1	32	1	16
EC19	DY_{92}	128	128	64	256	4	>256	4	128
EC20	DY_{98}	128	256	256	256	8	>128	4	128
EC21	DY_{102}	128	64	64	256	32	>256	16	>256
EC22	DY_{111}	32	32	32	64	4	256	32	128
EC23	DY_{113}	64	32	64	128	2	128	1	>256
EC24	DY_{211}	16	16	16	32	16	64	2	>256
EC25	DY_{222}	32	16	16	128	8	32	4	128
EC26	DY_{233}	128	128	256	>256	4	32	4	64
EC27	DY_{312}	16	8	16	16	4	16	4	64
EC28	MX_{17}	64	32	64	64	8	128	2	128
TC EC28	TC_{28}	0.5	0.5	1	0.5	2	0.5	0.25	32
EC 29	MX_{18}	8	8	16	16	32	128	1	64
TC EC29	TC_{29}	1	1	0.5	4	2	64	0.5	64
EC30	MX_{20}	8	8	16	8	16	128	1.0	32
E C31	MX_{26}	64	32	64	64	16	>256	32	>256
EC32	MX_{36}	128	64	>256	256	4	>256	32	.256
EC33	MX_2	16	32	32	64	64	128	64	128
TC EC33	TC_{33}	1	1	0.5	2	2	64	2	64
EC34	UR_9	64	64	128	128	32	>256	32	>256
EC35	UR_{12}	64	32	64	256	64	>256	8	128
Recipient E.coli BL21		0.03	0.06	0.06	0.13	0.5	16	0.26	16

Key: Cpx= Ciprofloxacin, Lev = Levofloxacin Ofx = Ofloxacin, Pef = Pefloxacin GN = Gentamicin, Amx = Amoxgcillin Ct= ceflriaxone, Doxy = Doxycycline

Appendix 11: Evaluating the susceptibility of PMQR *S. aureus* donors and their transconjugants to some commonly used antibiotics

Isolate	**lab Code**	**MIC (ug/ml)**							
		CPX	**Lev**	**Ofx**	**Pef**	**GN**	**Amx**	**CT**	**Doxy**
Sa 1	N_1	64	64	128	128	64	>256	32	>256
Sa2	N_3	128	64	128	128	128	>256	4	>256
Sa3	N_6	8	4	6	8	64	64	2	128
Sa4	N_7	4	4	16	8	1	64	1	32
Sa5	N_9	16	8	16	16	2	16	1	128
Sa6	NA_2	64	32	64	128	16	>256	32	>256
Sa7	NA_3	128	128	64	128	16	>256	16	128
Sa8	NA_6	64	32	32	32	64	128	8	64
Tc sa8	$TCsa_8$	1	0.5	1	1	2	16	2	32
Sa9	NA_8	8	16	16	16	1	128	4	>256
Sa10	NA_{10}	64	32	64	64	16	64	8	64
Sa11	NA_{11}	32	32	64	64	8	128	2	128
Sa12	NP_1	16	8	8	16	128	>256	2	128
TC	$TCsa_{12}$	0.5	0.5	0.5	0.25	2	32	4	32
Sa13	NP_2	32	16	16	32	1	12	16	>256
Sa14	UA_3	128	64	64	64	1	128	16	>256
Sa15	UA_5	64	32	32	64	8	32	32	128
Sa16	UC_1	64	64	64	32	64	16	16	256
Sa17	UC_4	16	16	32	16	4	64	2	128
Sa18	UP_5	32	16	16	32	32	128	16	256
TC	$TCsa_{18}$	2	1	1	1	2	8	4	256
Sa19	UP_9	32	32	32	64	4	64	16	128
Sa20	UQ_5	64	32	16	32	64	64	32	128
Sa21	UQ_6	8	8	8	16	64	128	1	64
Sa22	WA_1	8	4	16	16	2	128	0.5	64
Sa23	WA_5	16	16	32	64	1	128	0.5	64
Sa24	WA_{10}	128	128	128	128	16	64	16	128
Sa25	WA_{12}	16	32	32	32	16	256	4	128
Sa26	P_3	4	4	4	8	16	64	4	128
Sa27	P_4	64	32	64	64	4	128	4	8
Sa28	P_6	32	16	32	32	64	256	16	256
Sa29	Cat_1	64	32	32	32	16	128	16	256
Sa30	Cat_2	64	32	32	64	32	>256	16	256
TC	$TCsa_{30}$	1	0.5	0.5	1	2	16	2	64
Sa31	Cat_8	128	128	64	256	4	32	4	256
Sa32	Cat_9	64	64	128	128	8	32	4	256
Sa33	C_1	128	128	128	128	16	>256	2	64
TC	$TCsa_{34}$	1	0.5	0.5	0.5	1	8	4	16
Sa34	C_2	64	32	64	64	4	128	16	64
TC	$TCsa_{34}$	0.5	0.25	0.5	0.5	8	8	8	32
Sa35	C_4	64	32	64	128	>256	>256	32	>256
Sa36	C_5	128	64	128	128	64	128	16	>256
Recipient cell Staph.cohnii Subsp urealyticum		0.00	0.06	0.06	0.13	0.5	2	0.25	8

Key: Sa = Staph. Aureus, Cpx= Ciprofloxacin, Lev = Levofloxacin Ofx = Ofloxacin, Pef = Pefloxacin GN = Gentamicin, Amx = Amoxgcillin Ct= ceflriaxone, Doxy = Doxycycline Tc = Transconjugant

APPENDIX: 12 QUESTIONAIRE

To be filled by an adult (age : 12 years or above)

Please, provide the answers to the questions below as honestly and correctly as you can, to enable the researcher have basic data for his research analysis. Thank you.

Title of the Research: MOLECULAR IDENTIFICATION AND ANTIMICROBIAL STUDIES OF FLUOROQUINOLONE-RESISTANT STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI IN ENUGU STATE

DEMOGRAPHIC DATA

Sex: female [ ] male [ ]

Age: [ ]

Occupation: student [ ] or staff [ ]

MEDICAL QUESTION

Answer Yes or No

1. Have you heard about *Staphylococcus aureus* Yes [ ] No [ ]
2. Have you heard about *Escherichia coli* Yes [ ] No [ ]
3. Have you heard about Fluoroquinolone antibiotics Yes [ ] No [ ]
4. Have you heard about Fluoroquinolone-resistance Yes [ ] No [ ]

Choose one of these options below and Circle the correct answer

5. *Staphylococcus aureus* and *Escherichia coli* are
   a. Bacteria that cause infection
   b. Microorganism found in man, animals and inanimate object like handkerchief, soil, dirty spoon and plates,books, bed sheets etc
   c. Normal bacteria(flora) in one or more parts of body of man and animal
   d. Fungi like Mucor, candida and Rhizopus species.
   e. Infection that will change to Hiv/AIDS if not treated properly
   f. Non living things
   g. All of the above
   h. a & b above
   i. a, b & c above
   j. None of the above
6. What are Fluoroquinolone antibiotics
   a. They kill some bacteria and include Ciprofloxacin ,Pefloxacin, Ofloxacin and Levofloxacin
   b. They kill Candida and incude Nystatin and Ketoconazole
   c. They reduce pain sensation and include paracetamol and Ibuprofen

7. Have you gone for Widal test or Urine MCS or HVS MCS or Stool MCS or any other culture and sensitivity test i.e Infection test.Yes [ ] No [ ]

8. If **YES** to question No. **7**, was the **test** positive or negative?

9. If **Positive** to question No.8 **,** which among these organisms was isolated?

a. Staphylococcus aureus
b. Escherichia coli (E.coli)
c. Candida albicans
d. Salmonella spp
e. Shigella spp
f. Klebsiella pneumoniae
g. Enterobacter cloacae
h. Pseudomonas aeruginosa
i. Citrobacter freundii
j. Proteus mirabilis
k. I have forgotten

10. You were treated with?

a. Herbal drugs (plant MEDICINE)
b. Antibiotics (ORTHODOX MEDICINE)
c. Combination of both herbal drugs and antibiotics

11. Currently, you are taking the following drug

a. Herbal drugs (plant MEDICINE)
b. Antibiotics (ORTHODOX MEDICINE)
c. Combination of both herbal and antibiotics
d. None.

12. What is the **Name** of the antibiotic or Herbal drugs you are taking?.....

13. When was the last **time** you visited the hospital? Or Medical Laboratory? or pharmacy?

a. Within the past one week
b. Within the past three months
c. Within the past six months
d. Within the past one year
e. Within the past three year
f. Within the past five years

14. Have you ever been **admitted** into any hospital for a very long time?Yes [ ] No[ ]

15. If **yes** to No. **14**, how long did you stay in the hospital?

a. 3 to 5 days

b. 6 to 14 days

c. 15 to 30days

d. more than three months

e. one year and above

16. During your hospitalization, were catheters used on you for a very long time? Yes [ ] No [ ]

17. What is the **Name** of antibiotic you often take?...……

18. How do you usually **take** this antibiotic?

a. One tablet two times daily
b. One tablet three times daily
c. One tablet four times daily
d. Two tablet, two times daily
e. Two tablet three times daily
f. If otherwise please specify here……

19. How long do you usually take this antibiotics

a. 5 days
b. 7 days
c. 10 days
d. 14 days

20. Have you had resistance (treatment without getting well) with any antibiotics? Yes [ ] No [ ]

If **Yes** to question No.**20**, please answer questions **21**, **22**, **23**& **23**.

21. What is the Name of the antibiotic?.....

22. Which among these is the strength of the antibiotic you used?

a.100mg
b. 200mg
c. 250mg
d. 400mg
e. 500mg

23. How was the antibiotic taken?

a. One tablet twice daily
b. One tablet three times daily
c. One tablet four times daily
d. Two tablet, two times daily
e. Two tablet three times daily
f. If otherwise please specify here……

24. How long did you take the antibiotics?

a. 5 days
b. 7 days
c. 10 days
d. 14 days
e. If otherwise please specify here……

25. Have you had urinary tract infection in the last five years? Yes [ ] No[ ]

26. Did you take any antibiotics then? Yes [ ] No[ ]

27. If **yes**, who prescribed the antibiotic?

a. Doctor
b. Pharmacist
c. Nurse
d. Medical laboratory scientist

28. What is the name of the antibiotic that was used?.....

29. How many antibiotics did you use?

a. One
b. Two to three

c. Four
d. If otherwise, specify here…….

30. For how long did you take the antibiotic?……

31. How did you take the antibiotic?……

32. If **yes** to **No. 31**, did the urinary tract infection reoccur later? Yes [ ] No[ ]

33. If **yes** to No. **32**, did you take a different antibiotic? Yes [ ] No [ ]

34. If **yes** to No.**33,** What is the name of the antibiotic?**.....**

35. How long did you take the antibiotic……..

36. Where do you **live**?

a. Self contain apartment
b. flat apartment
c. one room apartment with public toilet

37. How many **people** do you live with?

a. 1-3 persons
b. 4-7 persons
c. More than 10 persons

38. Is the environment where you are living hygienic? Yes[ ] No [ ]

39. Presently, which of the **diseases** listed below are you suffering from?

a. Hiv/Aids
b. Infection but not HIV
c. Infection but the name is unknown
d. Typhoid that resists the drug you have been using.
e. Cancer
f. None

APPENDIX 13

STUDENTS CONSENT FORM

Name of School ----------

Local Government area in which the School is situated----

Health district in which the school situated----------

Centre or Health district Number- ***02-SS***

Subject Identification Number for this project----------

Title of Project: MOLECULAR IDENTIFICATION AND ANTIMICROBIAL STUDIES OF FLUOROQUINOLONE-RESISTANT *STAPHYLOCOCCUS AUREUS* AND *ESCHERICHIA COLI* FROM HUMANS AND FARM ANIMALS IN ENUGU STATE

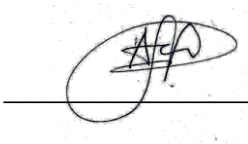
I confirm that I have read and understand the information sheet dated .....10/10/2014..... for the above study.

I have had the opportunity to consider the information, ask question and have these answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

I understand that relevant sections of any of my medical notes and data collected during the study, may be looked at by responsible individuals, hospital, company or from regulatory authorities or from the Health insurance scheme, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

I agree to take part in the above research study.

_____	_____	_____
Name of subject	Date	Signature
ADONU CYRIL C	10/**10/2014**	
Name of Researcher	Date	Signature